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Comparative studies of sperm cryopreservation of diploid and tetraploid Pacific Oysters

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COMPARATIVE STUDIES OF SPERM CRYOPRESERVATION OF DIPLOID AND TETRAPLOID PACIFIC OYSTERS

A Dissertation

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To my mom and dad, especially mom who has always encouraged education

To my husband who has always supported and inspired me to succeed

And to my 2-year old son who taught me how to love

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Table of Contents

List of Tables

List of Figures

Abstract

This dissertation addressed comparative studies of sperm cryopreservation of diploid and tetraploid Pacific oysters, *Crassostrea gigas*, with an emphasis on the development of standardized and optimized protocols. This includes comparative ultrastructural differences between sperm from diploid and tetraploid oysters, methods for the rapid estimation of sperm concentration, optimization of cryopreservation, and evaluation of the mechanisms for sperm agglutination (formation of clumps or elongated “noodles”) in thawed samples. Currently, cryopreserved sperm has not been commercialized in any aquatic species, and standardization and optimization could greatly benefit the potential commercialization of its use. In oysters specifically, cryopreserved sperm from tetraploids would facilitate the production of all-triploid seedstocks.

In this study, sperm from tetraploid oysters were 25% larger in linear dimensions (lengths and widths), and 53% had 5 mitochondria compared to 4 in diploids. Spectrophotometric methods for rapid estimation of sperm concentration were developed and validated. The effects of cooling rate, single or combined cryoprotectants at various concentrations, equilibration time (exposure to cryoprotectant), straw size, and cooling method were evaluated for protocol optimization. Combination of the cryoprotectants polyethylene glycol (PEG; formula weight of 200) and methanol (for sperm from diploids) or PEG and propylene glycol (for sperm from tetraploids) were effective in retaining post-thaw motility only when PEG was at low concentrations (2-6%). Such effectiveness was especially manifested with sperm from tetraploids, for example, post-thaw motility as high as 50% was obtained with combined cryoprotectant.

Sperm of tetraploid Pacific oysters were more susceptible to damage from cryopreservation procedures than were those of diploids, and male-to-male variation was significant for sperm from diploid and tetraploid oysters. Sperm agglutination was mainly due to the lack of sufficient cryoprotectant for specific sperm concentrations. These findings demonstrated the importance of standardization in sperm concentration and other procedures during cryopreservation. In addition, the systematic optimization of cryopreservation protocols involving interactions of multiple factors, recognition of male-to-male variation, and development of assays for sperm tolerance prior to freezing are all approaches important for the future potential commercialization of cryopreserved sperm in Pacific oysters and for other aquatic species as well.

Chapter 1
Foreword

The Pacific oyster, *Crassostrea gigas* (Figure 1.1), is one of the most important species of bivalves cultured worldwide being introduced from Japan to the west coast of the United States in 1903 (Glude and Chew, 1982). Except for large population crashes in the 1960s and 1970s from mass summer mortalities associated with high water temperatures (Pauley et al., 1988), the commercial fishery for Pacific oysters has grown rapidly. The total commercial landings in 2002 were 4,600 metric tons (a value of 27 million in US dollars) (Figure 1.2). Production of the Pacific oyster within the United States is exclusive to the Pacific Northwest with the state of Washington accounting for about 90% of the production and dollar value in the year 2002 (Figure 1.3).

Scientific name:	*Crassostrea gigas* (Thunberg)
Class:	Bivalvia
Order:	Anisomyaria
Family:	Ostreidae
Common name:	Pacific oyster
Other names:	Giant oyster, Japanese oyster
	Giant Pacific oyster

Figure 1.1 Nomenclature and taxonomy

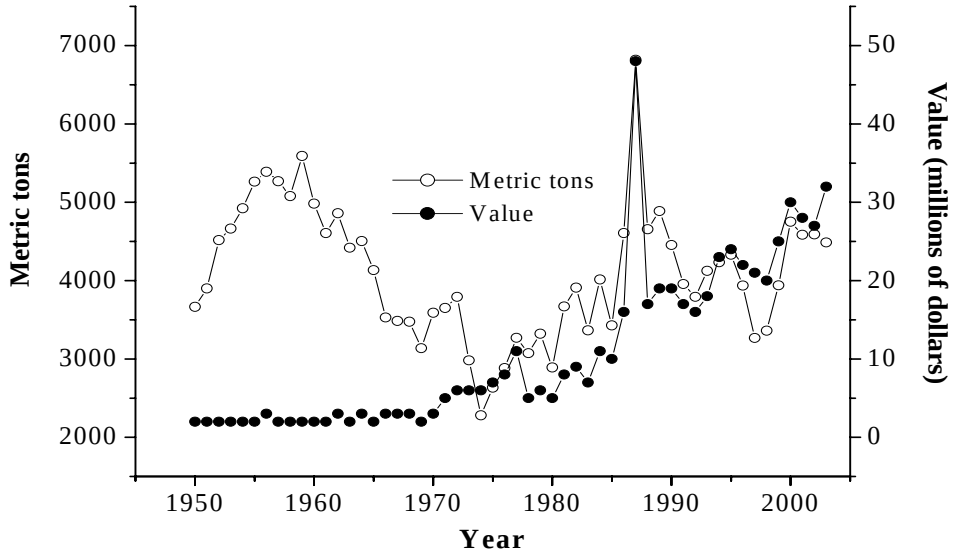

Figure 1.2 Commercial landings (1950-2003) for Pacific oysters in the United States (NMFS 2005). The large population crashes in the 1960s and 1970s were due to mass summer mortalities associated with high water temperatures (Pauley et al., 1988).

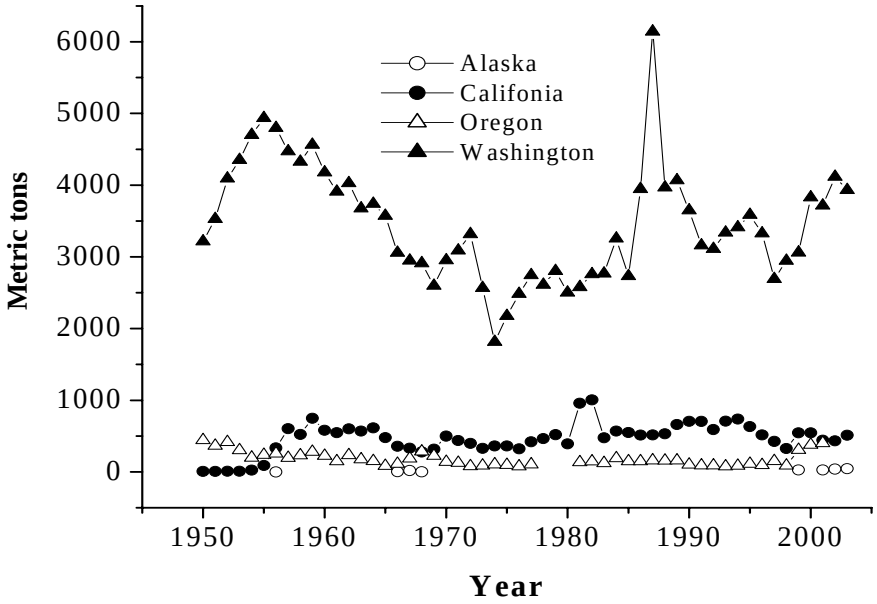

Figure 1.3 Commercial landings (1950-2003) for Pacific oysters by state (NMFS 2005). The large population crashes in the 1960s and 1970s were due to mass summer mortalities associated with high water temperatures (Pauley et al., 1988).

The induction of triploidy (possession of three chromosome sets instead of the normal two sets) is useful for aquaculture because reduced gamete output (functional reproductive sterility) leads to improved meat quality and growth. In the United States, triploid Pacific oyster have been commercialized mainly because they do not spawn during summer and thus are marketable throughout the year. Within the last 15 years, triploids have become an important segment of Pacific oyster aquaculture. For example, shellfish hatcheries in Washington and Oregon are producing about 37.5 billion ready-to-set or "eyed" larvae each year, of which about 12 billion or 33% are triploid (data based on 1999 and 2000, Nell, 2002).

The most reliable method of producing triploids, however, relies on the crossing of gametes from tetraploids (having four sets of chromosomes) and normal diploids (Guo et al., 1996; Eudeline et al., 2000 a, b). Theoretically, this cross produces 100% triploidy. The tetraploid technology was developed in 1993 with the first successful production of tretraploids by Guo and Allen (1994). The availability of tetraploid oysters creates opportunities for genetic improvement in addition to the direct production of triploid seedstocks (Guo and Allen, 1997). However, this tetraploid technology is patented by a commercial entity (4Cs Breeding Technologies, Inc.) and thus distribution of tetraploids for spawning presents problems for the protection of intellectual property. In addition, the management of tetraploid broodstocks is technically demanding. For example, tetraploids can only be obtained by chemical methods or by crossings between female and male tetraploids (Eudeline et al., 2000a, b), and the ploidy level must be identified from putative tetraploid spawns by techniques such as flow cytometry. Finally, the transportation of live broodstocks from one place to another is problematic due to

regulations in the United States and other countries. Cryopreservation of sperm from tetraploids could overcome these problems by facilitating transportation (e.g., frozen sperm could be shipped in liquid nitrogen vapor and be distributed throughout the world), and allowing control of this technology by release of gametes instead of broodstocks. Therefore, the production and distribution of cryopreserved sperm of tetraploids would facilitate the process of commercialization of triploid oysters worldwide.

In addition to the specific benefits of triploid production using cryopreserved sperm from tetraploid oysters, the use of cryopreserved sperm, in general, offers other benefits in genetic improvement programs, such as development and maintenance of inbred lines, selective breeding, hybridization, facilitation of gynogenesis and androgenesis, polyploidization, and domestication. Cryopreservation provides reliable supplies of sperm, without seasonal limitations and hatchery maintenance of adult males, and provides a safe repository for founder stocks or improved lines with desirable traits. For cultured animals, it is possible that selection of broodstock for traits appealing to consumers, or for rapid growth or disease resistance could unintentionally also select for low fertility and lead to poor quality broodstock over generations. For example, strains of Atlantic salmon, *Salmo salar*, selected for fast growth produced less than 0.1 mL of milt per kg body weight (Zohar, 1996) compared with 2 mL per kg in wild fish (Aas et al., 1991). In such case, sperm cryopreservation could help to maintain the genetic diversity.

The benefits mentioned above would be available if cryopreserved oyster sperm were available commercially. The ultimate goal of this project was to develop protocols for the commercial-scale production of sperm from diploid and tetraploid Pacific oysters. The research

reported here utilized comparative studies of sperm cryopreservation from diploid and tetraploid Pacific oysters, with an emphasis on laying the groundwork for commercialization by development of methods and identification of problems for future research. This project also provides a framework for the development of commercialization of cryopreserved sperm in other aquatic species. The specific objectives were to: 1) provide an overview of the current research of sperm cryopreservation in oysters; 2) examine ultrastructural differences between sperm from diploid and tetraploid oysters; 3) develop methods for rapid estimation of sperm concentration prior to cryopreservation; 4) optimize sperm cryopreservation from diploid Pacific oysters; 5) optimize sperm cryopreservation from tetraploid Pacific oysters; 6) evaluate the phenomenon of sperm agglutination in thawed samples, and 7) summarize and integrate the findings for future commercial application.

Results of this project showed that sperm from tetraploids were 25% larger in linear dimensions (lengths and widths) than those from diploids, and 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; sperm from tetraploid Pacific oysters were more susceptible to damage from cryopreservation procedures than were those of diploids; male-to-male variation was significant for sperm from diploid and tetraploid oysters; and sperm agglutination was mainly due to the lack of sufficient cryoprotectant for a specific sperm concentration. These findings underscore the importance of standardization in sperm concentration and other procedures during cryopreservation. In addition, the systematic optimization of cryopreservation protocols involving interactions of multiple factors, recognition

of male-to-male variation, and development of assays for sperm tolerance prior to freezing are all approaches important for the future potential commercialization of the use of cryopreserved sperm in Pacific oysters and for other aquatic species as well.

The results of this project represented a collaborative effort between the Louisiana State University Agricultural Center Aquaculture Research Station (ARS) (www.agctr.lsu.edu/inst/research/stations/Aquaculture/) in Baton Rouge, Louisiana, and the Taylor Resources, Inc. Quilcene Shellfish Hatchery (TRQSH) (www.taylorshellfish.com) in Quilcene, Washington. Because the Pacific oyster is a marine species, the hatchery facilities at TRQSH were utilized for conditioning and holding of broodstock, and for fertilization and rearing trials of larvae, while optimization of cryopreservation protocols were developed at the ARS and at Genex Cooperative, Inc. Custom Collection at the LSU T. E. Patrick Dairy Improvement Center in Baton Rouge. Work of this kind presents a number of challenges, including transport of live oysters and refrigerated sperm samples between facilities, finding balance between commercial production and research needs, temporal and spatial limitations on fertilization trials, and long distance travel. These challenges, however, reflect those faced by commercial enterprises, and thus, the technological and logistical problem solving developed here will be readily applicable to future production, facilitating progress towards the ultimate goal of commercialization of cryopreserved sperm from diploid and tetraploid Pacific oysters.

This work was supported in part by funding from the USDA-Small Business Innovation Research program, 4Cs Breeding Technologies, Inc., and the Louisiana Sea Grant College Program. The research follows up an earlier dissertation at the ARS entitled “Cryopreservation

of gametes and larvae of the eastern oyster *Crassostrea virginica*" by Carmen Paniagua-Chavez (1999) and is part of a continued effort to bring cryopreservation technology into practice for use in oyster hatcheries and for aquatic species in general.

The results of this project have been presented at several scientific meetings (Table 1.1). In addition, five papers related to this project, including Chapter 3 (Dong et al., 2005c, in press) and Chapter 4 (Dong et al., 2005b) have been published. Chapters 5, 6, and 7 are intended for submission for publication in peer-reviewed journals (Table 1.2). For consistency, all chapters of this dissertation have been presented in the format of the *Journal of the World Aquaculture Society* with specific formatting required to meet LSU dissertation format and style.

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Table 1.1 Conference presentations and abstracts based on the research presented in this dissertation.

Date	Title	Conference	Location
2005	Cumulative effects define quality of cryopreserved oyster sperm	Louisiana Chapter of the American Fisheries Society[1]	Baton Rouge, Louisiana
2005	Standardization of sperm concentration is necessary for cryopreservation	Aquaculture America	New Orleans, Louisiana
2004	Cryopreservation of sperm from tetraploid Pacific oysters	Gulf Coast Reproductive Biology Meeting	New Orleans, Louisiana
2004	A tale of tails: ultrastructure of spermatozoa from tetraploid Pacific oysters	Louisiana Chapter of the American Fisheries Society	Baton Rouge, Louisiana
2003	Surf and turf: cryopreservation with noodles	Gulf Coast Reproductive Biology Meeting	New Orleans, Louisiana
2003	Cryopreservation of sperm from tetraploid Pacific oysters	Aquaculture America[2]	Louisville, Kentucky
2003	The use of dairy methods of commercial-scale cryopreservation of sperm from Pacific oysters	Louisiana Chapter of the American Fisheries Society	Baton Rouge, Louisiana
2002	Cryopreservation of oyster spermatozoa	LSU/Audubon Joint Research Meeting	New Orleans, Louisiana
2002	Influence of osmolality, extender solution, pH and caffeine on sperm motility of tetraploid Pacific oysters	Louisiana Academy of Sciences	Baton Rouge, Louisiana
2002	Factors affecting sperm motility of tetraploid Pacific oysters	Louisiana and Mississippi Chapters of the American Fisheries Society	Biloxi, Mississippi

[1]Award received for Best Abstract (2nd place) from the Louisiana Chapter of the American Fisheries Society.

[2]Award received for Best Abstract from the United States Chapter of the World Aquaculture Society.

Table 1.2 Published papers and manuscripts in preparation based on the research presented in this dissertation.

Number	Title	Journal	Status	Chapter
1	Factors affecting sperm motility of tetraploid Pacific oysters	*Journal of Shellfish Research*	Published	--
2*	Variation in the membrane transport properties and predicted optimal rates of freezing for spermatozoa of diploid and tetraploid Pacific oyster *Crassostrea gigas*	*Biology of Reproduction*	Published	--
3	Commercial-scale sperm cryopreservation of diploid and tetraploid Pacific oyster, *Crassostrea gigas*	*Cryobiology*	Published	--
4	Spermatozoal ultrastructure of diploid and tetraploid Pacific oysters	*Aquaculture*	In press	3
5	Standardization of photometric measurement of sperm cell concentration from diploid and tetraploid Pacific oysters, *Crassostrea gigas* (Thunberg)	*Aquaculture Research*	Published	4
6	Optimization of sperm cryopreservation for diploid Pacific oysters	*Cryobiology*	In preparation	5
7	Optimization of sperm cryopreservation for tetraploid Pacific oysters	*Aquaculture*	In preparation	6
8	Agglutination of sperm from diploid and tetraploid Pacific oysters upon cryopreservation	*Biology of Reproduction*	In preparation	7
9	Fixation methods lead to artifacts of ultrastructure of spermatozoa from diploid and tetraploid Pacific oysters	*Journal of Experimental Cell Research*	In preparation	--

*Second author.

Eudeline, B., S. K. Allen, Jr., and X. Guo. 2000b. Delayed meiosis and polar body release in eggs of triploid Pacific oysters, *Crassostrea gigas*, in relation to tetraploid production. Journal of Experimental Marine Biology and Ecology 248:151-161.

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Chapter 2
Introduction

Cryopreservation is a process where biological materials such as cells and tissues are preserved by cooling to very low temperatures, typically, -196 °C (the boiling point of liquid nitrogen), yet remain viable after subsequent warming to temperatures above 0 °C. For sperm cryopreservation, this process typically includes gamete collection, suspension of sperm in an extender, quality assessment, addition of cryoprotectants, equilibration, freezing, thawing and fertilization, and subsequent development of early life stages for assessment of cryopreservation success (Tiersch, 2000). The term "extender" refers to a solution of salts, sometimes including organic compounds such as sugars that helps maintain sperm viability prior to and during the freezing process. This term has also been used to include cryoprotectant molecules (e.g., dimethyl sulfoxide or methanol) in some literature; however, its use in this dissertation refers to salt solutions only (e.g., calcium-free Hanks' balanced salt solution).

Cryoprotectants are chemicals used to protect cells from damage during the freezing and thawing processes, and are classified as to whether they penetrate the cell (referred to as permeating) or remain outside of the cell (non-permeating). Permeating cryoprotectants such as dimethyl sulfoxide are believed to help lower the freezing point of the solution, minimize osmotic shock by replacing the water inside the cell, and reduce formation of lethal intracellular ice (Doebbler, 1966; Rowe, 1966; Leung, 1991). Non-permeating cryoprotectants such as sugars and polymers are believed to help stabilize the membrane during the cryopreservation process (Meryman, 1971). However, cryoprotectants are often toxic to cells, and thus choice of the types of cryoprotectant and their optimal concentration (a balance between protection and

toxicity) has been the focus for numerous studies. Optimal equilibration time before freezing is necessary to allow permeating cryoprotectants to penetrate the sperm while minimizing toxicity. In practice, equilibration time refers to the period from the addition of cryoprotectant solutions to the initiation of the freezing process.

The choice of optimal cooling rate has been another major focus of numerous studies in the field of sperm cryopreservation. To be considered as optimal, a rate should be slow enough to minimize the amount of intracellular ice (below a damaging level) and yet be rapid enough to minimize the length of time cells are exposed to what is referred to as the "solution effect" (concentration of solutes and their precipitation if solubility limits are exceeded during the dehydration caused by ice formation). In general, rapid thawing is preferred to minimize the damage associated with recrystallization (the coalescence of small ice crystals into large crystals during the thawing process).

Numerous studies in sperm cryopreservation have been devoted to optimizing specific components of cryopreservation procedures. However, aside from those factors mentioned above, other factors such as sample density, freezing container, starting temperatures, final temperatures (before plunging into liquid nitrogen), and dilution and cryoprotectant removal after thawing could also affect results (Leibo, 2000). This dissertation also calls attention on the importance of the cumulative effects arising from all activities in the cryopreservation process. The remainder of this chapter is intended to provide an overview of sperm cryopreservation in oysters with an emphasis on identifying problems, variation, and lack of standardization among previous studies. This chapter also points out where and how this

research differs from previous studies, and the importance of standardization for the future potential commercialization of cryopreserved sperm in aquatic species.

The start of the science of cryobiology can be traced back to the 1950s after the discovery of the cryoprotective qualities of glycerol for fowl sperm (Polge et al., 1949). The first studies of fish sperm cryopreservation were published 4 years later (Blaxter, 1953), and since then more than 200 fish species have been studied (Rana, 1995; Tiersch, 2000). In contrast to the extensive studies in cryoproservation of fish semen, similar work for invertebrates has been limited to echinoderms (sea urchins, sand dollars, and starfishs), mollusks (oysters and abalone), polychaetes, and crustaceans (shrimps and crabs) (reviewed by Gwo, 2000). Currently, there are approximately 26 reports directly related to oyster sperm cryopreservation since the first study some 35 years ago (Lannan, 1971), comprising 16 peer-reviewed journal articles, 1 abstract, 2 book chapters, 2 conference proceedings, 1 thesis, 1 dissertation, 1 technical report, and 2 review articles (Table 2.1). Except for the review articles, 19 of the 24 research reports (~80%) were produced for sperm from the Pacific oyster, *Crassostrea gigas* (Table 2.1). These research efforts have yielded techniques with varying levels of success (Table 2.1). However, similar to the situation observed with other aquatic species, sperm cryopreservation in oysters has not yet found application in aquaculture on a commercial scale.

One of the major obstacles is the inconsistency of various components of cryopreservation technology among and within studies, such as initial sperm quality, gamete collection methods, extender formulation, cryoprotectant choice, cooling rate and method, thawing rate and method, insemination protocols, and evaluation of post-thaw sperm quality (Rana, 1995; Gwo, 2000;

Table 2.1 Literature review of sperm cryopreservation in oysters.

Reference number	Species	Summary of findings	Reference
1	*Crassostrea gigas*	0-10% fertility[a]; 0-3% larvae[a]	Lannan, 1971[1]
2	*C. virginica*	1-5% motility, 11% fertility[a] (2% normal fertility)	Hughes, 1973[1]
3	*C. gigas*	79% fertility[a]	Hwang and Chen, 1973[6]
4	*C. gigas*	Highest mean value: 36% fertility[a], 28% larvae[a]	Staeger, 1974[5]
5	*C. virginica*	7-91% fertility[a]	Zell et al., 1979[1]
6	*C. gigas*	0-26% fertility[a]	Van der Horst et al., 1985[2]
7	*C. gigas*	1-3 (0-5 scale) motility, 13-75% fertility[a], 47-92% fertility[b]	Bougrier and Rabenomanana, 1986[1]
8	*C. gigas*	0-106% fertility[b]	Iwata et al., 1989[1]
9	*C. gigas*	20-30% motility, 23-40% survival (19-57% normal shape)	Kurokura et al., 1990[1]
10	*C. tulipa*	0-71% fertility[a], 0-55% larvae[a], 87-93% survival at day 16.	Yankson and Moyse, 1991[1]
	C. iredalei	11-35% or 1-3 (0-5 scale) motility	Yankson and Moyse, 1991[1]
	C. gigas	48-93% fertility[a], 0-18% larvae[a]	Yankson and Moyse, 1991[1]
	Saccostrea cucullata	0-78% fertility[a], 0-51% larvae[a]	Yankson and Moyse, 1991[1]
11	*C. gigas*	(Protocols only)	McFadzen, 1995[3]
12	*C. gigas*	2-3 (1-4 scale) motility, 18-77% viability, 0-70% fertility[a], 0-70% larvae[b]	Usuki et al., 1997[1]
13	*C. virginica*	0-22% motility, 0-78% larvae[a]	Paniagua-Chavez, 1999[5]
14	*C. gigas*	Same as Number 12.	Usuki et al., 1999[4]
15	Invertebrates	(Review paper)	Gwo, 2000[7]
16	*C. virginica*	8-1316% survival[b] beyond settlement (juvenile)	Paniagua-Chavez et al., 2000[3]
17	Finfish and shellfish	(Review paper)	Chao and Liao, 2001[7]
18	*C. virginica*	Same as Number 13	Paniagua-Chavez and Tiersch, 2001[1]
19	*C. gigas*	0-100% fertility[a]	Smith et al., 2001[4]

Summary of findings: [a]absolute percentage; [b]relative percentage to controls

Report format: [1]journal article, [2]published abstract, [3]book chapter, [4]conference proceedings, [5]thesis or dissertation, [6]technical report, [7]review article.

Table 2.1 Continued.

Reference number	Species	Summary of findings	Reference
20	*C. gigas*	0-70% motility, 68% fertility[a], 64% hatch[a]	Li et al., 2002a[1]
21	*C. gigas*	(Morphological examination only)	Li et al., 2002b[1]
22	*C. gigas*	2 (0-4 scale) motility, 0-40% fertility[a]	Gwo et al., 2003[1]
23	*C. gigas*	0-90% fertility[a], 12% survival at settlement	Adams et al., 2004[1]
24	*C. gigas* (diploid)	(Theoretical prediction for cooling rate)	He et al., 2004[1]
	C. gigas (tetraploid)	(Theoretical prediction for cooling rate)	He et al., 2004[1]
25	*C. gigas*	0-59% regular D-stage larvae	Ieropoli et al., 2004[1]
26	*C. gigas* (diploid)	0-30% motility, 0-96% fertility[a]	Dong et al., 2005[1]
	C. gigas (tetraploid)	0-15% motility, 0-28% fertility[a]	Dong et al., 2005[1]

Summary of findings: [a]absolute percentage; [b]relative percentage to controls

Report format: [1]journal article, [2]published abstract, [3]book chapter, [4]conference proceedings, [5]thesis or dissertation, [6]technical report, [7]review article.

Tiersch, 2000). Lack of procedural standardization in the cryopreservation of oyster sperm is reviewed in detail in this Introduction (Table 2.2), but the same problems identified here would be equally applicable to other aquatic species.

Cryopreservation of oyster sperm involves many variables from broodstock condition to larval development, and for each step, various procedures have been used among different studies (Table 2.2). For gamete collection, the two most commonly used methods were dry stripping and aspiration using pipette or syringe. Non-destructive methods (without killing of the oyster), which would be especially useful for the purpose of self-fertilization (Lannan, 1971), include withdrawal of gonad material by use of a syringe through holes drilled in the shell, or induced spawning. Few studies have indicated what part of the gonad was sampled, and a recommendation was provided that no more than 50% of the gonad volume should be extracted to avoid including immature or nutritive cells (McFadzen, 1995). Milt from individual males or pooled milt from several males have been used for various studies. Fewer than half of the reports (excluding the two review articles) indicated a sperm quality assessment prior to freezing, and when quality was assessed, motility was the sole criterion used.

The most commonly used extender (when specified) was sterilized or filtered seawater, followed by artificial seawater (Table 2.2). Other extenders included Hanks' balanced salt solution (HBSS), calcium-free HBSS (C-F HBSS), DCSB4, Hanks' phosphate buffer, glucose, polysaccharide, and sodium citrate. The ion concentrations in various extenders (when reported) were expressed as salinity (parts per thousand), strengths (portion),

Table 2.2 Sources of variation in cryopreservation of oyster sperm

1. Broodstock condition	
Age	11-month-old[1], 1-3 yr-old[12], 2-3 yr-old[26],
Nutrient status	High food rations (mixed algal diet)[11]
Environmental conditions	Loosanoff and Davis method [2, 4], warm water[11], 18-20 °C[16], 25ppt and 20 °C[22],
Gonad maturity	Mature gamete[4, 22, 25], presence of prominent genital canals[13, 16, 26]
Seasonality	Reproduction season[19], January-September[22], November-December[23], August [24], April-August[26]
2. Gamete collection	
Collection methods	Withdrawn by syringe without killing[1, 4,]
	Spawned[2, 5, 19]
	Aspiration: pipette[5, 8, 10, 11, 20], syringe[6],
	Extracted by pressure on the genital gland[7]
	Chopping of gonad[9, 12, 22]
	Strip spawning[19, 23]
	Dry stripping[13, 16, 24, 26]
Part of gonad	Posterior-dorsal region of the right test (flat side)[4]
	No more than 50% gonad volume[11]
Milt pooled or not	Pooled[4, 5, 7, 8, 23, 25], Not pooled[1, 5, 11, 13, 22, 26]
Quality (threshold)	Motility: (Intensely active)[4, 23], (≥ 4 in 0-5 scale)[7, 10, 25], (> 80%)[9], (> 90%)[13]
3. Shipping	Intact oyster[13, 20, 24, 26]
4. Extender	
Artificial seawater (ASW)	(Not reported)[6], (22, 200, 203, 403, 602, **833** mOsm/kg)[13], (833 mOsm/kg) + 6% glycine[13], (1100 mOsm/kg)[22], (1/2, **2/3**, 5/6, and full strength)[8], (2/3 strength = 670 mOsm)[9, 12], (2/3 strength) + 50 mM sucrose + 6 mM reduced glutathione[12], (2/3 strength) + 36 mM sucrose + 4.3 mM reduced glutathione + 20% FBS[12]
Sterile seawater (SSW)	(25 ppt) [4, 22], (not reported)[1, 7, 20], (34 ppt)[25], (32 ppt) + 0.6% glycine[10]
Seawater (SW)	(not reported)[2, 3, 19]
DCSB4	(not reported)[7], (833 mOsm/kg) [13]
C-F HBSS	(475-679, 830 mOsm/kg)[13, 16], (671, **1000** mOsm/kg)[24, 26]
HBSS	(830, 833 mOsm/kg)[13]
Hanks' phosphate buffer	(13/5 strength)[8], (2.6 strength) + 80 mM glycine + 55 mM NaHCO[5],
Glucose	(0.2, 0.4, 0.6, 0.8, 1M)[8]
Polysaccharide	(not reported)[19]
Sodium citrate	(0.1, 0.15,0.2, 0.25M)[8]
pH	7.0[2], 7.0-8.0[4], 8.0[5], 8.5[7], 7.6[13], 8.2[22]
Preparation	Freshly made[7, 13], 2 h before use[11]
Storage temperature	4 °C[7], 25 °C[11]
Chemical source	Reagent grade[13, 26]

Numbers in superscripts indicate the reference number assigned in Table 2.1. Numbers in bold identify optimized protocols of that study.

Table 2.2 Continued.

5. Refrigerated storage	Undiluted[13, 26], Dilution ratio of sperm to extender (**1:0**, 1:1, 1:3, 1:7, 1:17, 1:31)[13]
Temperature (time)	4 °C (0-4 d)[13], 4 °C (7 d)[26]
6. Sperm concentration	
Initial sperm concentration (cells/ml)	(**2.7 x 10^9**, 3.0 x 10^8, 5.7 x 10^7)[4], (4.78 x 10^{10})[6], (2 x 10^{10})[19], (2 x 10^6, 2 x 10^7, 2 x 10^8, **2 x 10^9**)[26],
Dilution ratio (sperm to cryoprotectant, v/v)	(1:2, 2:1, 1:1)[4], (1:6)[5], (1:25)[6], (1:5, 1:10, **1:12.5**, **1:15**, 1:17.5, 1:20)[7], (1:8)[10], (1:1)[11], (**1:4**)[12], (1:1)[13], (1:1, **1:10**, 1:20)[19], (1:10)[23], (1:10)[25], (1:1)[26]
Freezing concentration (cells/ml)	(5 x 10^9)[8], (**5 x 10^8**, 5 x 10^7, 5 x 10^6)[12], (1 x 10^9)[16], (1 x 10^6, 1 x 10^7, 1 x 10^8, **1 x 10^9**)[26]
7. Cryoprotectant (CPA) and equilibration	
Dimethyl sulfoxide:	(20%)[1], (5, 10%)[2], (3.3, 5, 6.6, 7.5, 15, 20%)[3], (5, **10**, 20%)[4], (**8%**)[5, 12, 26], (6, **9**, 12%)[6], (10%)[7], (4, 6, **8**, 10, 12, 16%)[8], (6, **8,** 10 ,12%)[9], (5, **10, 15, 20%**)[10], (**5**, 10, 15%)[19], (8, **10**, 12, 14, 16, 18, 20%)[20], (**10%**)[21, 22], (2.5, 5, 7.5, 10, 12.5, 15%)[23], (5, 10, 15%)[25]
	(10% + 1 M trehalose)[11], (2.5, **5**, 7.5, 10, 12,5, 15% + 0.45 M trehalose)[23], (5% + 0.55 M trehalose)[23],
Glycerol	(3.3, 5, 6.6, 7.5, 15, 20%)[3], (5, 10, 20%)[4], (5, 10, 15%)[6], (5, 10, 15%)[25], (not reported)[19]
Ethylene glycol	(4, 6, 8, 10, 12, 16%)[8], (5, **10**, 15%)[25], (not reported)[19]
Propylene glycol	(5, **10**, 15, 20, 15%)[13], (5, 10, 15, 20, 15% each with 0.25 sucrose)[13], (**15**%)[16], (**5**, 10, 15%)[26], (5, 10, 15%)[25], (not reported)[19]
Methanol	(5, 10, 15%)[25], (not reported)[19]
Trehalose	(**0.45 M**)[23]
Glycine	(5, 10, 20%)[4]
Addition method	Single step[1,2, 4, 5, 6, 7, 8. 10, 13, 16, 19, 20, 21, 25, 26], step-wise addition[19, 23]
Equilibration temperature (duration)	0 °C (20 min)[5], (10-30 min)[19]
	On ice (< 30 min)[10]
	0-4 °C (20 min)[21]
	4 °C (5 min)[22], 5 °C (10-30 min)[26]
	10 °C (10-30 min)[19]
	20 °C (10-30 min)[19], 21 °C (20 min)[13, 16]
	25 °C (< 15 min)[11], 26 °C (10, 30 min)[25]
	Not reported (**0**, 5, 10min)[7], (**3**, 30, 60 min)[8], (3, 15 min)[9], (~45 min)[23]
Time between collection and freezing	10 min[22], 30 min[10], < 1.5 h[4], 4 h[19]

Numbers in superscripts indicate the reference number assigned in Table 2.1. Numbers in bold identify optimized protocols of that study.

Table 2.2 Continued.

8. Freezing container	Ampoules[4]: (1-ml)[1] (2-ml)[2]
	Plastic straw: (0.25-ml)[5, 6, 19, 23], (0.35-ml)[7], (0.5-ml)[8, 9, 11, 12, 19, 26], (2.5-ml)[19] (5-ml)[13, 16]
	Cryovials/cryotubes: (1-ml)[20], (1.5-ml)[22], (1.8-ml)[10], (2-ml)[3, 25], (4.5-ml)[23],
9. Freezing method	
Liquid nitrogen vapor :	2 min[1]
distance between sample	(8, 10, 12, 15, **17**, 20 cm above) x (2, **6**, 10, 14, 20, 30 min)[20]
and liquid nitrogen	3 cm above for10 min (13.5 °C/min)[23]
(recorded cooling rate)	5 cm above: (3 min)[7], 10 min (114.3 °C/min)[12], with exposed straws (**79.8** °C/min to -60 °C)[12], with straws in sheath (38.2 °C/min to -60 °C)[12]
	7 cm above (8.4 °C/min at -20 °C) [9]
	10 cm above with exposed straws (57.5 °C/min to -60 °C)[12]
	From 0 to -80 °C at 5-13.5 °C/min, then to liquid nitrogen[5]
	From RT to -30 °C at 15 °C/min, then to liquid nitrogen[22]
	4.7 °C/min to -70 °C, then to liquid nitrogen[10]
Directly to liquid nitrogen	(Not reported)[3], (106.8 °C/min)[23]
Methanol/dry ice bath	60 min (9.4 °C/min)[12], 10 min (26.8 °C/min)[23]
Graybill and Horton	(5, 30 °C/min)[4]
methods (1969):	
Controlled-rate freezer	Linde Model BF-4 Freezing chamber and controller[2, 5]: (1 °C/min to -8°C, -8°C to -25°C at 5.5°C/min, then to liquid nitrogen)[2]
	Planer Kryo 10 Mk II: (25 °C to -120 °C at 100 °C/min; at 15 °C/min to -150 °C, hold 1 min then to liquid nitrogen)[11], (15 °C to -30 °C at 2.5 °C/min; hold 5 min then to liquid nitrogen)[13, 16], (1, 5, 20, **50** °C/min)[19], (0 °C to -80 °C at 50 °C/min; held 10 min then to liquid nitrogen)[23], (9.5 °C/min)[23]
Deep freezer of -80 °C	60 min (-6.1 °C/min)[12]
Dairy commercial freezing	8 min run[26]
method	
Not specified	(**6**, 11, 16, 21 °C/min to -70 °C, then to liquid nitrogen)[25]
10. Storage	-196 °C: (90 d)[2], (5 min – 68 d)[5], (< 3 d)[7], (1-2 d)[8], (30-80 d)[9], (1h - 4 yr)[12],
temperature (time)	(14 d - 30 d)[13], (7d)[16], (2 h – 1 d)[20], (1 h)[23], (2 d)[26]
	-190 °C: (7, 12, 30, 168, 217 d)[10]
	-170 °C: (24 h)[4]
	-5, -20, -40, -80 °C (< 2 min)[5]

Numbers in superscripts indicate the reference number assigned in Table 2.1. Numbers in bold identify optimized protocols of that study.

Table 2.2 Continued.

11. Thawing	
Water bath	4 °C (3 min)[4]
	16-17 °C running water[20],
	16-17 °C running water, then move to 0 – 4 °C (10-14 min)[20]
	Room temperature (not reported)[8, 9], (> 1min)[12]
	20 °C (1 min)[7], (not reported)[11], (15 s)[19], (15-20 s)[23], (5-8 min)[23]
	22 °C (2 min)[4], 25 °C (30 s)[13], 40 °C (7 s)[26],
	48 °C (10 s)[5], 55 °C (20 s)[10], **60 °C (10 s)**[5], **70 °C (15 s)**[13, 16], (1 min)[22], 75 °C (2 s)[19]
Air bath	21 °C[2], 22 °C(5 min)[4]
Not specified	74 °C/min up to 26 °C[25]
12. Removal of CPA	Diluted in SW[4, 11, 16], Diluted in C-F HBSS[13], Step-wise removal[19, 23]
13. Post-thaw sperm quality assay	
Motility	Percentage[2, 9, 11, 13, 20, 26]
	Scale: (0-5)[7, 10], (1-4)[12], (0-4 ≈ 0-75%)[22], (0-5 at an increment of 0.5)[25]
Morphology	Cytogenetic examination[2], Scanning electron microscopy[9, 21]
Viability/survival	Eosin-nigrosine[9], Dye exclusion (0.3% trypan blue)[12], Comet assay[22]
Fertility	Absolute[1, 2, 4, 5, 6, 7, 8, 10, 12, 22, 23, 26], Relative to controls[7, 8]
	Polar body formation or appearance of first cleavage furrow[11]
	Count embryos at (1.5 h)[8, 25], (2-3 h)[1, 5, 6, 12, 26], (4 h)[7], (24 h)[22], (4-cell stage)[23]
	Subtraction of unfertilized eggs 6 h post-fertilization[4]
	Pooling abnormal embryos and normal D-larvae at 24 h[10]
Hatch	Absolute[1, 4, 13, 25, 26,], Relative to controls[12]
	Count straight-hinge larvae after (6 h)[12], (12 h)[13], (24 h)[4, 10, 26], (40 h)[1] (48 h)[25]
Larval growth	(2, 11 d)[5], (16 d)[10], (6 d)[12], (> 4 month)[16], (10 mm spat)[19], (metamorphosed spat)[23]
14. Fertilization method	
Methods	34 ml sperm to 5-15 million eggs in 250 ml SW[2]
	0.25 ml sperm to 30-300 ml SW with 200-900 eggs/ml[5]
	0.35 ml sperm to 1 ml of ova[7]
	0.5 ml sperm to 2000 eggs in 200 ml SW[10]
	0.5 ml sperm to 14,000 eggs in 500-mL plastic beaker[13]
	5 ml sperm to 2000 -12000 eggs in 200 ml SW[26],
	12-well tissue culture plates (30 µl sperm at 10^4- 10^7 cells/ml to 600 eggs in 3 ml SW of each well)[19, 23]
Eggs pooled or not	Pooled[4, 5, 7, 13, 16, 22, 23, 26] , Not pooled[1, 5, 13,
Sperm-to-egg ratio	(7300)[4], (10^4-10^5)[8], (10^3)[12], (~18000)[13], (10, 10^2, 10^3, **10^4,** 10^5, 10^6)[22], (10^2-10^5)[19, 23],
Controls	Positive control[1, 2, 4, 5, 7, 8, 10, 12, 13, 19, 20, 22, 23, 25, 26], Negative control[1, 5]
	Initial sperm quality control[4, 5, 23], Initial egg quality control[5]
	Toxicity control for fresh sperm[1], Toxicity control for fresh egg[4]

Numbers in superscripts indicate the reference number assigned in Table 2.1. Numbers in bold identify optimized protocols of that study.

or osmolalities (mOsm/kg). The pH values (when reported) ranged from 7.0 to 8.5. Only two or three studies specified the method of extender preparation, storage temperature, and the grade of chemicals used (Table 2.2). Refrigerated storage of fresh sperm was evaluated in only two studies with sperm samples either in undiluted or diluted form and stored at 4 °C for 0 to 7 d.

In terms of sperm concentration, only four studies explicitly identified the final sperm concentration in each freezing trial (Staeger, 1974; Usuki et al., 1997; Paniagua-Chavez et al., 2000; Dong et al., 2005). Most reports indicated the dilution ratio of sperm volume to cryoprotectant solution, of which three reports identified the original sperm concentrations for sampled milt. Final sperm concentrations ranging from 5×10^8 to 1.4×10^9 cells mL^{-1} were considered optimal for freezing (Table 2.2).

Cryoprotectants included dimethyl sulfoxide (DMSO), glycerol, ethylene glycol, propylene glycol, methanol, trehalose, and glycine. Among these, DMSO was the one most commonly used (in 19 reports); the concentrations that were tested ranged from 2.5 to 20% in various studies. The concentration of DMSO considered to be optimum varied among studies, ranging from 5 to 20%, with most reports referring to either 8 or 10% (Table 2.2). In addition, ethylene glycol at 10%, propylene glycol at 5, 10, or 15%, and trehalose at 0.45 M were also considered to be effective in maintaining post-thaw fertility. The addition of a cryoprotectant was usually performed in a single step. However, step-wise additions were suggested to avoid osmotic injury (Adams et al., 2004). Temperatures at which sperm samples were equilibrated with cryoprotectant before freezing varied from 0 to 26 °C with time intervals ranging from 0 to 60 min. In general, shorter equilibrations time were considered to be more effective in retaining

post-thaw sperm quality, but as shown in Chapter 6, long equilibration (e.g., 60 min) with the cryoprotectant at low concentrations (< 10%) may not decrease percent fertilization. The longest time reported between gamete collection and freezing was 4 h in all previous studies.

For freezing trials, glass ampoules, plastic straws, and cryovials were used as freezing containers with volumes ranging from 0.25 ml to 5 ml, but most studies used plastic straws of 0.25-ml or 0.5-ml volumes (Table 2.2). Liquid nitrogen vapor was most commonly used to freeze samples, followed by controlled-rate freezers. Other freezing methods included mixtures of methanol and dry ice (-75 °C), deep freezers (-80 °C), dairy commercial freezing methods (Dong et al., 2005), and direct plunging into liquid nitrogen. Various rates of success were reported with each method (Table 2.1), but comparisons among them were made difficult due to inconsistency in methods and reporting of other components (e.g., cryoprotectant and concentrations, equilibration time, and thawing methods) of the cryopreservation procedures. The cooling rates of samples frozen in liquid nitrogen vapor were affected by the distance between samples and the surface of liquid nitrogen, the exposure time, as well as the freezing container itself. For liquid nitrogen vapor, reported cooling rates ranged from 4.7 to 114.3 °C per min in different studies. Differences in freezing containers played an important role in the rate of cooling regardless of the freezing method. For example, when samples in 4.5-ml cryovials were cooled at a desired rate of 50 °C per min using a controlled-rate freezer, that actual cooling rate was 9.5 °C/min (Adams et al., 2004). A wide range (6 to 80 °C/min) of optimal cooling rates was reported in various studies.

For storage, frozen samples were stored at -190 °C or lower in most studies except one, in which samples were stored at -170 °C (Staeger, 1974) (Table 2.2), but storage time varied from 5 min to 4 years before thawing. Spermatozoa of Pacific oysters cryopreserved for 4 years yielded 78% normal D-shaped larvae and no negative effect was found in mean shell length of the larvae at 6 days after fertilization (Usuki et al., 1997). For thawing, samples were thawed in a water bath in most studies, but the temperature of the water bath varied from 4 to 75 °C. Thawing at higher temperature (60 °C versus 48 °C) was suggested to more effectively preserve the post-thaw fertility for samples in 0.25-ml straws (Zell et al., 1979) and 5-ml macro-straws (Paniagua-Chavez et al., 2000) of *C. virginica*. Studies with *C. gigas* indicated no difference between thawing at 20 °C for 15 s and 75 °C for 2 s for samples in 0.25-ml straws (Smith et al., 2001). Incomplete thawing of samples in 1-ml cryovials in 16-17 °C running water followed by a complete thawing at 0 to 4 °C for 10 to 14 min was found to retain higher post-thaw motility (>40%) in *C. gigas* (Li et al., 2002a). Samples thawed in the air (21 to 22 °C) were considered to be sub-optimal (Staeger, 1974). Only one study reported the warming rate, but no thawing method was specified (Ieropoli et al., 2004). Few studies reported dilution or serial dilutions for thawed samples.

Various criteria were used to estimate post-thaw sperm quality (Table 2.2), but specific terms had different meanings in different studies. For example, what was defined as percent fertilization in one study (Gwo et al., 2003) was defined as percent hatch in other studies (Staeger, 1974; Yankson and Moyse, 1991; Dong et al., 2005). Motility was also expressed in several methods such as percentage, or scales of 0 – 4, 1 – 4, or 0 – 5 at increments of 0.5 or 1.0.

Similarly, percent survival or viability referred to results derived from different assays (Table 2.2). In addition, results for percentage fertilization, larvae produced, or survival were reported as absolute values or as values relative to controls (Table 2.1). Fertilization methods used in various studies were also different from one another in many aspects, such as sperm-to-egg ratio, the use of eggs from individual females or pooled eggs from several females, trial scale (12-well tissue culture plates versus 500-ml plastic beakers), and different types of control treatments. Despite these differences, for sperm-to-egg ratios, generally a 100-fold increase was suggested for cryopreserved sperm compared to fresh controls (Iwata et al., 1989; Gwo et al., 2003; Adams et al., 2004). Larval development beyond the settlement stages (the ending of planktonic existence of larvae by attachment to suitable substrates) was also evaluated in six studies, but no adverse effects were reported for larvae produced with cryopreserved sperm.

To summarize this review of sperm cryopreservation in oysters, a lack of standardization was observed in each step involved in the cryopreservation process (Table 2.2). Comparisons among different studies were difficult to perform and could be invalid in most cases due to the procedural and reporting variations across studies observed at each cryopreservation step. The results presented in this dissertation call attention to the requirement for researchers to standardize sperm concentration and methods during cryopreservation. Optimization of protocols without standardization offers little value for the improvement of existing methods and results, especially for the future development of commercial application. On the contrary, controversy and inconsistency would be reduced if more congruent approaches were utilized and results among various studies could be directly compared.

The ultimate goal of this project was to develop a practical model for commercial application of cryopreserved sperm from diploid and tetraploid Pacific oysters. Currently, human and livestock are the only worldwide industries that have incorporated cryopreservation of semen into commercial artificial insemination practices (Crister, 1998; Curry, 2000; Centola, 2002). If cryopreservation of fish or shellfish sperm is to be integrated into hatchery operations, the use of specialized cryopreservation centers such as dairy facilities should be considered as a standardized, time-saving, and cost-effective option (Tiersch et al., 2004). The first most important component involved in utilizing a central cryopresevation facility is shipment of broodstock or milt. Previous studies of sperm cryopreservation from oysters have rarely (4 studies) involved shipping (Table 2.2). In contrast, the research in this dissertation exclusively involved the use of shipped sperm and oysters.

The research in this project involved activities at three locations: a commercial hatchery (Taylor Resources, Inc.), a research laboratory (Aquaculture Research Station, ARS), and a commercial livestock sperm freezing facility (Genex Cooperative, Inc.) (Figure 2.1). Samples were shipped chilled overnight from the hatchery to the ARS. In the case of shipment of oysters, the time between sperm collection and freezing ranged from 2 to 3 h depending on where the freezing trials were performed. In the case of shipment of sperm samples, the time between sperm collection and freezing was more than 26 h (with the addition of 24 h shipment time), which was more than 6 times greater than the longest time (4 h) reported in previous studies (Smith et al., 2001). Sperm samples frozen at Genex Cooperative were appropriately labeled, sorted, stored, and inventoried in the database at Genex Cooperative, Inc.

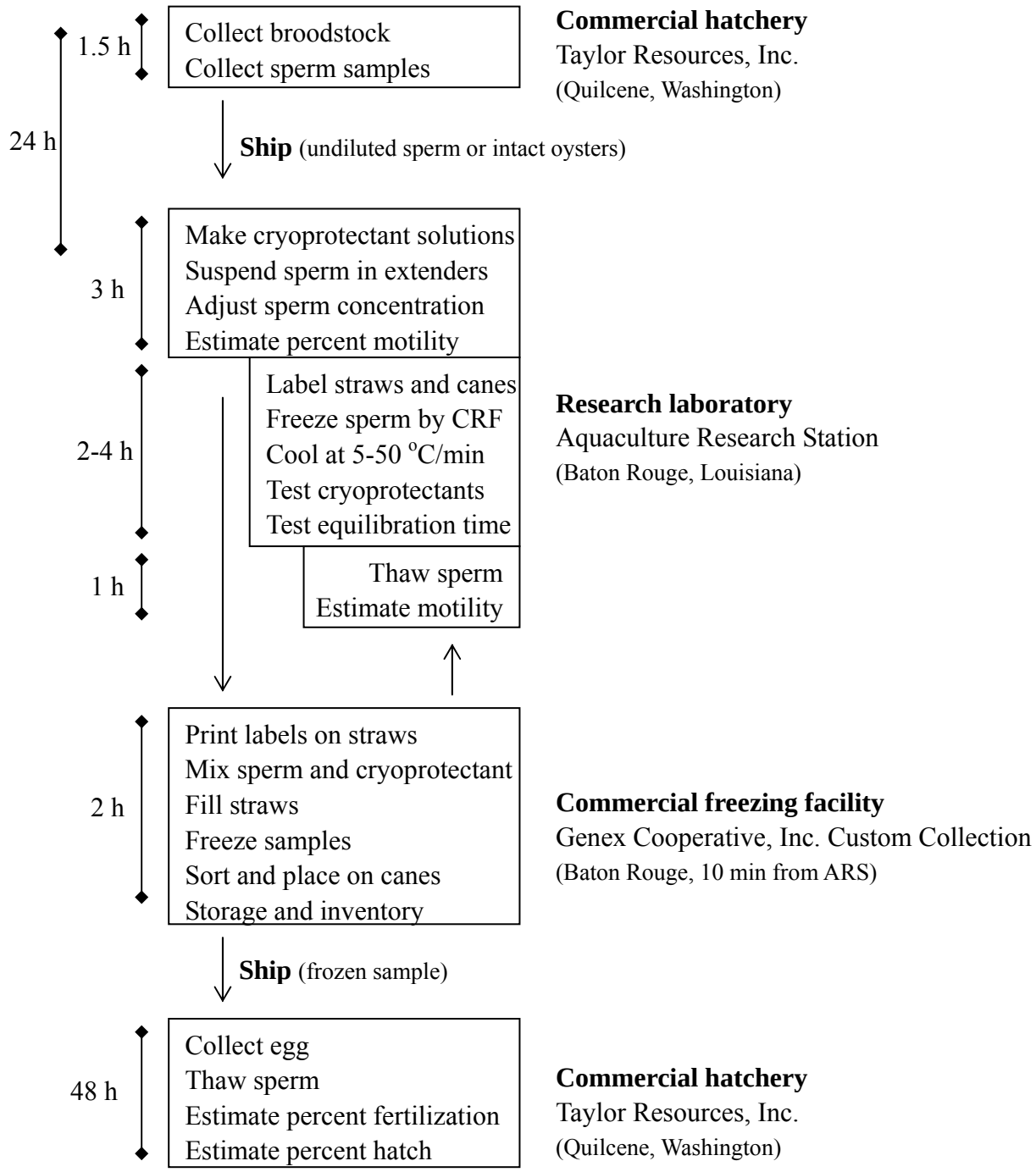

Figure 2.1 Overview and chronology of activities at the commercial hatchery (Taylor Resources, Inc.), research laboratory (Aquaculture Research Station, ARS), and commercial livestock sperm freezing facility (Genex Cooperative, Inc.) for cryopreservation of sperm from diploid and tetraploid Pacific oysters, *Crassostrea gigas*. Sperm samples were frozen either at the ARS using a controlled-rate freezer (CRF) or at Genex using the commercial freezing method. (Figure adapted from Tiersch et al., 2004)

For fertilization trials, samples were shipped frozen from Genex Cooperative to the Taylor Resources Quilcene hatchery (Figure 2.1). The process described in this dissertation is readily applicable to commercialization for cryopreserved sperm, and the experiments were conducted to establish standardized and optimized protocols for practical application. In addition, this project also identified problems requiring future improvements.

Preliminary studies of sperm cryopreservation of tetraploid oysters showed limited success (Dong et al., 2005) compared to that of diploid oysters. Differential response to cryopreservation by sperm from tetraploid and diploid oysters may be due to the differences in their gonadal development, or differences between sperm architecture of tetraploids and diploids. Thus, the ultrastructure of spermatozoa in tetraploid oysters was described in Chapter 3 and compared to that of diploid oysters by use of light and electron microscopy. The implications of ultrastructural findings on susceptibility of sperm from diploid and tetraploid oysters to damage from cryopreservation were discussed.

One of the earlier efforts in this project was to standardize sperm concentration prior to freezing. Accurate estimations of sperm concentration are necessary for the development of standardized techniques for cryopreservation of sperm of any aquatic species, and can be used to facilitate toxicity studies of cryoprotectants before freezing, fertilization studies of sperm after thawing, and to standardize the "freezing unit" (number of sperm per freezing container) for future commercial-scale production. Development of a method for rapid estimation of sperm concentration using spectrophotometry is presented in Chapter 4.

The use of a spectrophotometer and standard curves (described in Chapter 4) enabled timely standardization of sperm concentrations prior to freezing for subsequent studies. For this project, the sperm concentrations were all adjusted to 1 x 10^9 cell mL^{-1} for each freezing trial except for experiments specifically addressing sperm concentration. Due to the limitations on numbers of fertilization trials (Chapter 1), motility was used as the initial screening criterion for range-finding experiments, reducing the number of treatments necessary for testing with fertilization. Research described in Chapters 5 and 6 focused on optimization of protocols used for sperm cryopreservation of diploid and tetraploid Pacific oysters. With regard to protocol optimization, the commercial freezing methods presented several constraints such as fixed cooling protocols and the requirement of large numbers of straws (e.g., 600 straws) for a single freezing run (SOP-10, Appendix A). The controlled-rate freezer, on the other hand, provided greater research flexibility in various cooling rates and no requirement for a minimum number of straws. Thus, the approach was to use the controlled-rate freezer to optimize protocols (including evaluation of cooling rate, cryoprotectant, equilibration time, straw size, and interactions among them) and to later test the optimized protocols using the commercial freezing method. Most experiments in these two chapters were performed in parallel with the only difference being ploidy of the broodstock. They were prepared as separate chapter to improve the clarity of presentation.

In the early studies with cryopreserved sperm from diploid and tetraploid oysters, sperm were frequently found to agglutinate after thawing with the formation of elongated “noodles” in extreme cases (J. Buchanan and C. Paniagua-Chavez, unpublished; Dong et al., 2005). Sperm

agglutination for thawed samples has also been reported in fish species such as common carp, *Cyprinus carpio* (Sneed and Clemens, 1956; Miskolczi et al., 2005), and Atlantic croaker, *Micropogonias undulatus* (Gwo and Arnold, 1992). Despite these observations, no attempts have been made to explain this phenomenon. In addition, agglutination is generally considered to be a negative outcome and is often thought to be an indicator of failure in cryopreservation (Bougrier and Rabenomanana, 1986; Kurokura et al., 1990). However, Pacific oyster sperm samples with "noodle" formation yielded fertilization as high as 50% in my studies (Dong et al., 2005). In Chapter 7, factors such as the type of cryoprotectant, cryoprotectant concentration, sperm concentration, cooling rate, and thawing methods were examined for their effects on sperm agglutination of thawed samples, and the relationships among them emphasized the importance of standardization of sperm concentration and systematic optimization of protocols involving multiple interacting factors. In Chapter 8, the findings for future commercial application are summarized and integrated.

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Chapter 3
Ultrastructural Differences of Spermatozoa from Diploid and Tetraploid Pacific Oysters

The Pacific oyster, *Crassostrea gigas*, is one of the most successful models for ploidy manipulation in bivalves. Tetraploid broodstock (possessing four chromosome sets) of the Pacific oyster have been developed either through chemical shocks or by breeding tetraploid females with tetraploid males, and are available for commercial application for triploid seed production (Guo et al., 1996; Eudeline et al., 2000a, b). Cryopreservation of sperm from these Pacific oysters has been initiated to fulfill the goal of expanding commercial-scale application of tetraploid stocks and improving tetraploid breeding programs. Preliminary studies of sperm cryopreservation of tetraploid oysters showed limited success (Dong et al., 2005a) compared to that of diploid oysters. Differential response to cryopreservation of sperm from tetaploid and diploid oysters may be due to the differences in their gonadal development or differences in the sperm architecture of tetraploids and diploids. For Pacific oysters, sperm ultrastructure has been studied for diploids (Bozzo et al., 1993) and triploids (Komaru et al., 1994), but sperm ultrastructure in tetraploids is unexplored.

The goal of this study was to examine by light and electron microscopy the ultrastructure of spermatozoa in tetraploid oysters compared to that of diploid oysters. The specific objectives were to compare the ultrastructural differences of sperm from diploid and tetraploid oysters in: (1) sperm architecture; (2) the sizes (length and width) of sperm components; (3) the number of mitochondria, and (4) to discuss the relationship between sperm morphology differences and the susceptibility to cryopreservation damage.

Materials and Methods

Oysters

Tetraploid and diploid Pacific oysters were obtained in August 2003, from Taylor Resources Quilcene Shellfish Hatchery in Quilcene, Washington (47° 49' 133" N, 122° 49' 523" W) and were shipped chilled at 5-10 °C by overnight delivery to the Louisiana State University Agricultural Center, Aquaculture Research Station (ARS). Ploidy level of individual oysters was verified by flow cytometry (Allen, 1983).

Sample Preparation

For scanning electron microscopy (SEM), sperm were collected by dry stripping of the gonad (Allen and Bushek, 1992) and suspended in calcium-free Hanks' balanced salt solution at 1000 mOsmol/kg (Dong et al., 2002). Two fixation methods were used for two batches of sperm samples: the first batch (two diploid and two tetraploid males) were fixed with 2% glutaraldehyde and 1% osmium tetroxide (OsO_4) in 0.1M sodium cacodylate buffer (CB, pH 7.4); the second batch of samples (one diploid and one tetraploid male) was fixed with 4% glutaraldehyde in 0.1 M CB with the addition of sucrose to bring the final osmolality to 1110 mOsmol/kg. Sperm were collected on 0.2-µm polycarbonate membrane filters (Osmonics, Inc. Minnetonka, Minnesota) during fixation, rinsed with 0.1 M CB, and dehydrated by exposure to an ethanol series (20, 30, 50, 70, 75, 80, 85, 90, 100, 100, and 100%) for 10-20 min each. Samples were critical point dried and sputter coated with gold (60%) and palladium (40%) at a thickness of less than 100 Å. A total of six SEM filters (one filter per oyster) were examined using scanning electron microscopy (Cambridge 260 Stereoscan). Observations were made for all areas of the filter for each sample.

For transmission electron microscopy (TEM), the gonads were dissected and minced into small fragments (< 1 mm) while immersed in 2% glutaraldehyde in 0.1M CB (first batch) or 4% glutaraldehyde at 1110 mOsmol/kg (second batch). Fixation occurred over 1 h at room temperature (RT), during which the fixative was replaced twice. The tissues were rinsed in 0.1M CB (194 mOsmol/kg) three times for 15 min each, and post-fixed in 2% OsO4 in 0.1M CB for 1 hr at RT. Tissues were rinsed twice with distilled water, stained with 0.5% uranyl acetate in the dark for 1 hr at room temperature, rinsed in distilled water and dehydrated through an ethanol series (20, 30, 50, 70, 75, 80, 85, 90, 100, 100, and 100%) for 10-20 min each. Samples were infiltrated in equal volumes of LR white resin (London Resin Company Ltd., England) to 100% ethanol on a rotating shaker (Vari-Mix, Thermolyne) for 1 hr at RT, and 100% LR white resin for 1 hr. Flat embedment in LR white resin was carried out by using two aluminum 43-mm (diameter) weighing dishes (VMR Scientific, West Chester, Pennsylvania). The sample pieces and 7 ml of resin were distributed in one dish and the other dish was nested into it as an air-excluding cover, and a tiny hole (~1 mm in diameter) was punched along one edge to allow air bubbles to escape while the two dishes were pressed together. The resin was polymerized at 60°C overnight and the sample blocks were shaped by sawing. Thin sections (80 nm) were cut with a ultramicrotome (DuPont MT 5000, Sorvall® Ultra Microtome), stained with Reynolds' lead citrate (Bozzola and Russell, 1992) for 1 min, and five grids per oyster were examined using TEM (JEOL 100CX). All chemicals were of reagent grade (Electron Microscopy Sciences, Fort Washington, Pennsylvania)

Spermatozoa Measurements

Measurements were made after fixation but prior to dehydration from the first batch, and the length and width of sperm heads, and the length of the main piece and end piece of flagella were

measured at 800-× magnification with light microscopy (Figure 3.1). Digital images were captured through a diagnostic instrument CCD camera (SPOT RT Slider, SpectraCore, Inc. Webster, New York). Unfixed sperm samples from 10 tetraploid and 3 diploid males were measured for the height and width of the acrosome, the length and width of the sperm head, the height of the mitochondria, and the total length of flagellum at 1000-× magnification by light microscopy (Figure 3.1). The number of mitochondria per spermatozoa was counted from two samples by use of TEM (from two oysters of each ploidy in the first batch) showing transverse sections of the midpiece, and one sample by use of SEM with exposed mitochondria (from two oysters in the second batch).

Nomenclature and Image Processing

The detailed structures of spermatozoon from invertebrates and vertebrates have been described by terms used with different meanings by different authors. In the present study, the terms used were based on Franzén (1956), and the flagellum length includes the main piece and end piece (Figure 3.2). For fixed samples, prior to measurement, images were processed with different enhancements to locate the tip of the end piece (Figure 3.2). The *invert* command reversed a positive black-and-white image into a negative. The *equalize* command redistributed the brightness values of the pixels within an image to more evenly represent the entire range of brightness levels. The flagellum components were clearly distinguishable after these enhancements (Figure 3.2B). However, this processing did not enhance analysis of images produced with unfixed samples in this study.

Data Analysis

Data were analyzed using either the Student's t-test (when there were two means) or by one-way analysis of variance (ANOVA) when there were more than two means. The Tukey-Kramer

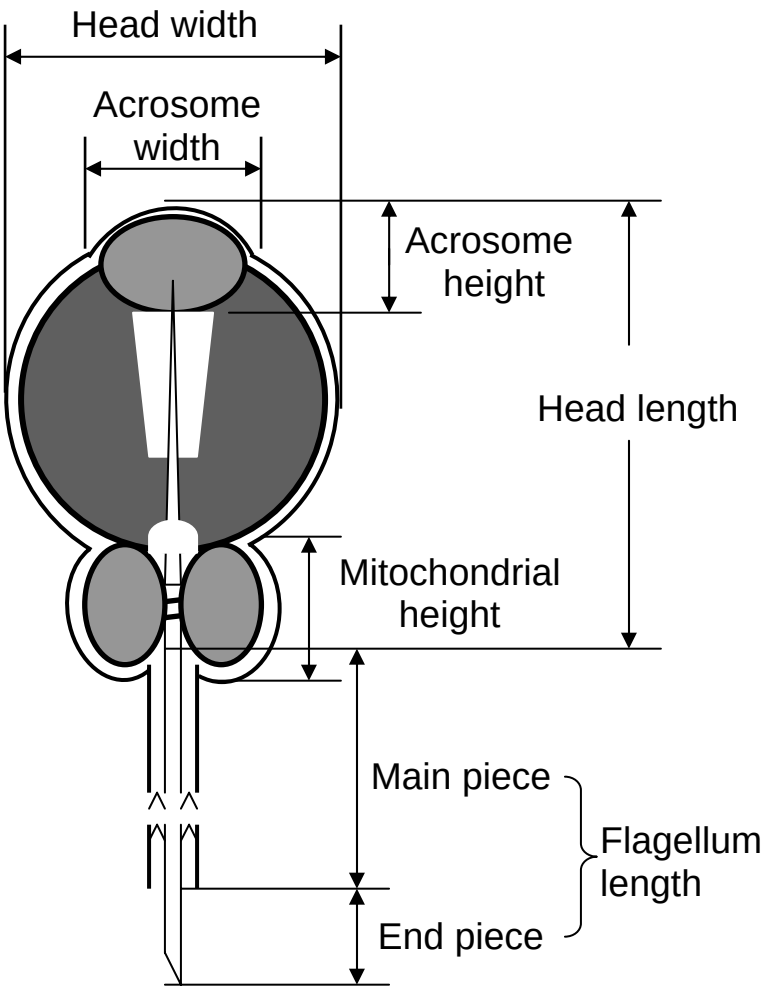

Figure 3.1 Identification of spermatozoal components used for measurement of unfixed sperm samples with light microscopy. Diagram adapted from Galtsoff and Philpott (1960).

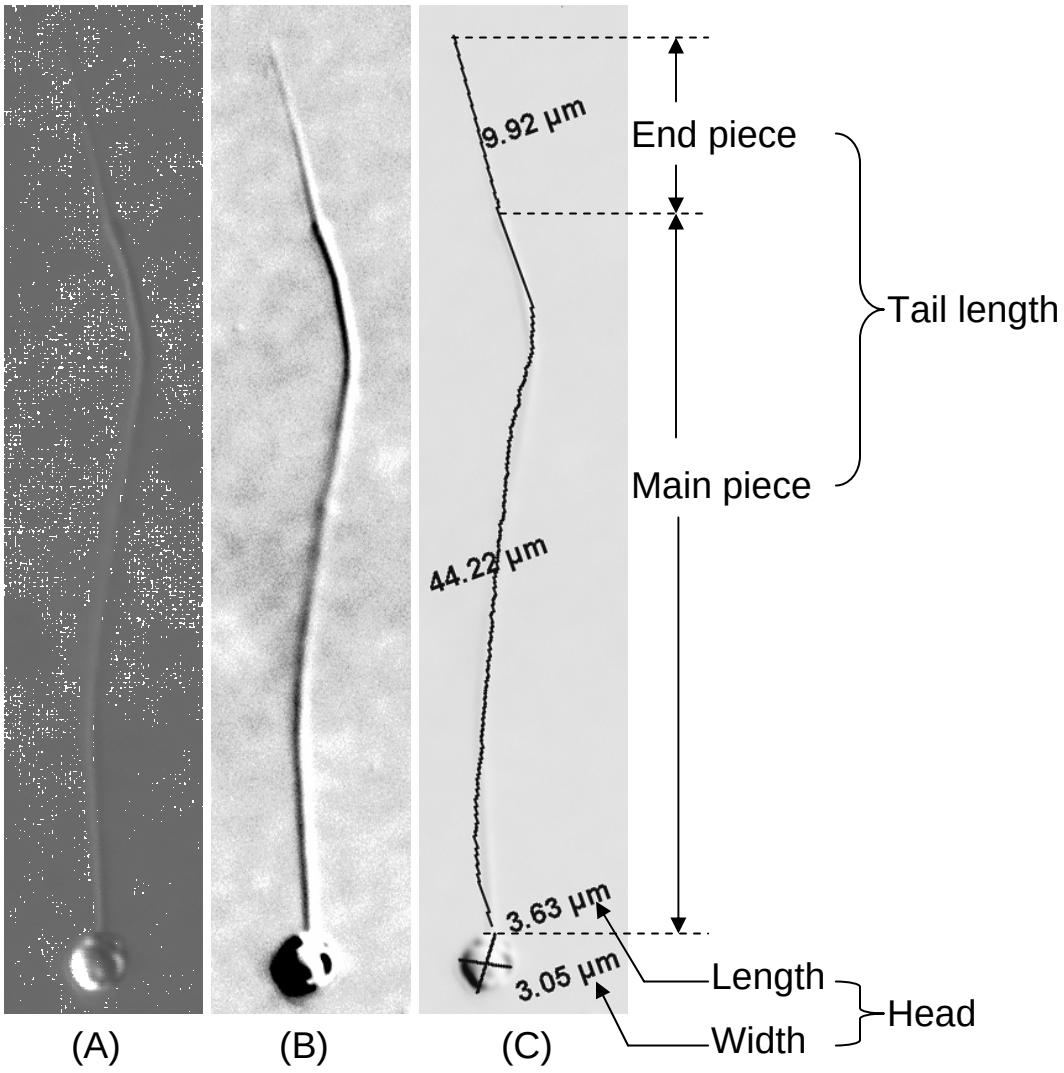

Figure 3.2 Examples of image processing and measurements made for spermatozoal components with samples after fixation (prior to dehydration) at 800-× magnification with light microscopy. (A): image after enhancement of brightness and contrast to reveal the sperm morphology; (B): image after "invert", "equalize", and "brightness/contrast" adjustments to show details of the end piece; (C): image showing the measurement made with cursor on computer screen.

method for unequal cell sizes was used to test for differences ($\alpha = 0.05$) among results for the various parameter estimates. Chi-squared test statistics were applied to test whether the number of mitochondria was independent of sample preparation methods for SEM and TEM, and ploidy levels. Due to the presence of cell counts of zero (e.g., for sperm from diploid oysters, cell counts for mitochondrial numbers of 5 and 6 were zero), data were transformed by the addition of a constant value of 0.5 prior to the chi-square test (Agresti, 1996).

Results

Sperm Architecture

General ultrastructural architecture of spermatozoa produced by tetraploids (Figures 3.3A, 3.4A) was similar to that of spermatozoa produced by diploids (Figures 3.3C, 3.4B, 3.4C) except for differences in size. Spermatozoa from diploids and tetraploids possessed a cap-shaped acrosome filled with fine granular substances with the axial body extended into the nucleus. The nucleus was more electron dense than the acrosome in spermatozoa from both ploidies, and the typical 9 + 2 microtubule structure of the flagellum observed in diploids (Bozzo et al., 1993) was also found in tetraploids (picture is not shown).

Sizes of Sperm Components

The sizes (length and width) of sperm components were significantly larger in spermatozoa from tetraploids than in those from diploids (Table 3.1). The height (0.76 ± 0.10 µm) and width (1.38 ± 0.10 µm) of the acrosome of sperm from tetraploids were significantly ($P < 0.001$) larger than the height (0.69 ± 0.08 µm) and width (1.22 ± 0.10 µm) of acrosomes in diploids. However, the ratio of acrosome height to width in spermatozoa was not significantly different ($P = 0.606$) between ploidies. Similarly, the lengths and widths of the sperm head in

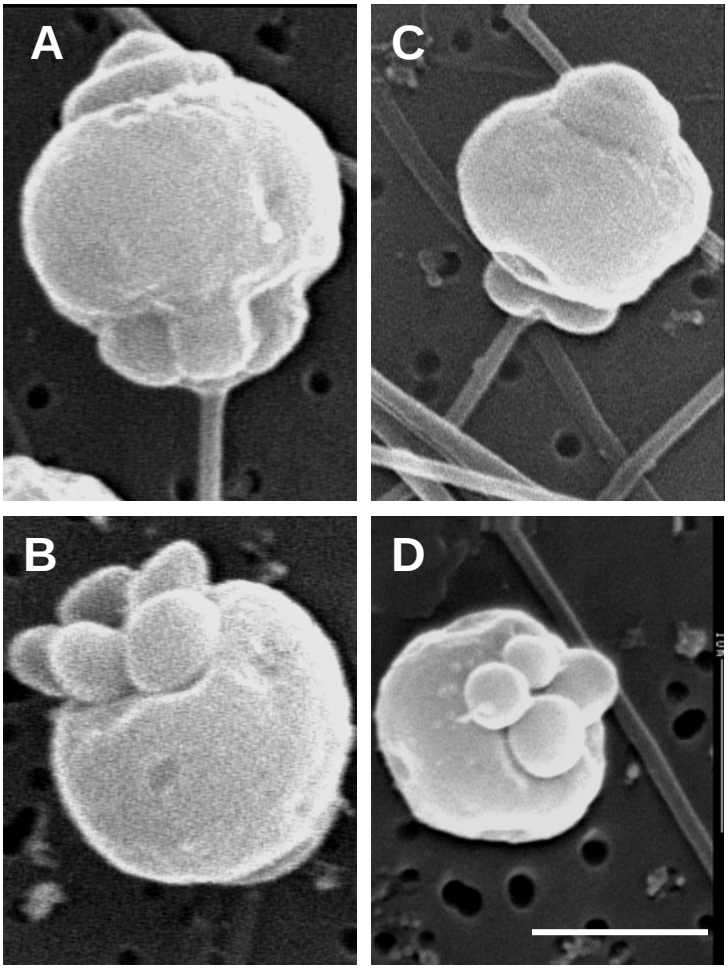

Figure 3.3 Scanning electron micrographs of spermatozoa from tetraploid (A, B) and diploid (C, D) Pacific oysters, *Crassostrea gigas*. A: sperm head from tetraploid. B: five mitochondria exposed after membrane disruption under hypertonic condition in tetraploids. C: sperm head from diploid. D: four mitochondria exposed after membrane disruption under hypertonic condition in diploids. Bar equals 1 μm.

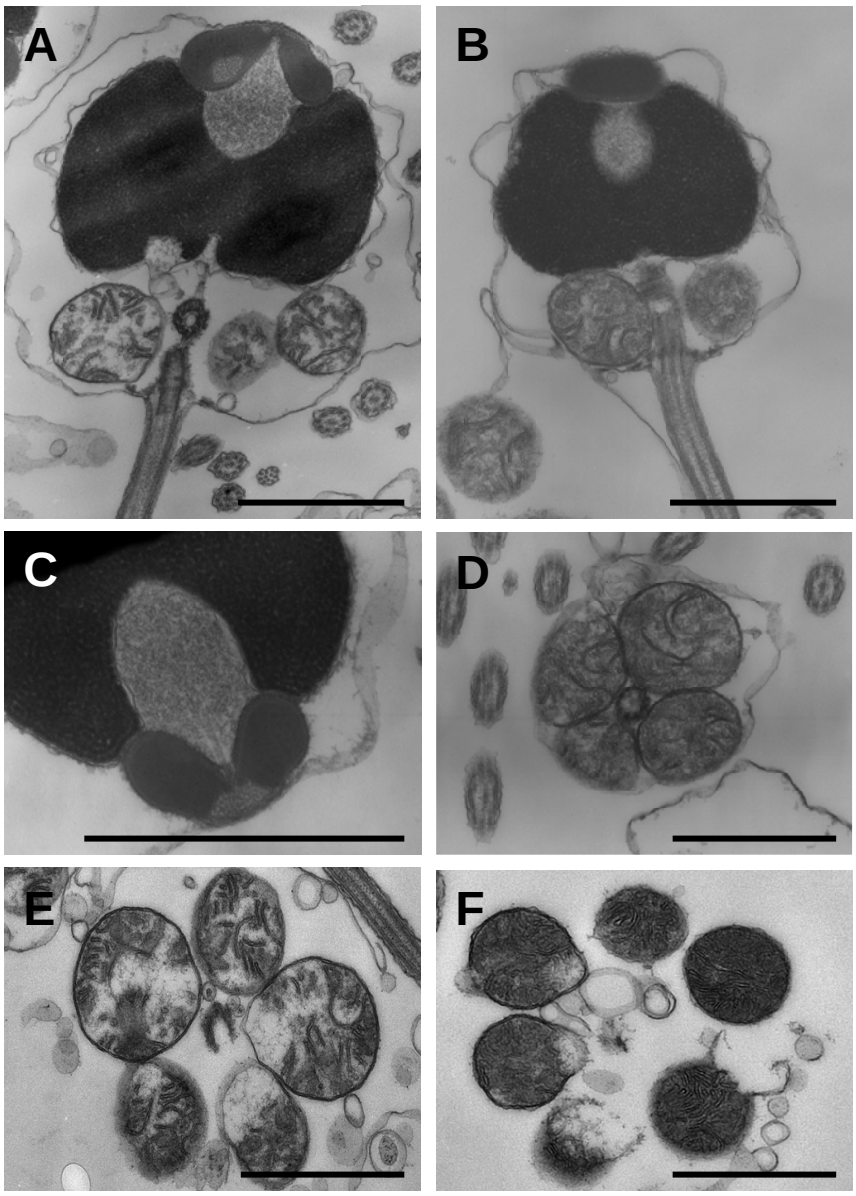

Figure 3.4 Transmission electron micrographs of spermatozoa from tetraploid (A, E, F) and diploid (B-D) Pacific oysters, *Crassostrea gigas*. A: longitudinal section through spermatozoa from tetraploid. B: longitudinal section through spermatozoa from diploid. C: longitudinal section through acrosome showing axial body and axial rod from diploid. D: transverse section through midpiece showing four mitochondria from diploid. E, F: transverse section through midpiece showing five (E) and six (F) mitochondria from tetraploid. Bar equals 1 μm.

Table 3.1 Size (μm) and ratio of spermatozoal components (mean ± SD) of diploid and tetraploid Pacific oysters *Crossastrea gigas*

		Diploid		Tetraploid	
Component	Parameter	Fixed (n = 40)	Live (n = 30)	Fixed (n = 40)	Live (n = 90)
Acrosome	Height	--	0.69 ± 0.08^{a}	--	0.76 ± 0.10^{b}
	Width	--	1.22 ± 0.10^{a}	--	1.38 ± 0.10^{b}
	Height/Width	--	0.56 ± 0.07^{a}	--	0.55 ± 0.09^{a}
Head	Length	2.61 ± 0.12^{a}	2.40 ± 0.14^{b}	3.42 ± 0.17^{c}	3.19 ± 0.22^{d}
	Width	2.32 ± 0.07^{a}	2.26 ± 0.16^{a}	3.04 ± 0.19^{b}	2.79 ± 0.21^{c}
	Length/Width	1.12 ± 0.06^{a}	1.06 ± 0.07^{b}	1.13 ± 0.06^{a}	1.14 ± 0.08^{a}
Mitochondria	Height	--	0.72 ± 0.08^{a}	--	0.82 ± 0.08^{b}
Flagellum	Main piece	33.2 ± 1.1^{a}	--	43.4 ± 1.6^{b}	--
	End piece	7.9 ± 0.7^{a}	--	9.7 ± 2.0^{b}	--
	Total length	41.1 ± 1.3^{a}	37.3 ± 1.9^{b}	53.1 ± 2.9^{c}	50.4 ± 2.0^{d}
	Main/End piece	4.23 ± 0.46^{a}	--	4.70 ± 1.18^{b}	--
Acrosome height/Head length		--	0.29 ± 0.04^{a}	--	0.31 ± 0.05^{b}
Acrosome width/Head width		--	0.54 ± 0.06^{a}	--	0.50 ± 0.04^{b}
Head length/Flagellum length		0.064 ± 0.003^{a}	0.064 ± 0.005^{a}	0.057 ± 0.005^{b}	0.063 ± 0.005^{a}

Numbers within the parenthesis indicates the sample size (n).
Numbers in rows sharing the same superscript were not significantly different at $P = 0.05$

tetraploids were significantly larger than those in diploids ($P < 0.001$). The dimensions of the sperm head changed with fixation method, except for width in diploids, with larger values observed with fixed samples (Table 3.1). The ratios of sperm head length to width from fixed and live samples of both ploidies were not significantly different ($P > 0.150$) except for spermatozoa from live samples in diploids ($P < 0.001$).

The height of mitochondria (Figure 3.1) was significantly larger ($P < 0.001$) for spermatozoa from tetraploids (0.82 ± 0.08 µm) than from diploids (0.72 ± 0.08 µm) (Table 3.1). For the flagellum, the length of the main piece and end piece (Figure 3.2) were significantly larger ($P < 0.001$) for spermatozoa from tetraploids than from diploids. Significant differences were observed for the total tail length in fixed and live samples from both ploidies ($P < 0.001$). The ratio of the length of the flagellum main piece to the end piece was similar in spermatozoa from tetraploids (4.70 ± 1.18) and diploids (4.23 ± 0.46) though they were significantly different ($P = 0.020$). The ratio of acrosome height to head length was significantly ($P = 0.010$) larger in spermatozoa from tetraploids than from diploids, while the ratio of acrosome width to head width was significantly ($P < 0.001$) larger in spermatozoa from diploids than from tetraploids. However, ratios of sperm head length to total flagellum length in fixed and live samples from both ploidies were all close to 0.06 and were not significantly different ($P > 0.276$) except for the fixed samples from tetraploids ($P < 0.001$).

Number of Mitochondria

The number of mitochondria per spermatozoa produced by tetraploids ranged from four to six (Table 3.2; Figures 3.3B, 3.4E, 3.4F) with a modal number of five, while the number of mitochondria in diploids was always four (Table 3.2; Figures 3.3D, 3.4D). Chi-squared test statistics for the counts of mitochondrial number in spermatozoa from diploids

Table 3.2 Frequency distribution (percent) of the number of mitochondria in spermatozoa observed in diploid and tetraploid Pacific oysters (one male for SEM, and two males for TEM for each ploidy with 100-102 sperm counted for each male)

		Number of mitochondria		
Ploidy[1]	Preparation[2]	4	5	6
Diploid	SEM (n = 100)	100	0	0
	TEM (n = 200)	100	0	0
	Average	100 ± 0	0 ± 0	0 ± 0
Tetraploid	SEM (n = 102)	47	51	2
	TEM (n = 202)	42	54	4
	Average	44 ± 3	53 ± 2	3 ± 1

[1]Chi-squared test for ploidy level: $\chi^2 = 242.950$; $P < 0.001$

[2]Chi-squared test for preparation method in diploids ($\chi^2 = 0.246$; $P = 0.884$) and tetraploids ($\chi^2 = 1.087$; $P = 0.581$).

($\chi^2 = 0.246$; $P = 0.884$) and tetraploids ($\chi^2 = 1.087$; $P = 0.581$) indicated that the number of mitochondria was independent of the SEM and TEM preparation methods, which means the frequency distribution of the number of mitochondria observed from SEM samples was not different from the two TEM samples for each ploidy. Therefore, the depressions (dented appearance) sometimes observed on the SEM micrographs (Figures 3.3B, 3.3D) suggested other effects, perhaps from hypertonic fixative treatment, rather than missing mitochondria. Subsequent combination of the data into a single table indicated that the mitochondrial number was strongly related to ploidy levels ($\chi^2 = 242.950$; $P < 0.001$).

Discussion

Generally, the architecture of sperm produced by tetraploid Pacific oysters was larger but similar to sperm from diploids with respect to the presence of a broad, cup-shaped acrosome, subacrosomal material including an axial rod, a relatively spherical nucleus, and a typical 9 + 2 microtubule structure. These characteristics of sperm from diploids and tetraploids were also consistent with previous ultrastructural studies of sperm from diploids within the family Ostreidae of *C. virginica* (Galtsoff and Philpott, 1960), *C. angulata* (Sousa and Oliveira, 1994), *C. gigas* (Bozzo et al., 1993), and *Saccostrea commercialis* (Healy and Lester, 1991).

The primary differences in spermatozoal ultrastructure between tetraploids and diploids were size related. The linear dimensions (e.g., length and width of acrosome and sperm head, and flagellum length) of the spermatozoa components ranged from 1.10 to 1.35 (approximately 1.25 on average) times that of sperm from diploids. Similar findings were observed for sperm from diploid and tetraploid rainbow trout *Oncorhynchus mykiss*, in which the average width of sperm from tetraploids was 1.30 (and length was 1.20) times that of sperm from diploids (Chourrout et al., 1986). These values correspond to a doubled sperm volume with the assumption of a

spherical configuration of the sperm head, and a theoretical value of $\sqrt[3]{2}$ (~1.26 times) for the increase in radius given a double-fold increase of volume ($V = 3/4\pi R^3$, where V is the volume and R is the radius). Earlier studies have indicated that tetraploid males of *C. gigas* produced 2.7 ± 0.5 × 10^{10} sperm g^{-1} of gonad wet weight, which was half the number produced by diploid males (Dong et al., 2005b). The fewer number of sperm per gram of gonad wet weight in tetraploids is also explainable by the doubled sperm volume, which is similar to the findings with egg production from tetraploid and diploid females in *C. gigas* (Guo and Allen, 1997).

The length and width of the sperm heads from diploids in the present study were in good agreement with those reported for diploid Pacific oysters in Japan (Komaru et al., 1994). However, a difference was observed for the length of the flagellum: 34.2 ± 1.2 μm (Komaru et al., 1994) compared to 41.1 ± 1.3 μm in the present study. It is possible that measurement in the previous study only included the main piece of the flagellum (excluding the poorly visible end piece) because the flagellum length in the previous study was close to the length of main piece (33.2 ± 1.1 μm) in the present study. It is also possible that the flagellum was defined only as the main piece (the definition of this study) as controversies on nomenclature existed in early studies (Franzén, 1956). Unfortunately, no nomenclature was provided for clarification in the previous study. The end piece of the flagellum was difficult to visualize under light microscopy without image processing, especially with unfixed samples. However, there is also a possible geographical or genetic difference between the populations studied as the observed acrosome height was also smaller (0.51 ± 0.03 μm) in the previous study, compared to that of the present study (0.69 ± 0.08 μm). Another study from Spain, however, reported a smaller head size (2 μm diameter) and longer flagellum length (~48 μm) for mature spermatozoon from *C. gigas* (Bozzo et al., 1993) compared to either the previous or present study. Despite the larger values observed

with spermatozoa from tetraploid Pacific oysters compared with the diploids in the present study, the ratios of the different sperm components were not different, such as for the ratios of head length to width, and head length to flagellum length.

In addition to the differences in linear dimensions of spermatozoa, the other major difference was of number of mitochondria. Variation from the conventional four mitochondria in oyster sperm was documented in *C. virginica* "on rare occasions" (Eckelbarger and Davis, 1996). This study found no evidence of deviation in sperm from diploids; however, in tetraploids more than half of the spermatozoa had 5 or 6 mitochondria instead of the 4 observed for spermatozoa from diploid Pacific oysters and other oysters (Galtsoff and Philpott, 1960; Healy and Lester, 1991; Sousa and Oliveira, 1994). An ultrastructural study of spermatozoa from diploid and tetraploid Mediterranean mussels (*Mytilus galloprovincialis* Lamarck) revealed similar findings for the number of mitochondria, in which 5 to 7 mitochondria were observed in tetraploids compared to only 5 in diploids (Komaru et al., 1995). The increased number of mitochondria in the spermatozoa of the tetraploid mussels was suggested to compensate for the size increase of the sperm head with resultant motility similar to sperm from diploids because the majority (75%) of spermatozoa has six or seven mitochondria. Previous study of spermatozoa from triploid Pacific oysters, however, did not show an increase in the number of mitochondria, and it was suggested that motility of spermatozoa produced by triploid Pacific oysters could be reduced because the head was significantly larger (compared to spermatozoa from diploids) and the number of mitochondria was the same (Komaru et al., 1994). If the number of mitochondria does play a role in sperm motility, the increased number of mitochondria in only roughly half (56%) of the spermatozoa from tetraploid Pacific oysters may explain their consistently relatively lower motility (45 ± 18%) compared to the sperm from diploids (57 ± 27%) (Dong et al., 2005a;

Chapters 5 and 6). In other words, spermatozoa with four mitochondria might be less motile than those with five or six mitochondria. Increases in the number of mitochondria may be regulated by increased nuclear size, but why the increased nuclear size of triploid Pacific oysters was not associated with an increased number of mitochondria such as in tetraploids merits further study.

In this study, although the height of mitochondria was estimated by use of light microscopy, mitochondria were sometimes observed of different sizes in the SEM micrographs (e.g. Figure 3.3D) although measurement of the sizes was not attempted here for SEM samples. Future efforts may be required to compare the sizes (e.g. diameter or volume) of individual mitochondria prepared with SEM among spermatozoa with 4, 5 or 6 mitochondria within tetraploid oysters, and between tetraploid and diploid oysters especially if a comparison of total volume or metabolic capacity could be made. It would also be informative to evaluate differences among spermatozoa having four mitochondria and those with five or six in tetraploid oysters, for example, in motility speed and duration, and their susceptibility to cryopreservation damage. Flow cytometric assays have been used to estimate the mitochondrial function of sperm samples prior to freezing and after thawing (e.g., Graham et al., 1990; Sutovsky et al., 1996; Segovia et al., 2000). If mitochondrial size was uniform and their differences in numbers would reflect differences in fluorescence intensity, it might be possible to sort spermatozoa with five or six mitochondria from those with four prior to freezing. Therefore, subsequent freezing and thawing could evaluate differential susceptibility to cryopreservation. An alternative approach would be to examine mitochondrial number distribution in spermatozoa that survive cryopreservation compared to the distributions prior to freezing.

With respect to cryobiological theory, the larger linear dimensions of the sperm found in tetraploid Pacific oysters yielded a smaller ratio of surface area to volume compared with sperm from diploids. Therefore, for a specific time period, water or permeating cryoprotectants will move across the cell membrane more slowly in sperm from tetraploids than from diploids. This is associated with differences in membrane permeability parameters between these two types of sperm cells when studied with differential scanning calorimetry (He et al., 2004), a physical method of estimating water movement based on thermal properties. Although this same study suggested similar optimal cooling rates for sperm from diploid (~44°C/min) and tetraploid (~43°C/min) oysters, the previous empirical trials have shown that sperm from tetraploids are more susceptible to cryopreservation at these cooling rates than are those from diploids (Dong et al., 2005a). It is possible that variables other than surface area-to-volume ratio may play important roles in sperm cryopreservation, for example, membrane fluidity. A recent study of human spermatozoa suggested that sperm adaptability to the stresses induced by freezing and thawing could be dependent on initial membrane fluidity (Giraud et al., 2000). It is also possible that spermatozoa from tetraploids are more sensitive to osmotic shock than those from diploids. This would be especially important if plasma membrane thickness or composition was not different between the ploidies, although cell size was, yielding effectively a compromised membrane in tetraploids. Based on these and other questions, diploid and tetraploid oysters provide a useful model for study of a variety of factors, and future research can seek improved experimental control of variables to examine the different susceptibility to cryopreservation between sperm from diploid and tetraploid Pacific oysters.

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Chapter 4
Standardization of Photometric Measurement of Sperm Concentration from Diploid and Tetraploid Pacific Oysters[*]

Presently, the cryopreservation of sperm from diploid oysters is limited to research reports (e.g. Hughes, 1973; Usuki et al., 1997; Paniagua-Chavez and Tiersch, 2001), and cryopreservation of sperm from tetraploid oysters has just began (Dong et al., 2005). Moreover, most previous reports (Lannan, 1971; Zell et al., 1979; Kurokura et al., 1990; Yankson and Moyse, 1991) on diploid oysters failed to identify sperm concentrations, instead reporting sperm volumes, thereby hindering or preventing reproducibility of experimental conditions. Accurate estimates of sperm concentrations are necessary for the development of standardized techniques for the cryopreservation of sperm of oysters and other aquatic species for several reasons. First, dilution of sperm suspensions into specific concentration ranges required for cryopreservation is critical (Paniagua-Chavez and Tiersch, 2001). Second, the accurate estimation of concentration would facilitate cell toxicity studies of cryoprotectants prior to freezing, and fertilization studies of sperm after thawing. Third, accurate estimation of sperm concentration would enable standardization of the "freezing unit" (number of sperm per freezing container) for future commercial-scale production. Finally, accurate estimations would improve the utility of cryopreservation through establishment of optimal sperm-to-egg ratios for fertilization (Zell et al., 1979).

Several methods exist for determining sperm concentration. Counts by hemacytometer can give unbiased estimations if done properly, but they are tedious and time consuming (Salisbury et al., 1943), these being attributes not suitable for cryopreservation, which often requires rapid handling between sperm collection and freezing. Automated cell counters, such as Coulter

[*]The contents of this chapter were published prior to the completion of this dissertation (Aquaculture Research 2005, 36:86-93).

counters and flow cytometers, offer high precision, but can be inaccurate (Foote, 1968; Foote et al., 1978). Furthermore, they are not routinely available because of the high cost associated with their purchase and maintenance, and the need for trained technicians to operate them. Spectrophotometry, however, is a reliable, rapid, simple, and inexpensive method to estimate sperm concentration given that an accurate initial calibration has been established. This method has been used to estimate semen concentration for use in artificial spawning of fish (Suquet et al., 1992; Ciereszko and Dabrowski, 1993), and has been widely used for sperm cryopreservation in the dairy industry (Foote et al., 1978).

Applying this technique to oyster sperm would help to standardize freezing protocols, especially for commercial-scale cryopreservation of sperm from tetraploid Pacific oysters. Tetraploid oysters were developed to efficiently produce triploid offspring by crossing their gametes with normal diploids (Guo and Allen, 1994; Guo et al., 1996; Wang et al., 1999; Eudeline et al., 2000a, b). Induced triploid oysters are useful in aquaculture because they are essentially reproductive sterile and the reduced investment in gamete output improves meat quality and growth (Allen and Downing, 1991; Wang et al., 2002). Refrigerated and frozen storage of tetraploid oyster sperm at a commercial scale would effectively expand the market of triploid seed worldwide (see Chapter 1 for details).

The goal of this study was to develop a standardized photometric measurement of sperm concentration from diploid and tetraploid Pacific oysters. The specific objectives of this study were to: 1) identify appropriate wavelengths for the spectrophotometric estimation of sperm concentrations; 2) develop standard curves for these spectrophotometric estimations of sperm concentrations; 3) validate the adequacy of the regression models in sperm concentration

estimations, and 4) investigate the applicability of the standard curves for use in cryopreservation practices.

Materials and Methods

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 from Willapa Bay (46° 29' 885" N, 124° 01' 810" W) in Washington. Oysters were shipped chilled by overnight delivery to the Louisiana State University Agricultural Center, Aquaculture Research Station (ARS) from June to August 2002. Sperm samples were collected by dry stripping of the gonad (Allen and Bushek, 1992) and were weighed to the nearest 0.01g. Calcium-free Hanks' balanced salt solution (C-F HBSS) (Paniagua-Chavez et al., 1998) was used as an extender at a concentration of 1000 mOsmol/kg based on previous work (Dong et al., 2002). All chemicals used for solutions were reagent grade (Sigma Chemical Corporation, St. Louis, Missouri). A dilution of 1:20 (weight:volume; g:ml) of sperm to extender was used for initial suspension. Sperm suspensions were filtered through a 40-µm cell strainer (BD Biosciences Discovery Labware, Bedford, Massachusetts). A second dilution of 10:1000 (v/v) of sperm:extender was used for hemacytometer (Hausser Scientific, Horsham, Pennsylvania) counts. To facilitate counting, sperm were immobilized by using extender at a low osmolality of 200 mOsmol/kg. The spectrophotometer used was a single-beam instrument (Genesys^{TM} 20, Thermo Spectronic, Rochester, New York). Calcium-free HBSS was used as the blank to obtain the readings of absorbance.

Oysters were received on June 11, July 16, and July 30 2002 for calibration of different sperm suspension. Four wavelengths within the visible range (380, 550, 581 and 780 nm) were chosen for comparisons. Concentrations of sperm suspensions of diploid oysters were estimated

by hemacytometer counts, and serial dilutions were prepared by diluting the next higher concentration solution (e.g., a concentration of 4×10^8 cells mL^{-1} was prepared by diluting sperm solutions at 6×10^8 cells mL^{-1}). The serial dilutions included 2×10^9, 1×10^9, 8×10^8, 6×10^8, 4×10^8, 2×10^8, 1×10^8, 8×10^7, 6×10^7, 4×10^7, 2×10^7, 1×10^7, 8×10^6, 6×10^6, 4×10^6, 2×10^6, 1×10^6, 1×10^5 cells mL^{-1}. Four readings from four wavelengths (one reading for each wavelength) were obtained for the same sample (oyster) at each concentration, and were replicated eight times with sperm from eight oysters, therefore eight readings were generated for any single dilution concentration.

For sperm suspensions from tetraploid oysters, the same serial dilutions were used except for 2×10^9 cells mL^{-1}, because the tetraploid oysters provided fewer sperm than did diploids. Readings were also obtained at the aforementioned wavelengths for each dilution, and seven replicates, rather than eight, were used for readings at 380, 550 and 780 nm. For testing of the standard curves at 581 nm, oysters were received on June 4, June 11, July 3, July 10, July 16, July 23, July 30, August 13, and August 20 2002. A total of 137 sperm dilutions from 22 diploid oysters, and 127 sperm dilutions from 33 tetraploid oysters were used for hemacytometer counts and for spectrophotometric readings.

Basic parameters such as body wet weight, shell height, and gonad wet weight were recorded (Table 4.1). For diploids, only oysters with ripe gonads were used for experiments, which were determined by the presence of prominent genital canal (Supan and Wilson, 2001). All tetraploids were used except for those with little or no gonadal tissue, or spent gonads.

To determine if there was variation in gonadal development during the normal spawning season (June to August 2002), the sperm concentration per gram of gonad wet weight (sperm g^{-1} gonad) was approximated by assuming a sperm density of 1.0 g mL^{-1}. Net gonad wet weight

was approximated by subtraction of the mass of the digestive gland from the gross wet weight of the gonad after dissection from other tissues (SOP-3, Appendix A). Thus, after obtaining the net wet weight (W) of gonad tissue and suspension with extender (V) at 1:20 (w/v) of sperm to extender, and estimating the sperm concentration (C) in the solution by hemacytometer, the sperm g^{-1} gonad were obtained by use of the following formula:

$$Sperm / g\ gonad = \frac{C \times (V + W / D)}{W}$$

C: sperm concentration, cell mL^{-1}

V: extender volume, mL

W: gonad wet weight, g

D: sperm density, 1.0 g mL^{-1}

The values obtained from the spectrophotometer were single readings of absorbance, and for the hemacytometer were the average of duplicate counts. Correlation and simple linear regression (SLR) were used for data analysis (SPSS 10.0 for windows, 1999, SPSS Inc. Chicago, Illinois). Data for sperm concentrations were logarithmic (common logarithm) transformed prior to SLR analysis. The average of the absorbance readings was used to construct the standard curves. Two-way ANOVA was used to test the difference of the number of sperm per gram of gonad between diploid and tetraploids, and for differences among different shipments.

Results

The standard curves showed a curvilinear relationship within the tested concentration range for readings obtained at all four wavelengths for sperm from diploid oysters (Figure 4.1). A linear relationship was observed between the log of sperm counts and absorbance when sperm concentrations were between 2 x $10^7$and 2 x 10^9 cells mL^{-1} (4 x 10^6 to 2 x 10^9 cells mL^{-1} for

Table 4.1 Basic parameters (Mean ± SD) of diploid (2C) and tetraploid (4C) Pacific oysters received and used for experiments during June and August 2002

	Sample size		**Body wet weight (g)**		**Shell height (mm)***		**Gonad net wet weight (g)****	
Ploidy	received	used	received	used	received	used	received	used
2C	34	22	52.4 ± 21.5	58.0 ± 23.6	93.1 ± 14.8	95.6 ± 15.0	3.62 ± 2.03	4.10 ± 2.23
4C	38	25	138.6 ± 107.0	100.1 ± 92.5	105.6 ± 21.9	98.2 ± 19.0	5.31 ± 3.96	4.15 ± 3.60

*Shell height: distance between umbo and posterior margin of the valves (Carriker, 1996)
**Net gonad wet weight was approximated by subtraction of the mass of digestive gland from the gross wet weight of the gonad after dissection from other tissues (SOP-3, Appendix A).

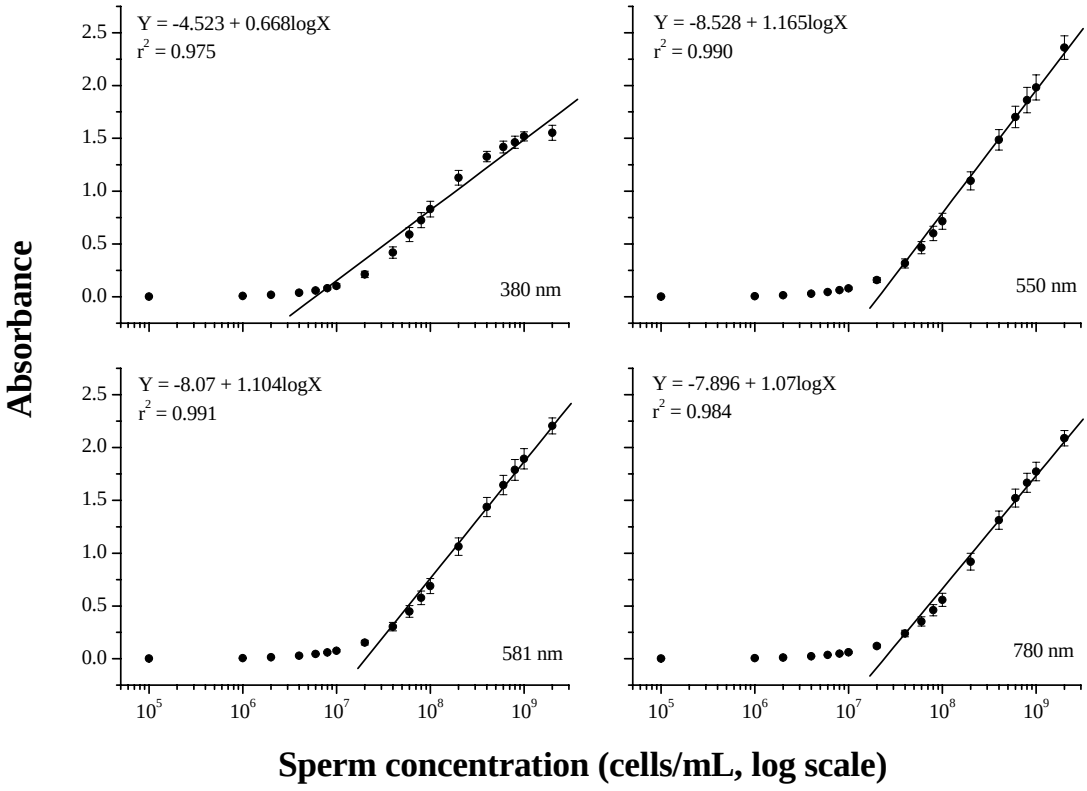

Figure 4.1 Standard curves for sperm suspensions from diploid Pacific oysters. Each point represents the mean of eight replicates of sperm from eight diploid oysters. Error bars indicate standard deviations. Linear regressions were constructed for concentrations between 4 x 10^6 and 2 x 10^9 cells mL^{-1} for readings at 380 nm, and between 2 x $10^7$and 2 x 10^9 cells mL^{-1} for readings at 550, 581 and 780 nm.

readings at 380 nm), and the regression equations for the standard curve were calculated (Figure 4.1). Concentrations below 2 x 10^7 cells mL^{-1} were not reliably measured by spectrophotometry, and concentrations above 2 x 10^9 cells mL^{-1} were at the upper limit of the photometric range of the instrument. The standard curve at 550 nm had the same coefficient of determination (r^2 = 0.988) as that of 581 nm (r^2 = 0.988). Sperm concentrations between 4 x 10^6 and 1 x 10^9 cells mL^{-1} were used to generate the standard curve at 380 nm, which showed a lower coefficient of determination (r^2 = 0.975) than did analysis at 550, 581 and 780 nm.

Similar findings were observed for the standard curves for sperm from tetraploid oysters (Figure 4.2). Linear regressions were constructed between sperm concentrations of 2 x 10^7 and 1 x 10^9 cells mL^{-1} at wavelengths of 550, 581 and 780 nm. The standard curve at 550 nm had a coefficient of determination (r^2 = 0.997) similar to that of 580 nm (r^2 = 0.998). The standard curve at 380 nm also could be applied to a wider range (4 x 10^6 to 1 x 10^9cells mL^{-1}), but had a lower coefficient of determination (r^2 = 0.981).

Plotting the observed values against the standard curves generated from readings at 581 nm indicated good agreement for concentration estimates of sperm suspensions from diploid and tetraploid Pacific oysters (Figure 4.3). Models constructed with the observed values showed coefficients of determination similar to those of standard curves for sperm from diploid (r^2 = 0.983) and tetraploid (r^2 = 0.980) Pacific oysters.

Within the concentration range of 1 x 10^5 to 2 x 10^9 cells mL^{-1}, higher correlations were found at higher wavelengths between the hemacytometer counts and absorbance readings for sperm suspensions from diploid and tetraploid oysters (Table 4.2). High correlations were also observed within the four wavelengths, especially those between 550 nm and 780 nm (correlation coefficient, r = 0.999), 581 nm and 780 nm (r = 0.999), 550 nm and 581 nm (r = 1.000).

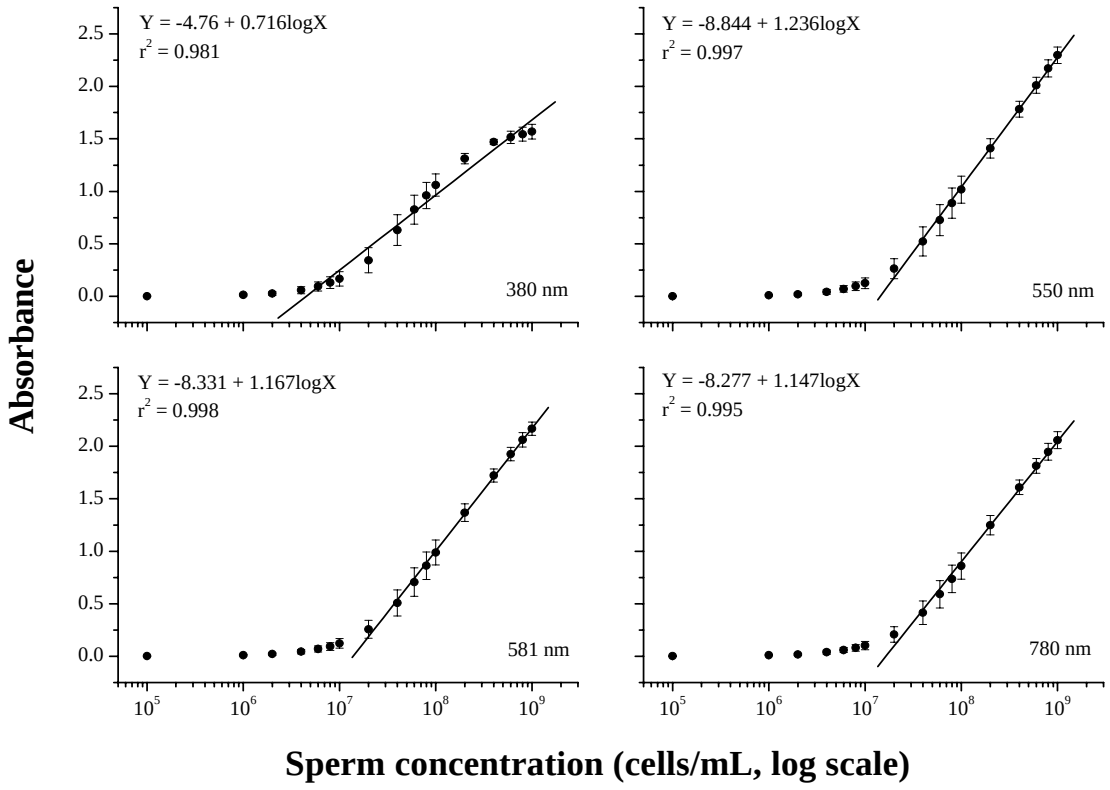

Figure 4.2 Standard curves for sperm suspension from tetraploid Pacific oysters. Each point represents the mean of seven replicates (eight replicates at 581 nm) of sperm from seven (or eight) tetraploid oysters. Error bars indicate standard deviations. Linear regressions were constructed for concentrations between 4 x 10^6 and 1 x 10^9 cells mL^{-1} for readings at 380 nm, and between 2 x $10^7$and 1 x 10^9 cells mL^{-1} for readings at 550, 581 and 780 nm.

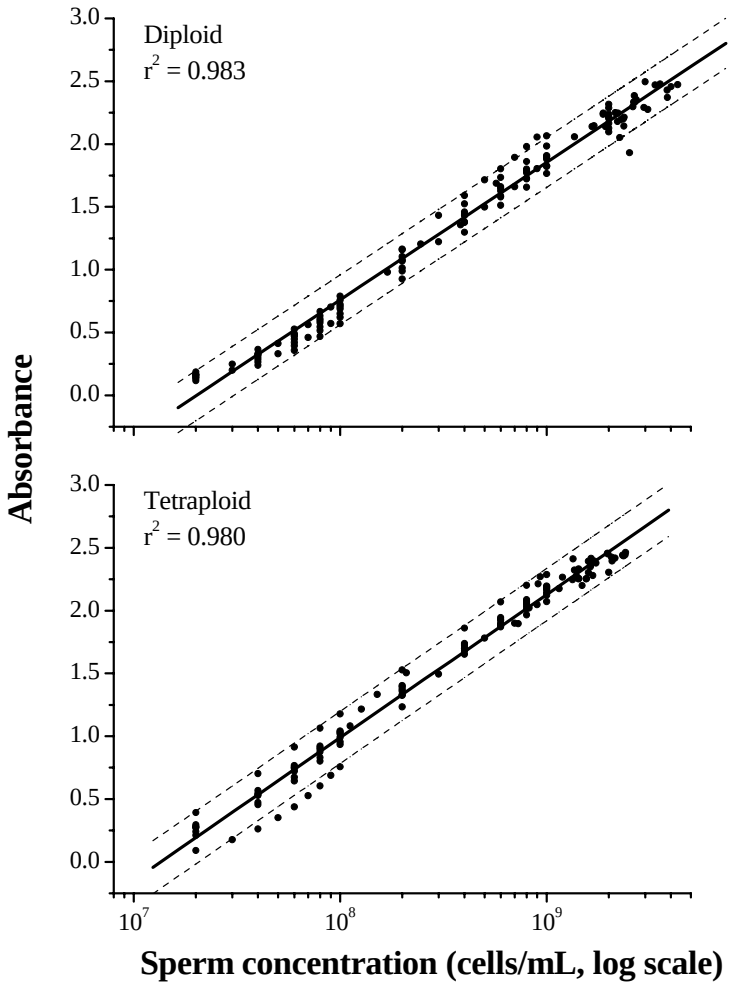

Figure 4.3 Plot of observed values against the standard curves generated using spectrophotometry at 581 nm for sperm from diploid and tetraploid Pacific oysters. Upper panel: data for 137 sperm dilutions collected from 22 diploid oysters; lower panel: data for 127 sperm dilutions collected from 33 tetraploid oysters. Dashed lines indicate the 95% confidence intervals for predicted individual points.

Table 4.2 Correlations among estimates of sperm cell concentrations from diploid (2C) and tetraploid (4C) Pacific oysters performed by spectrophotometric analysis at four wavelengths or by hemacytometer counts.

	Correlations with other estimates							
	380 nm		**550 nm**		**581 nm**		**780 nm**	
Method (wavelength)	**2C**	**4C**	**2C**	**4C**	**2C**	**4C**	**2C**	**4C**
Hemacytometer	0.869	0.810	0.940	0.914	0.938	0.908	0.950	0.921
Spectrophotometer* (380 nm)	-	-	0.976	0.976	0.979	0.979	0.969	0.970
Spectrophotometer (550 nm)	-	-	-	-	1.000	1.000	0.999	0.999
Spectrophotometer (581 nm)	-	-	-	-	-	-	0.999	0.999

*Spectronic 20 (Genesys^{TM} 20, Thermo Spectronic, Rochester, New York)

Readings at 380 nm were found to be least correlated with the hemacytometer counts and readings at other wavelengths (Table 4.2).

The overall average of sperm per gram of gonad for oysters received during the normal spawning season was $(5.4 \pm 0.9) \times 10^{10}$ for diploids, and $(2.7 \pm 0.5) \times 10^{10}$ for tetraploids. A significant difference of sperm per gram of gonad was observed between the ploidy levels ($P < 0.001$), but there was no significant difference in the values for sperm per gram of gonad among shipments within diploids ($P = 0.270$) or within tetraploids ($P = 0.067$) (Figure 4.4).

Discussion

There is a lack of standardization in protocols and reporting of results for cryopreservation in aquatic species. Researchers new to the field are often confronted with successful protocols that cannot be repeated, unsuccessful experiments that cannot be interpreted and contradictory findings even within a single species (Tiersch, 2000). A lack of standardized sperm concentrations is a major source of this uncertainty within studies. For example, when specific volumes or percentages of cryoprotectant are applied to different sperm concentrations, the observed variation is usually attributed to genetic or environmental differences among males. The use of sperm or gonadal volumes instead of sperm concentrations make experiments difficult to repeat even for the same population. In aquaculture, when there is need to expand sperm cryopreservation from research to commercial production, as in the case of sperm from tetraploid Pacific oysters, standardization of sperm concentration would reduce quality variation and improve consistency after thawing. Such standardized practices have been incorporated for decades with sperm cryopreservation in the dairy industry, for example (Foote et al., 1978). Wavelengths in the range of 375 to 630 nm have been used to determine semen concentrations in the dairy industry (Foote et al., 1978). In the present study, we tested four wavelengths (380,

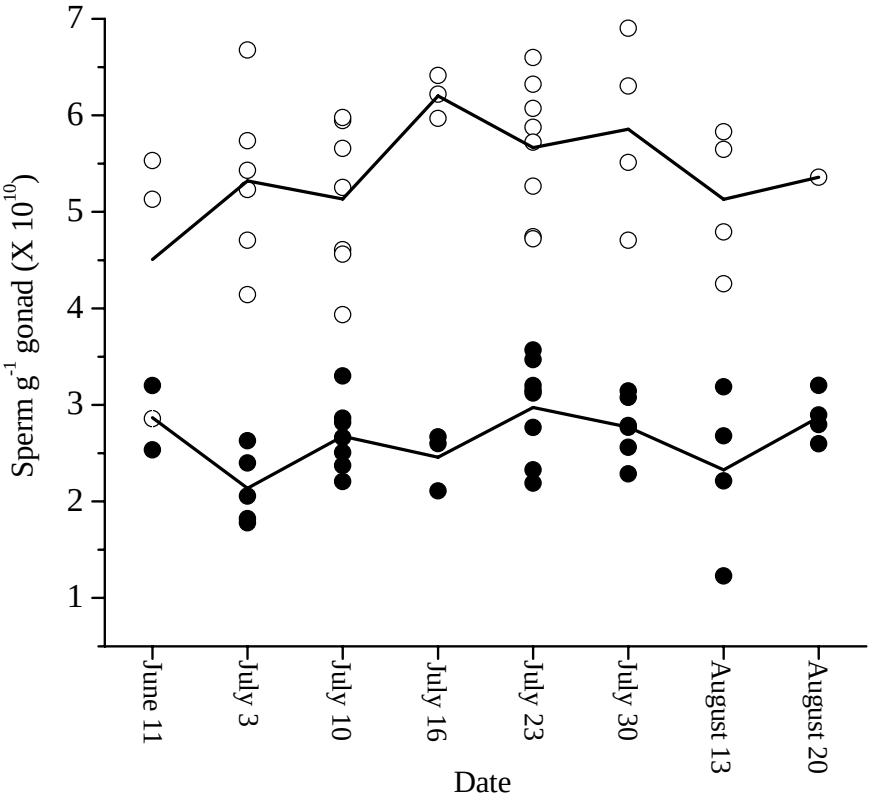

Figure 4.4 Mean sperm concentration per gram of wet gonad weight (sperm g^{-1} gonad) of diploid (n = 36, open circles) and tetraploid (n = 39, filled circles) oysters received during shipments in June through August, 2002. Each circle represents the number of sperm g^{-1} of gonad of a single oyster. The lines represent the average values for each shipment.

550, 581 and 780 nm), representing four different portions of the visible range, and wavelengths of 550 and 581nm appeared to be the most suitable because of the highest coefficients of determination. In addition, for the same series of standard solutions, absorbances of 550 and 581 nm had wider reading ranges than did 380 and 780 nm. Thus, the change in absorbance at 550 and 581 nm for a given concentration change was greater, leading to enhanced sensitivity and accuracy in measurement.

The present study also showed high correlations ($r > 0.969$) among readings at 380, 550, 581 and 780 nm, indicating that each of these wavelengths was appropriate for determining sperm concentrations as long as accurate standard curves were generated. This is explainable because sperm suspensions are mixtures of many components (e.g., carbohydrates, proteins, lipids). Consequently, the observed absorption at each wavelength is the sum of the individual absorption spectra of the components, which makes discrete peak discrimination in the absorption specta difficult. However, differences were evident between the absorbance at 380 nm and the higher wavelengths. Lower correlations were observed between the absorbance values at 380 nm and the hemacytometer counts for sperm from the diploid and tetraploid oysters. Also, for the same series of standards of known sperm concentrations, absorbance values at 380 nm showed the narrowest reading range.

The standard curves were non-linear over the concentration range from 1×10^5 to 2×10^9 cells mL^{-1} at all four wavelengths for sperm from diploid and tetraploid oysters. Although a polynomial regression (a cubic model) could be used to construct a best-fit calibration, a simple linear relationship was observed between the absorbance and log transformation of sperm concentration between 2×10^7 and 2×10^9 cells mL^{-1}. This is simpler and expedient for practical purposes. Most importantly, this was within the useful concentration range for the

cryopreservation of oyster sperm, because motility is lost if concentrations are too dilute (Gray, 1928; Bougrier and Rabenomanana, 1986; Usuki et al., 1997; Paniagua-Chavez et al., 1998; Dong et al., 2005). Of the 26 studies in the literature that directly address cryopreservation of oyster sperm, only five indicate an approximate sperm concentration for freezing, and these concentrations were within the range of 1×10^7 to 1×10^9 cells mL^{-1} (Staeger, 1973; Usuki et al., 1999; Paniagua-Chavez and Tiersch, 2001; Smith et al., 2001; Dong et al., 2005).

Based on the principles of spectrophotometer operation, as long as the size, shape and opacity of the particles are similar from sample to sample, concentration estimation based on standard curves should be accurate given accurate calibration. In general, oyster sperm of the same species with the same ploidy level were quite homogenous. This was especially true for sperm collected during the normal spawning season, which was demonstrated by the constant sperm concentration per gram of gonad wet weight during the present study (Figure 4.4). The good agreement between the predicted and observed values also indicated the general applicability of the standard curves developed at 581 nm for estimation of sperm concentration in Pacific oysters across the spawning season.

Sperm from tetraploid oysters were different from those of diploids in size (Chapter 3), density and motility (Chapters 5 and 6), and thus a separate calibration was necessary for the two ploidy levels. Another important factor that may influence the applicability of the standard curves was the extender solution used to suspend sperm. Sperm from tetraploid Pacific oysters were found to exhibit variable motility when suspended in different extender solutions (Dong et al., 2002), which may affect the absorbance readings because sperm with low motility may tend to precipitate sooner than those with higher motility.

Other factors that can affect the accuracy of spectrophotometry to estimate sperm concentration include dilution ratios (Foote et al., 1978), and the accuracy of sampling and dilutions when making hemacytometer counts for calibration (Ciereszko and Dabrowski, 1993). Care should also be taken when collecting the sperm, because contamination with other substances such as gut contents could affect calibration and concentration estimates. Thorough mixing of sperm suspensions before measurement was also important for accurate readings as precipitation can occur when samples are held prior to measurement. Overall, to use the standard curves effectively and accurately, sperm sample preparation and measurement should always follow the exact procedures used for calibration. In the present study, standard curves generated at wavelengths of 550 and 581 nm within the range of 2×10^7 and 2×10^9 cells mL^{-1} are recommended for determining the concentration of sperm from diploid and tetraploid Pacific oysters. It is important to note that the specific standard curves developed in this study may not be applicable to all populations within the species (such as in oysters grown in different environments), and validation of standard curves should be performed when working with any aquatic species. Thus, the process outlined in this report can serve as a model for establishment of widespread use and reporting of sperm concentration estimates for work in areas such as cryopreservation, genetic manipulation (such as in ultraviolet irradiation of sperm for induction of gynogenesis), and in artificial spawning.

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Chapter 5
Optimization of Sperm Cryopreservation for Diploid Pacific Oysters

Sperm cryopreservation in aquatic species offers many benefits in genetic improvement programs such as hybridization, selective breeding, gynogenesis and androgenesis, development of inbred lines, polyploidization, and domestication. Cryopreservation provides reliable supplies of sperm, without seasonal limitations and costly hatchery maintenance of adult males, and provides a safe repository for improved lines with desirable traits or founder stocks. The first studies of fish sperm cryopreservation were published 50 years ago (Blaxter, 1953), and since then more than 200 fish species have been studied (Rana, 1995; Tiersch, 2000). In contrast to the extensive studies in cryopreservation of fish semen, similar work for invertebrates has been attempted for only about 30 species, and has been limited to echinoderms (sea urchins, sand dollars, and starfishs), mollusks (oysters and abalone), polychaetes, and crustaceans (shrimps and crabs) (for review see Gwo, 2000). Among these, research has been primarily concentrated on spermatozoa from oysters, specifically focusing on the Pacific oyster, *Crassostrea gigas*.

Although these research efforts have yielded protocols that are being applied with varying levels of success in fish and invertebrates, they essentially are limited to laboratory scale, and sperm cryopreservation has not yet found application in aquaculture on a commercial scale (Lang et al., 2003). The benefits of sperm cryopreservation can be fully realized only if this technology is commercialized. In the case of oysters, tetraploid broodstocks have been developed to facilitate the commercial production of triploid seedstock; thus, commercialization of cryopreserved sperm from tetraploid oysters would be especially beneficial for this industry (Dong et al., 2005a). Currently, human and livestock are the only worldwide industries that have incorporated cryopreservation of semen into commercial artificial insemination practices (Crister, 1998; Curry, 2000; Centola, 2002). If cryopreservation of fish or shellfish sperm is to

be integrated into hatchery operations, the use of specialized cryopreservation centers such as dairy facilities should be considered as a time-saving and cost-effective option (Tiersch et al., 2004). In fact, dairy protocols have been adapted to freeze sperm from blue catfish, *Ictalurus furcatus* (Lang et al., 2003), red snapper *Lutijanus campechanus* (Riley et al., 2004), and diploid and tetraploid Pacific oysters (Dong et al., 2005a). In order to achieve commercial-scale application of cryopreserved sperm in aquatic species, research studies in commercial settings are necessary. An understudied, but especially important component involved in utilizing a central cryopresevation facility is shipment of broodstock or milt. Unlike other studies in oyster sperm cryopreservation, the present study employed the use of shipped sperm or sperm collected from shipped oysters.

Despite the fact that the Pacific oyster has been the focus of 26 sperm cryopreservation studies, with dimethyl sulfoxide (DMSO) as the primary cryoprotectant in the majority of these, the results have varied widely and there has been inconsistent reporting of cryoprotectant concentrations, equilibration time, cooling rates, and thawing conditions (see review in Chapter 2). A lack of procedural standardization has been considered to be the most important factor responsible for the inconsistencies in results among various studies, not only in sperm cryopreservation of oysters *per se*, but of aquatic species in general (Rana, 1995; Gwo, 2000; Tiersch, 2000). Optimization of protocols without standardization offers little value for the improvement of existing methods and results, especially for the future development of commercial application. Researchers have begun to pay attention to this problem and recent studies address or incorporate standardization in protocol optimization (Lahnsteiner et al. 1995; Dong et al., 2005a, b). The present study continued the efforts to optimize protocols for sperm cryopreservation for the Pacific oyster through use of a systematic approach. Due to the marked

effects of male variation observed in this study, males with broad, intermediate, and narrow tolerances of cryopreservation procedures, in combination with the stress related with shipping, were evaluated and this principle was extended to species variation in general.

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

Materials and Methods

Sperm Collection and Motility Estimation

Diploid Pacific oysters were obtained in June and July, 2004, from Taylor Resources Quilcene Shellfish Hatchery (TRQSH; www.taylorshellfish.com) in Quilcene, Washington (47° 49' 133" N, 122° 49' 523" W), and were shipped chilled at 5-10 °C by overnight delivery to the Louisiana State University Agricultural Center, Aquaculture Research Station (ARS). For Experiments 1 and 2, intact oysters were shipped and sperm were collected by dry stripping of the gonad upon arrival (SOP-3, Appendix A). For other experiments, sperm samples were collected with the same method and placed separately in one 15-mL centrifuge tube per male, and each week 8 to 10 samples (undiluted) were shipped in a foam shipper (SOP-4, Appendix A)

from TRQSH to the ARS. Because this project also included study of sperm from tetraploid oysters (reported in Chapter 6), the ploidy level of individual oysters was verified by flow cytometry (Allen, 1983; Dong et al., 2005a). 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 mOsm/kg (Dong et al., 2002) and suspensions were filtered through a 40-µm cell strainer (BD Biosciences Discovery Labware, Bedford, Massachusetts). The concentrations of sperm suspensions were adjusted to 2 x 10^9 cells/mL using readings at 581 nm from a spectrophotometer (Genesys^{TM} 20, Thermo Spectronic, Rochester, New York) and derived standard curves (Dong et al., 2005b; Chapter 4). A total of 27 males were used in this study, and each oyster or sperm sample was identified by a code (e.g., CG04M01) with the species designation (CG: *Crassostrea gigas*), year (04: 2004), sex (M: male) and the order in which it was processed (01: the first oyster of 2004). These codings were used for database entries, and was part of the information permanently printed on the French straws with a specialized laser printer (Domino Codebox 2, Domino Amjet, Inc. Gurnee, Illinois).

Motility was used as one of the indicators of sperm quality. The sperm were motile after suspension and retained continuous motility after activation for hours to days. Sperm motility was estimated visually at 200-× magnification using darkfield microscopy (Optiphot 2, Nikon Inc., Garden City, New York) and was expressed as the percentage of cells actively moving in a forward direction (SOP-5, Appendix A). Sperm vibrating in place were not considered to be motile. Post-thaw motility was estimated immediately after thawing.

Cryoprotectant

The cryoprotectants tested included permeating and non-permeating compounds. The permeating cryoprotectants were methanol, ethylene glycol, propylene glycol, dimethyl sulfoxide, N, N-dimethyl acetamide, and glycerol. The non-permeating cryoprotectants were polymers of polyethylene glycol at formula weights of 200 or 600. Single or combined cryoprotectants were used (abbreviations are listed in Table 5.1). All solutions were prepared within 2 h of use with C-F HBSS at 1000 mOsm/kg as the diluent and were stored at 4 °C. All chemicals used for preparation of solutions were of reagent grade (Sigma Chemical Corporation, St. Louis, Missouri).

Freezing and Thawing Procedures

For freezing with a controlled-rate freezer (Kryo 10 Series II; Planer Products, Sunbury-on-Thames, UK) (SOP-9, Appendix A), aliquots of sperm suspensions with cryoprotectant (detailed below) were drawn into 0.5-ml or 0.25-ml French straws (IMV International, Minneapolis) manually as described in SOP-8 (Appendix A). Straws were held (equilibrated) for 10 min (except for the experiment addressing equilibration time) at room temperature (23 – 25 °C), for 2 min at 4 ^{o}C in the controlled-rate freezer before initiation of the various cooling rates. For the cooling rates of 0.5, 5, 16, and 30 ^{o}C per min, samples were cooled in two steps, initially to -30 ^{o}C at these rates, followed by cooling at 45 ^{o}C per min from –30 ^{o}C to –80 ^{o}C. For cooling rates of 45 and 50 ^{o}C per min, samples were cooled in a single step from 5 ^{o}C to –80 ^{o}C directly at the specified rate. All straws were held at –80 ^{o}C for 5 min before being plunged into liquid nitrogen in a storage Dewar. After a minimum of 12 h, four straws were thawed for 7 s (for 0.5-ml straw) or 6 s (for 0.25-ml straw) in a 40 ^{o}C water bath (Model 1141, VWR Scientific, Niles, Illinois) to estimate the post-thaw motility.

Table 5.1 Abbreviations for cryoprotectants

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

[1]polyethylene glycol at formula weight of 200.
[2]polyethylene glycol at formula weight of 600.
[3]example of abbreviation for combined cryoprotectants with specific concentrations.

Existing commercial freezing methods developed for dairy bulls (SOP-10, Appendix A) were also evaluated in this study. Sperm samples were cryopreserved at the Genex Custom Collection Center in Baton Rouge. Sperm samples were mixed with the appropriate cryoprotectant before freezing and allowed 15 min for equilibration in a walk-in cooler held at 5°C. The sperm solutions were placed into pre-labelled 0.5-mL French cryopreservation straws using an automated straw filler (model MRS 1, IMV Int. Corp., 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°C 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 (Chandler et al., 1984). Once frozen, the samples were removed and placed under liquid nitrogen for sorting and preparation for long-term storage. After 2 d, 2 to 4 straws from each male were thawed in a 40°C water bath for 7 s to estimate the post-thaw motility. General observations of sperm morphology, such as broken tails or sperm agglutination (for details see Chapter 7) were also documented for thawed samples.

Fertilization and Larval Evaluation

Ten straws of each treatment were transported in a shipping Dewar (CP35, Taylor-Wharton, Theodore, Alabama) to the TRQSH for fertilization trials within 2 months after freezing. Diploid females were used for fertilization trials. Eggs from individual females were obtained by dissection, sieved, washed on a 25-μm mesh, and suspended in filtered seawater (34 ppt) at 25°C. The number of eggs per ml was determined by Coulter counter (Z1 series, Beckman Coulter, Inc. Fullerton, California). After counting, the eggs were held in seawater at 25°C for at

least 30 min to observe germinal vesicle breakdown at 100-× magnification brightfield. In general, unfertilized eggs (fresh) were pooled from three females and separated into beakers, and fertilization trials were conducted by mixing 5 ml of thawed sperm suspension (the pooled contents of ten 0.5-ml straws) with 500,000 eggs (fresh) held in 250 ml of seawater. The gametes were incubated at 25°C and percent fertilization was calculated by counting developing embryos at 2 h after insemination (SOP-13, Appendix A). Treatments held for further evaluation of percent hatching were transferred to 100-L tanks filled with fresh seawater. Twenty-four h after fertilization, these tanks were drained through a 45-µm mesh and percent hatching was calculated by counting normal straight-hinge larvae with a dissecting microscope (SOP-13, Appendix A). For a negative control, eggs were monitored after treatment as described above without addition of sperm.

For controls of egg quality, fresh (non-frozen) sperm from diploid males were collected using the techniques described above and the sperm were washed through a 70-µm mesh and added to fresh eggs to obtain about 100 spermatozoa per egg. Sperm counts were performed with a spectrophotometer (DR/2000, Hach Company, Loveland, Colorado) at TRQSH based on the techniques developed in Chapter 4 (Dong et al., 2005b). For controls of cryoprotectant toxicity, fresh sperm at the same concentration as those with thawed sperm samples were exposed to the same treatments (concentration, equilibration time, batch of eggs), and percent fertilization was estimated. To avoid contamination of gametes among individuals, the animals were handled with care and all surfaces were washed with 0.01% bleach. The sexes were held separately in different containers to avoid unintended fertilization.

Experimental Design and Data Analysis

A total of eleven experiments (Figure 5.1) were carried out and the experimental design began with a preliminary evaluation of five cooling rates with four cryoprotectants (Experiment 1). Cooling rates of 5 and 30 °C per min were selected to test on eight cryoprotectants each at two concentrations (Experiment 2). Cooling rate of 5 °C per min was selected for subsequent extensive evaluations of single or combined cryoprotectants at various concentrations. Among eight cryoprotectants tested in Experiment 2, six were selected to form 16 single or combined treatment levels to see possible combinations between different cryoprotectants (Experiment 3). Subsequently, three combinations (PEG/MeOH, PEG/P-glycol, 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 per treatment per 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 that shown consistently higher post-thaw motility from Experiments 2, 3, 4 and 5 were selected to evaluate straw size (Experiment 6), equilibration time (Experiment 8), and re-evaluate cooling rate (Experiment 7). For all 8 experiments, samples were cooled with the controlled-rate freezer and the criterion used for selection was post-thaw motility. Subsequently, in addition to post-thaw motility, percent fertilization or hatch was used to evaluate the selected cryoprotectants and cooling rate with the controlled-rate freezer (Experiment 9), the selected cryoprotectants with commercial freezing method (Experiment 10), and the difference between these two cooling methods with the same males (Experiment 11).

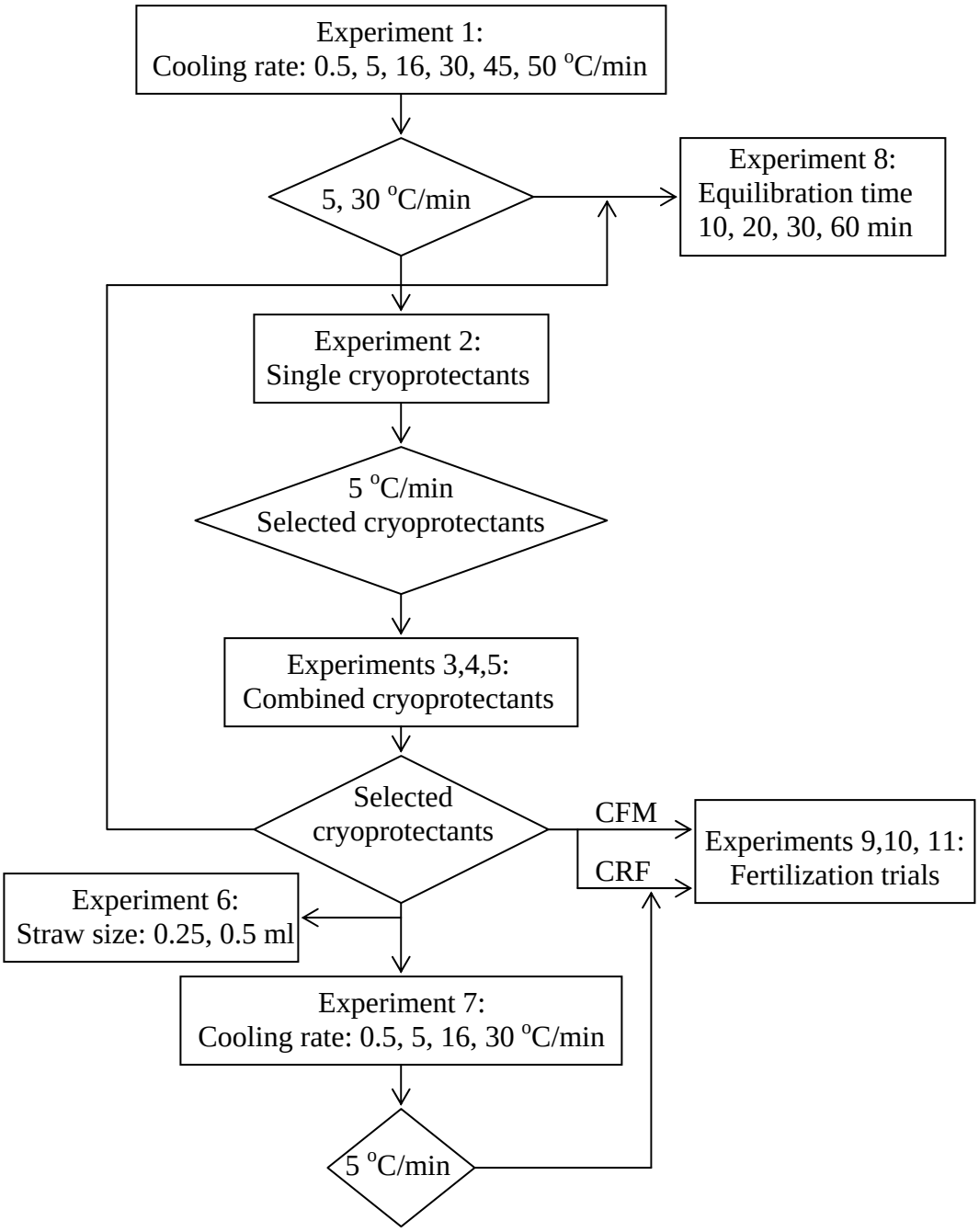

Figure 5.1 Design of experiments for optimization of sperm cryopreservation from diploid Pacific oysters. Post-thaw motility was used as the main criterion for procedure optimization and percent fertilization and hatch were used to test the results of optimized procedures. CRF: cooled at 5 °C/min using a controlled-rate freezer; CFM: cooled using a commercial freezing method developed for dairy bulls. All fertilization trials were conducted in the hatchery at Quilcene, Washington. Rectangles indicate experiments, rhomboids indicate decisions made based on experiments.

To minimize the effects of seasonality on the sperm quality of oysters used for different experiments, all 11 experiments were performed within the peak spawning month (June 4 to July 7). Due to the large number of treatment factors and levels to be evaluated in initial range-finding experiments within this time constraint, most experiments were replicated only with two males (Table 5.2). However, the experimental design outlined above (Figure 5.1) actually allowed factors such as cooling rate and cryoprotectant to be repeatedly evaluated in successive experiments (Table 5.2), and in some cases the same treatment (e.g., 2% PEG/4% MeOH) was tested on as many as 17 males.

Experiments involving two or three factors were all factorial design (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 5.2). 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, North Carolina). When a significant difference ($\alpha = 0.05$) was observed among treatments, Tukey's Honestly Significant Difference Procedure was used for pair-wise comparisons. Results were presented as means ± SD, and probability values of $P < 0.05$ were considered to be significant. Data for sperm motility, percent fertilization and percent hatch were arcsine-square root transformed prior to analysis (Sokal and Rohlf, 1995).

Experiment 1: Effect of Cooling Rate

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

Table 5.2 Experimental design and model statement for the eleven experiments.

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

[1]Cooled at 5 °C/min using a controlled-rate freezer; [2]Cooled using a commercial freezing method developed for dairy bulls
*The full coding would include the designation "CG04M" preceding each number; ***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, lower case letters indicate random factors.

Experiment 2: Effect of Single Cryoprotectant

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

Experiment 3: Effect of Combined Cryoprotectant (First Selection)

Sperm from two males (CG04M69, 70) were used to test 16 single or combined cryoprotectants at various concentrations (Table 5.3). Sperm suspensions were placed in 0.5-ml straws and cooled at 5 °C per min. Motilities were estimated after suspension and after thawing.

Experiment 4: Effect of Combined Cryoprotectant (Second Selection)

Based on the results of Experiment 3, PEG200 at 0, 2, 4, 6, and 8% was chosen as the non-permeating agent for use in combination with the permeating cryoprotectants MeOH, P-glycol, and DMSO each at 0, 4, 6, or 8% for a total of 48 combinations (Table 5.4). Based on the availability of sperm volume, sperm from five males were allocated to these three groups: PEG/MeOH (CG04M80, 81), PEG/P-glycol (CG04M77, 78), and PEG/DMSO (CG04M78, 79). Sperm suspensions were placed in 0.5-ml straws and cooled at 5 °C per min. Motilities were estimated after suspension and after thawing.

Experiment 5: Effect of Combined Cryoprotectants (Final Selection)

Single or combined cryoprotectants that yielded the highest post-thaw motility at the lowest concentration combinations in Experiment 4 were chose for use in this experiment (Table 5.5). Sperm from two males (CG04M87, 88) were used and suspensions were placed in 0.25-ml straws and cooled at 5 °C per min. Motilities were estimated after suspension and after thawing.

Experiment 6: Effect of Straw Size (0.25-ml versus 0.5-ml)

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

Experiment 7: Effect of Interactions between Cooling Rate and Cryoprotectant

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

Experiment 8: Effect of Equilibration Time

Sperm from two males (CG04M106, 111) were used to evaluate equilibration times of 10, 20, 30, and 60 min. Six percent of MeOH and 2% PEG/4% MeOH were used as cryoprotectants. Sperm suspensions were placed in 0.25-ml straws and cooled at 5 and 30 °C per min, and thawed in a 40 °C water bath for 6 s. Motilities were estimated after suspension and after thawing.

Experiment 9: Evaluation of Selected Cryoprotectants on Percent Fertilization and Hatch

Sperm from three males (CG04M87, 88, 92) were placed in 0.5-ml straws and used to test 7 selected single or combined cryoprotectants at a cooling rate of 5 °C per min in the controlled-rate freezer. The selected cryoprotectants and their concentrations were 2% PEG/4% MeOH, 6%

MeOH, 2% PEG/6% P-glycol, 8% P-glycol, 2% PEG/6% DMSO, 8% DMSO, and 8% PEG200. Motilities were estimated after suspension and after thawing, and percent fertilization and hatch were estimated as described above.

Experiment 10: Evaluation of Selected Cryoprotectants with Commercial-scale Freezing Method

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

Experiment 11: Evaluation of Cooling Methods

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

Results

Initial Sperm Motility After Shipment

A total of 27 Pacific oysters were transported in 7 shipments from June 4 to July 7, 2004. For the first two shipments, intact oysters (n = 8) were shipped and sperm were collected upon arrival. For the other shipments (n = 19), undiluted sperm samples were shipped and the samples were diluted in C-F HBSS immediately prior to freezing. The initial motility ranged from 5 to 95% with an average of 82 ± 22%. Except for three males, the sperm had initial motilities of >80% (Figure 5.2). The lowest initial motility (5%) was found in male CG04M26, followed by

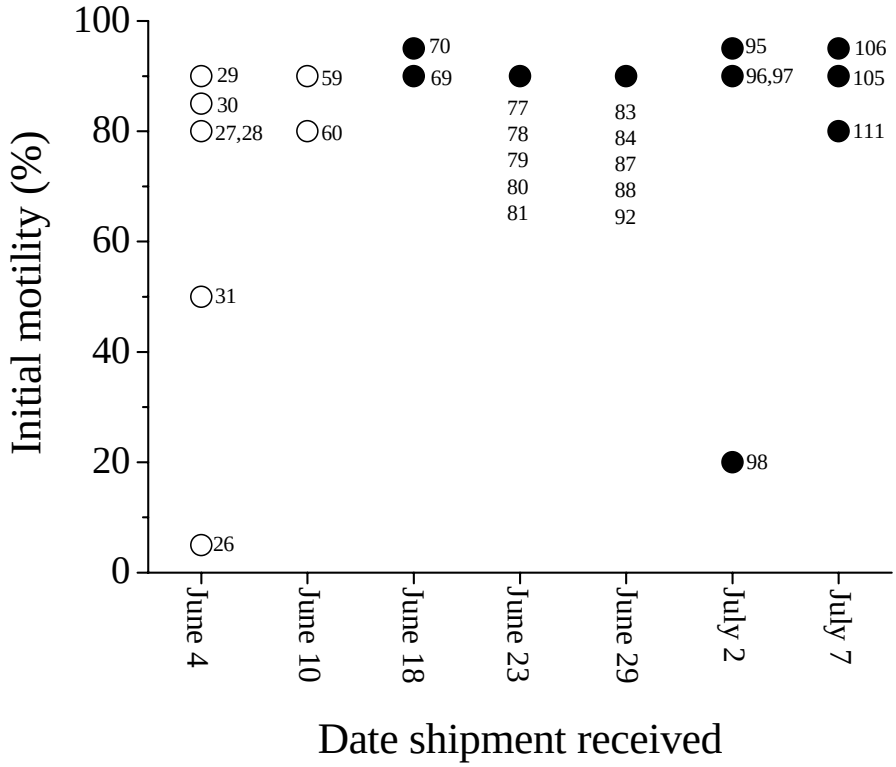

Figure 5.2 The motility of sperm from 27 diploid oysters transported in 7 shipments from June 4 to July 7, 2004. Intact oysters (open circles) were transported in the first two shipments (n = 8), and undiluted sperm (filled circles) were transported in the other shipments (n = 19). The numbers identified each individual oyster at the order of their usage in experiments (the full coding would include the designation "CG04M" preceding each number).

CG04M98 (20%), and CG04M31 (50%). The initial motility of sperm samples collected from shipped oysters (70 ± 29%) had marginally (P = 0.046) lower initial motility than shipped undiluted sperm samples (87 ± 16%).

Experiment 1: Effect of Cooling Rate

Post-thaw motility of sperm cooled at rates ranging from 0.5 to 50 °C per min were not significantly different from one another ($P = 0.098$), although highest post-thaw motility was obtained with the rates of 5 and 30 °C per min (Figure 5.3). There was a significant difference among the four cryoprotectants ($P < 0.001$) with 10% MeOH yielding the lowest post-thaw motility. A significant interaction was detected between the cryoprotectant and cooling rate ($P < 0.001$). The four males used were significantly different in post-thaw motility ($P < 0.001$) with the lowest post-thaw motility found for male CG04M26 (< 10% for all treatments). After thawing, the highest motility (37 ± 6%) was found for samples suspended in 10% DMSO and cooled at 30 °C per min, followed by samples suspended in 10% DMSO (30 ± 0%), 10% E-glycol (30 ± 0%), 10% P-glycol (28 ± 8%) and cooled at 5 °C per min. Therefore, cooling rates of 5 and 30 °C per min were chosen for subsequent experiments.

Experiment 2: Effect of Single Cryoprotectant

For samples cooled at 5 °C per min (Figure 5.4), no significant differences were observed among the eight cryoprotectants ($P = 0.160$) or between the concentrations of 5 and 10% ($P = 0.837$). However, the interaction between cryoprotectant and concentration was significant ($P < 0.001$). Differences among males were significant ($P = 0.002$) with CG04M30 yielding the highest overall post-thaw motility. The highest post-thaw motility (20 ± 12%) was found in samples suspended with 10% DMSO, followed by samples suspended in 5% methanol (15 ± 0%), 5% E-glycol (13 ± 3%), and 10% P-glycol (11 ± 6%).

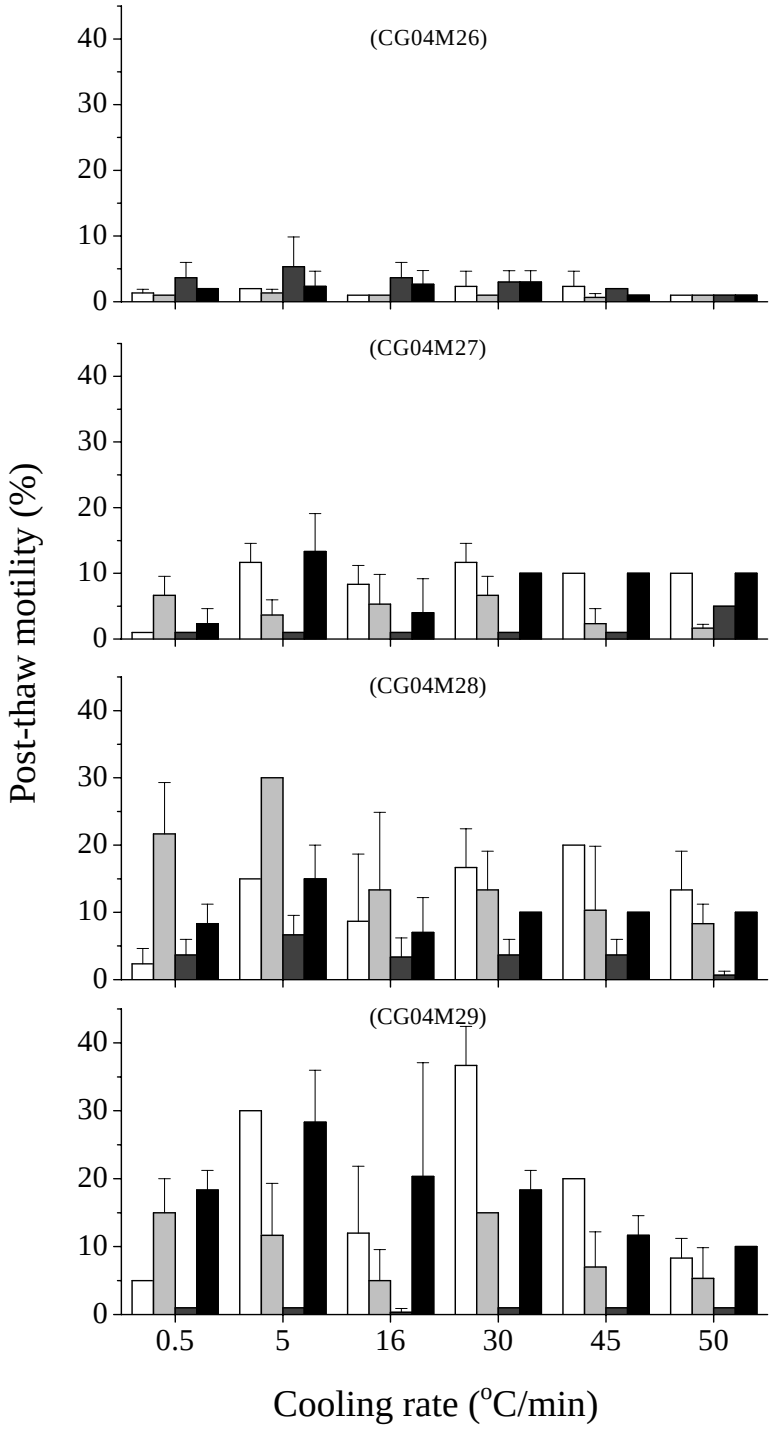

Figure 5.3 Post-thaw motility (mean ± SD) of sperm samples suspended in 10% DMSO (white bars), E-glycol (light gray bars), MeOH (dark gray bars), P-glycol (black bars), and cooled at 0.5, 5, 16, 30, 45, and 50 °C per min. Four males were used (CG04M26, CG04M27, CG04M28, and CG04M29).

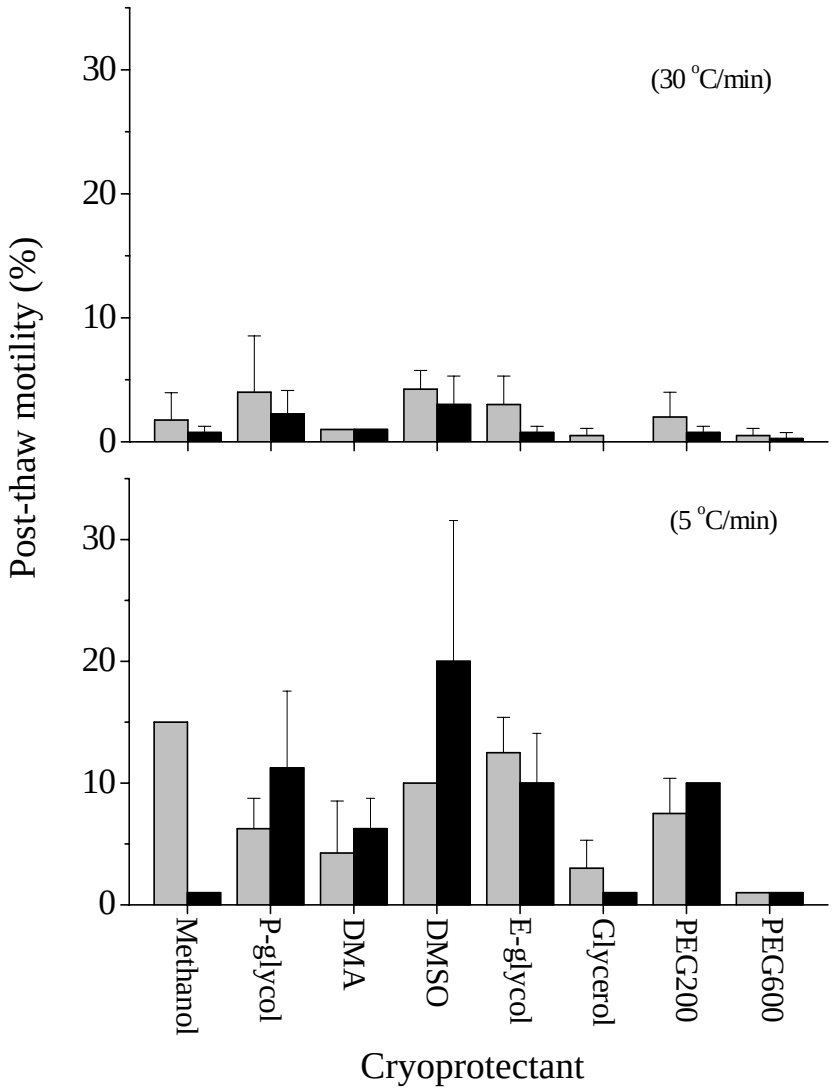

Figure 5.4 Post-thaw motility (mean ± SD) of sperm samples suspended in eight cryoprotectants: MeOH, P-glycol, DMA, DMSO, E-glycol, glycerol, PEG200 and PEG600 each at 5 (light gray bars) and 10% (black bars), and cooled at 5 (CG04M30 and CG04M31) and 30 °C per min (CG04M59 and CG04M60).

For samples cooled at 30 °C per min (Figure 5.4), significant differences were observed among the eight cryoprotectants ($P < 0.001$), and post-thaw motility of samples suspended in DMSO and P-glycol was found to be significantly higher than those suspended with glycerol and PEG600. There was also a significant difference between the concentrations of 5 and 10% ($P = 0.004$), but the interaction between cryoprotectant and concentration was not significant ($P = 0.930$). Both males yielded low post-thaw motility (< 10%) and there was no significant difference between them ($P = 0.432$). Glycerol, DMA and PEG600 yielded the lowest post-thaw motility among the eight cryoprotectants tested in both trials. However, better sperm morphology (sperm with most tails attached) was observed for samples cryopreserved with PEG200 and PEG600. Thus, glycerol and DMA were excluded from subsequent experiments.

Experiments 3: Effect of Combined Cryoprotectants (First Selection)

Significant differences ($P < 0.001$) were observed among the 16 single and combined cryoprotectants, with the combined cryoprotectants of PEG/P-glycol (23 ± 13%), PEG/DMSO (16 ± 14%), DMSO/E-glycol (13 ± 12%), and PEG/MeOH (10 ± 12%) yielding the highest post-thaw motility (Table 5.3). The two males used in this experiment did not show significant variation ($P = 0.066$). Combinations of non-permeating agent (PEG200) and the permeating agents MeOH, P-glycol and DMSO were chosen for further optimization in the subsequent experiments.

Experiments 4: Effect of Combined Cryoprotectants (Second Selection)

Significant differences ($P < 0.001$) were found for various concentration combinations within each combined cryoprotectant group (Table 5.4). Of the single cryoprotectants and cryoprotectant combinations of PEG200 and MeOH, the highest post-thaw motility was found in samples suspended in 2% PEG/6% MeOH (40 ± 8%) and 6% MeOH (39 ± 6%), followed by

Table 5.3 Post-thaw motility (mean ± SD) of sperm samples cryopreserved in 16 single or combined cryoprotectants and cooled at 5 °C per min (males: CG04M69 and CG04M70).

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

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

Table 5.4 Post-thaw motility (mean ± SD) of sperm samples suspended in single or combined cryoprotectants and cooled at 5 °C per min.

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

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

*Male numbers.

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

Experiment 5: Effect of Combined Cryoprotectants (Final Selection)

Significant differences ($P < 0.001$) were observed among the re-grouped 16 single or combined cryoprotectants with the highest post-thaw motility observed in samples cryopreserved with 6% MeOH (29 ± 14%), followed by 2% PEG/4% MeOH (27 ± 11%), 4% PEG/4% MeOH (26 ± 15%), and 2% PEG/6% MeOH (23 ± 14%) (Table 5.5). The two males used in this experiment were significantly different in post-thaw motility ($P = 0.0001$). Based on the combined results of Experiments 3 through 5, 2% PEG/4% MeOH, 6% MeOH, 2% PEG/6% P-glycol, 8% P-glycol, 2% PEG/6% DMSO, 8% DMSO, and 8% PEG200 were selected for subsequent experiments.

Table 5.5 Post-thaw motility (mean ± SD) of sperm samples suspended in 16 single or combined cryoprotectants and cooled at 5 °C per min.

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

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

Experiment 6: Effect of Straw Size (0.25-ml versus 0.5-ml)

To evaluate possible effects from straw size differences, 0.25-ml and 0.5-ml straws were compared in this experiment (Figure 5.5). A significant difference in post-thaw motility was not observed for either straw size ($P = 0.465$) or thawing method (40 vs. 60 °C, $P = 0.208$). All interactions among straw size, thawing method and cryoprotectant were found to be non-significant ($P > 0.125$). Significant differences were observed among the seven cryoprotectants ($P = 0.034$) and two males ($P < 0.001$) used in this experiment (Figure 5.5). Similar to the previous experiment, samples suspended in 2% PEG/4% MeOH and 6% MeOH yielded the highest post-thaw motility, and those suspended in 8% PEG200 yielded the lowest post-thaw motility.

Experiment 7: Effect of Interactions between Cooling Rate and Cryoprotectant

Samples cooled at 5 °C per min yielded the highest post-thaw motility, which was significantly different from that observed for cooling at 0.5, 16, and 30 °C per min ($P < 0.044$) (Figure 5.6). Samples cooled at 16 and 30 °C per min were not significantly different from one another ($P = 0.082$), but these cooling rates yielded higher post-thaw motility than did samples cooled at 0.5 °C per min ($P < 0.001$). The interaction of cooling rate and cryoprotectant was not significant ($P = 0.645$). However, significant differences were observed for the cryoprotectant ($P = 0.002$) and the two males ($P < 0.001$) used in this experiment. The highest post-thaw motilities were found with samples from male CG04M97 suspended in 2% PEG/6% P-glycol (31 ± 6%), 2% PEG/6% DMSO (31 ± 3%), and 2% PEG/4% MeOH (28 ± 5%) and cooled at 5 °C per min. Consequently, a cooling rate of 5 °C per min was chosen for subsequent experiments.

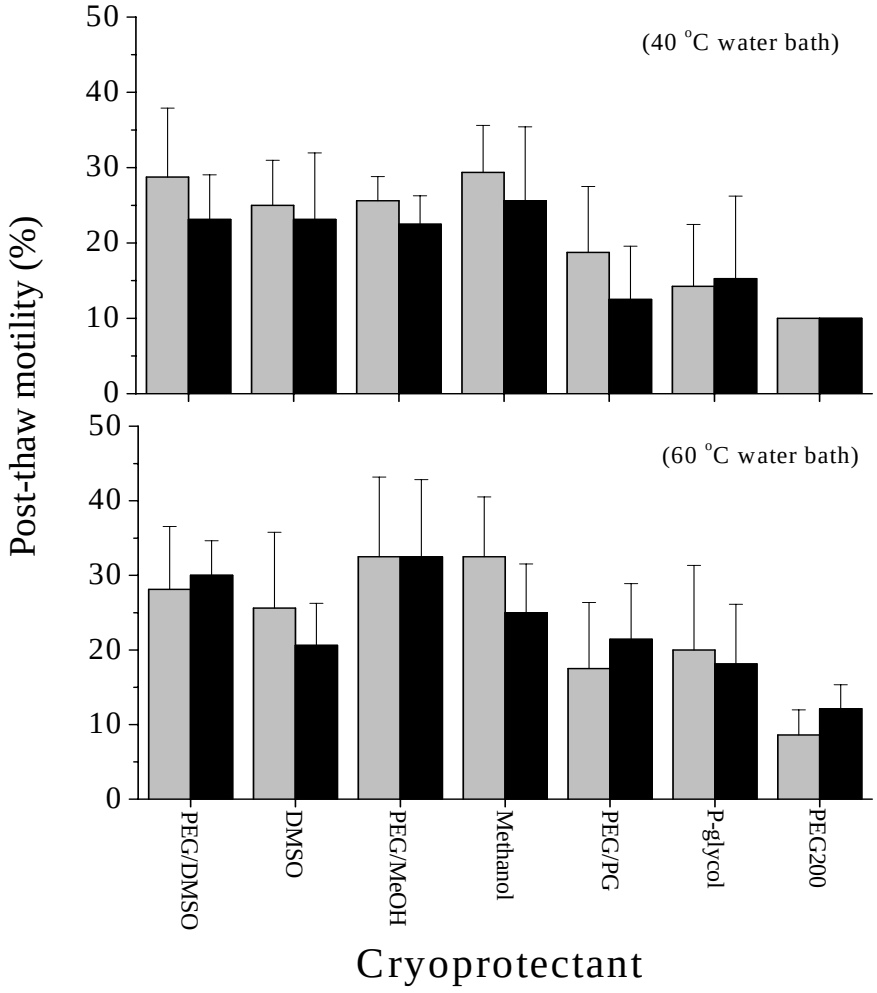

Figure 5.5 Post-thaw motility (mean ± SD) of sperm samples suspended in 2% PEG/6% DMSO, 8% DMSO, 2% PEG/4% MeOH, 6% MeOH, 2% PEG/6% P-glycol, 8% P-glycol, and 8% PEG200, and equilibrated for 12 min in 0.25-ml (light gray bars) or 0.5-ml straws (black bars). Samples were cooled at 5 °C per min and thawed in a water bath at 40 °C (6 s for 0.25-ml straws, and 10 s for 0.5-ml straws), and 60 °C (5 s for 0.25-ml straws, and 7 s for 0.5-ml straws). Two males were used (CG04M83 and CG04M84).

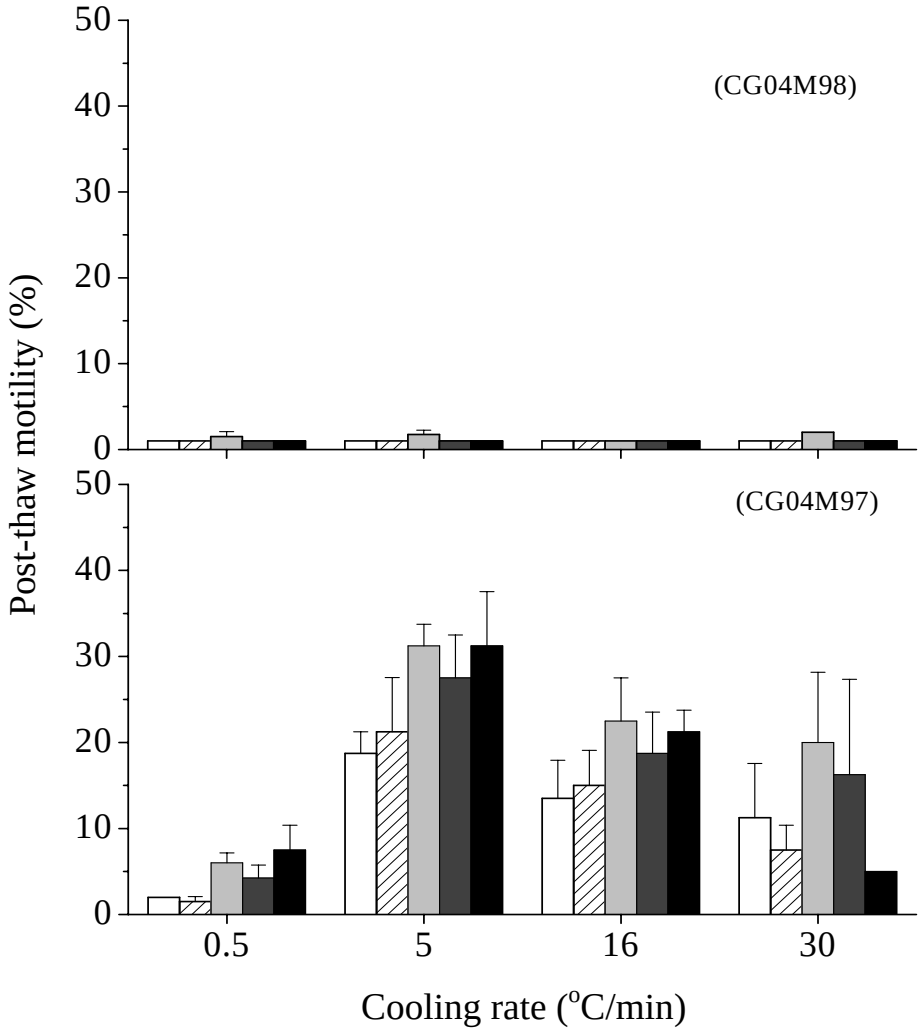

Figure 5.6 Post-thaw motility (mean ± SD) of sperm samples suspended in 6% MeOH (white bars), 8% PEG200 (hatched bars), 2% PEG/6% DMSO (light gray bars), 2% PEG/4% MeOH (dark gray bars), 2% PEG/6% P-glycol (black bars), and equilibrated for 12 min in 0.25-ml straws. Samples were cooled at 0.5, 5, 16, and 30 °C per min, and thawed in a 40 °C water bath for 6 s. Two males were used (CG04M97 and CG04M98).

Experiment 8: Effect of Equilibration Time

Samples with equilibration times of 30 and 60 min yielded higher post-thaw motility than did those of 10 and 20 min, although they were not significantly different from one another (P = 0.468) (Figure 5.7). All interactions among equilibration time, cooling rate, and cryoprotectant were found to be non-significant ($P > 0.051$). Cooling rates of 5 and 30 °C per min were not significantly different in this experiment ($P = 0.790$), but samples suspended in 2% PEG/4% MeOH yielded higher post-thaw motility than did those in 6% MeOH alone ($P < 0.001$). Post-thaw motility was significant for the two oysters used in this experiment ($P < 0.001$).

Experiment 9: Evaluation of Selected Cryoprotectants on Percent Fertilization and Hatch

For a cooling rate of 5 °C per min with the controlled-rate freezer (Table 5.6), the highest post-thaw motilities were obtained with samples suspended in 6% MeOH (35 ± 19%) and 2% PEG/4% MeOH (33 ± 15%), which were significantly higher than those of the other cryoprotectants although a significant difference in post-thaw motility ($P < 0.001$) was observed among the three males used. The highest percent fertilizations were also found in samples suspended in 6% MeOH (60 ± 35%) and 2% PEG/4% MeOH (50 ± 39%), which were not significantly different from the other cryoprotectants except for 6% PEG200 (9 ± 10%). Percent fertilization as high as 98% was observed for sperm samples cryopreserved with 6% MeOH for male CG04M87. Percent hatch was high (> 50%) for treatments with fertilization above 80% (Table 5.6).

Experiment 10: Evaluation of Selected Cryoprotectants with Commercial-scale Freezing Method

The selected cryoprotectants and their combinations were also tested with the commercial freezing methods developed for dairy bulls (Table 5.7). Although the post-thaw motility was generally low (< 5%), fertilization of greater than 50% was obtained with samples suspended in

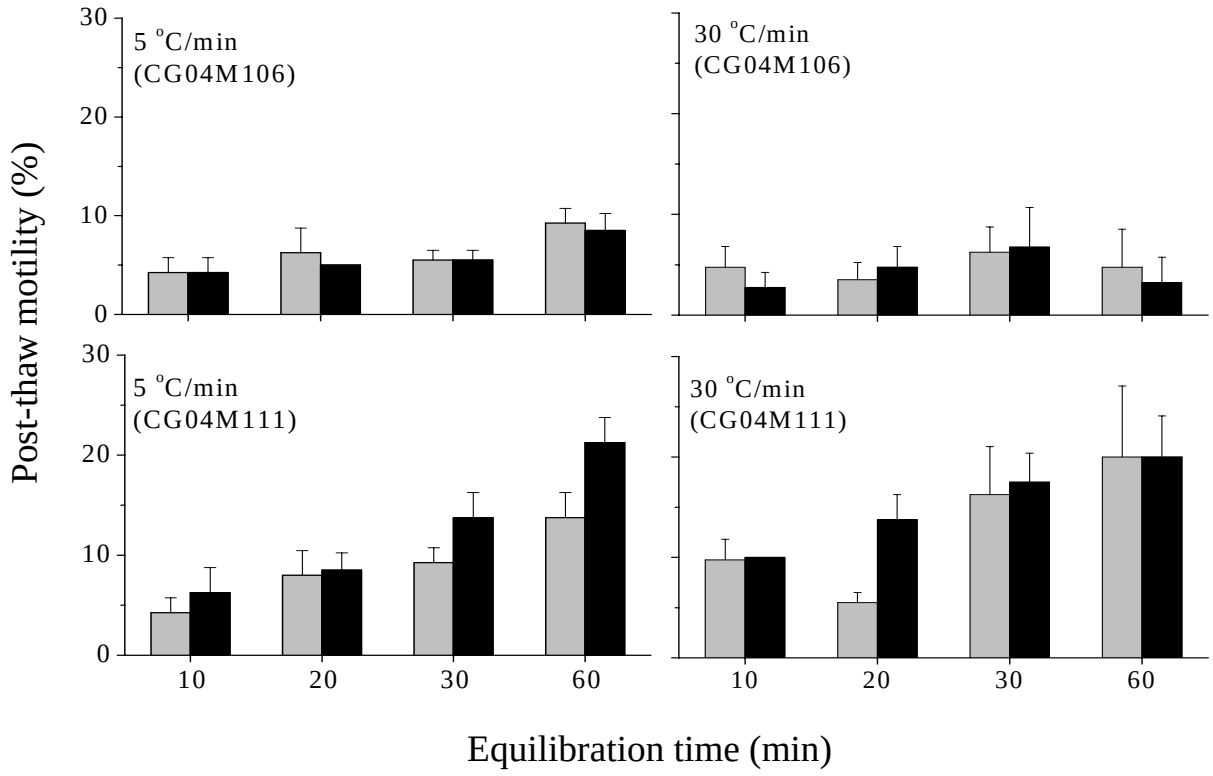

Figure 5.7 Post-thaw motility (mean ± SD) of sperm samples suspended in 6% MeOH (light gray bars) and 2% PEG/4% MeOH (black bars), equilibrated for 10, 20, 30 and 60 min, and cooled at 5 and 30 °C per min. Two males were used (CG04M106 and CG04M111).

Table 5.6 Post-thaw motility (mean ± SD), percent fertilization and hatch of sperm samples suspended in 2% PEG/4% MeOH, 6% MeOH, 2% PEG/6% P-glycol, 8% P-glycol, 2% PEG/6% DMSO, 8% DMSO, or 8% PEG200, equilibrated for 15 min, and cooled at 5 °C per min with a controlled-rate freezer.

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

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

Table 5.7 Post-thaw motility (mean ± SD), and percent fertilization of sperm samples suspended in 2% PEG/4% MeOH, 6% MeOH, 2% PEG/6% P-glycol, 8% P-glycol, and 2% PEG/6% DMSO, equilibrated for 15 min, and cooled using a commercial freezing method developed for dairy bulls.

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

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

2% PEG/4% MeOH for male CG04M96. The treatment effects of different cryoprotectants were found to be significant for post-thaw motility ($P < 0.001$) despite their low values, but were found to be non-significant for percent fertilization ($P = 0.540$), which may be due to the significant difference ($P < 0.001$) observed for the two males used in this experiment.

Positive controls using fresh sperm without addition of cryoprotectant yielded 99% fertilization, and controls using fresh sperm equilibrated with the same cryoprotectants for the same equilibration time all yielded > 90% fertilization (Table 5.6), indicating cryoprotectants at these concentrations were only mildly toxic or non-toxic to sperm and eggs. Fresh eggs without sperm addition yielded 0% fertilization, indicating that eggs were not contaminated with extraneous sperm.

Experiment 11: Evaluation of Cooling Methods

To directly compare the controlled-rate freezer (CRF) and commercial freezing method (CFM), these two methods were evaluated with same males (Table 5.8). Samples frozen with the CRF were found to be significantly higher in post-thaw motility ($P < 0.001$) and marginally different in percent fertilization ($P = 0.050$) than those frozen with CFM. No differences were observed within cryoprotectants for post-thaw motility ($P = 0.393$) or for percent fertilization ($P = 0.702$). Interactions between cooling methods and cryoprotectants were found to be significant for post-thaw motility ($P = 0.003$), but not for percent fertilization ($P = 0.550$). The two males used in this experiment showed significant differences in post-thaw motility ($P < 0.001$) and percent fertilization ($P = 0.025$). Positive controls using fresh sperm without addition of cryoprotectant yielded 92% fertilization, and controls using fresh sperm equilibrated with the same cryoprotectants for the same equilibration time all yielded > 94% fertilization (Table 5.8),

Table 5.8 Post-thaw motility (mean ± SD), percent fertilization and hatch of sperm samples suspended in 2% PEG/4% MeOH, 6% MeOH, and 5% E-glycol, and cooled at 5 °C/min using a controlled-rate freezer (CRF) or a commercial freezing method (CFM) developed for dairy bulls.

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

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

indicating cryoprotectants at these concentrations were not toxic for sperm and eggs. Again, fresh eggs without any sperm addition yielded 0% fertilization.

Discussion

A wide range of cooling rates has been reported for oyster sperm, from 1 °C per min (Hughes, 1973) to immediate plunging in liquid nitrogen (Hwang and Chen, 1973). In the present study, direct comparison of 0.5, 5, 16, 30, 45, 50 °C per min did not show significant differences although the highest post-thaw motility was obtained with 5 and 30 °C per min. However, subsequent experiments with optimized cryoprotectants and concentrations showed that motility of samples cooled at 5 °C per min was significantly higher than those cooled at 0.5, 16 and 30°C per min. Previous studies with oyster sperm have reported that cooling rates of ~ 5 °C per min (Yankson and Moyse, 1991; Ieropoli et al., 2004) and 50 °C per min (Smith et al., 2001) have yielded fertilization success. Optimized cooling rates could depend on the type of cryoprotectant and concentrations, as a significant interaction was observed between cryoprotectant and cooling rate in the present study. Optimized cooling rates can also depend on other factors involved in the cryopreservation process such as the type of extender (Babiak et al., 1999) or the type of cooling methods. In this study with the controlled-rate freezer and the cryoprotectants studied, more consistent results were observed with samples cooled at 5 °C per min. In contrast to the controlled-rate freezer, results from the present study showed lower post-thaw motility and fertilization resulted from the commercial protocols. Despite that, high fertilization (> 90%) was obtained with the commercial protocols (e.g., for male CG04M105). Unlike cooling rate, cooling method rarely receives much attention in controlled studies. It would be informative to evaluate the differences among various cooling methods with the same nominal cooling rates.

Choosing an appropriate cryoprotectant has been a primary focus in gamete cryopreservation for mammals and aquatic species (Leibo, 2000; Tiersch, 2000). 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 enabling the cells to avoid the otherwise irreversible effects of excessive osmotic gradients (Meryman, 1971). A classic formula of combining sugars (e.g., glucose) or proteins (e.g., egg yolk) with permeating cryoprotectants such as DMSO (usually at a concentration of 5-10%) has been applied in fish species and with variable success (e.g., Scheerer and Thorgaard, 1989; Piironen, 1993; DeGraaf and Berlinsky, 2004). Contrary to that, few studies have been conducted to evaluate the combined effects of polymers (e.g., polyethylene glycol, or polyvinyl pyrrolidone) with permeating compounds. The present study tested combinations of polyethylene glycol with MeOH, P-glycol, and DMSO and found that a low concentration (2%) of polyethylene glycol (FW 200) was effective in retaining post-thaw motility and fertilizing capability for sperm of diploid Pacific oysters when combined with permeating compounds. However, polyethylene glycol alone was not as effective as permeating compounds such as MeOH, DMSO and P-glycol when using the same methods.

Previous studies with oyster sperm have excluded methanol from the list of suitable cryoprotectants (e.g., Smith et al., 2001). However, consistently higher post-thaw motility was obtained with MeOH or the PEG/MeOH combination in the present study, and the highest post-thaw motility (70%) and percent fertilization (98%) were obtained for samples cryopreserved with 6% MeOH. Other than DMSO, methanol is one of the most widely used cryoprotectants for sperm from aquatic species (Tiersch, 2000) with successful semen cryopreservation reported in a variety of species such as zebrafish *Danio rerio* (Harvey et al., 1982), tilapia *Oreochromis* spp.

(Rana and McAndrew, 1989), channel catfish *Ictalurus punctatus* (Tiersch et al., 1994), Siberian sturgeon *Acipenser baeri* (Glogowski et al., 2002), rainbow trout *Oncorhynchus mykiss* (Lahnsteiner et al., 2002), and common carp *Cyprinus carpio* (Miskolczi et al., 2005). Controversies concerning the effectiveness of cryoprotectants for particular species frequently occur. For example, 20% glycerol was found to be better than 10% DMSO for semen cryopreservation of Arctic charr *Salvelinus alpinus* (Piironen, 1993), but the opposite was reported for the same species in another study (Richardson et al., 2000).

Despite the fact that MeOH and the PEG/MeOH combination yielded the highest percent fertilization in the present study, it is difficult to conclude that MeOH is a better overall cryoprotectant than the others tested for oyster sperm. Similar to the optimization of cooling rate, optimization of cryoprotectant and its concentration is interlinked with the other factors involved in the overall process of cryopreservation. Given optimized conditions for all other aspects (e.g. extender composition, cooling rate, and thawing method), several cryoprotectants could provide adequate protection for a given species. For example, DMSO and MeOH were each found to be equally efficient in sperm cryopreservation of Northern pike, *Esox lucius* (Babiak et al., 1995; Lahnsteiner et al., 1998) and paddlefish, *Polydon spathula* (Bean et al., 2005). In the case of Pacific oyster sperm, high fertilization rates (> 90%) or D-stage larvae (>50%) have also been reported for samples cryopreserved with DMSO, P-glycol and E-glycol (Ieropoli et al., 2004; Dong et al., 2005a). Compared with the wide concentration range reported for DMSO, methanol may have a narrower effective range in terms of cryoprotection and toxic effects (Lahnsteiner et al., 1996). In the present study, methanol at 10% was found to be less effective in retaining post-thaw motility than was 5%. All told, protocols that provide equal protection for a specific cell population may vary with the type of cryoprotectants. Therefore,

the effectiveness of different cryoprotectants for a specific species can be only evaluated through the comparisons across different protocols.

Various sizes of straws or cryovials have been used for different purposes in previous studies of aquatic species. In general, large volume containers were studied for the purpose of scaling up production for hatchery application (e.g. Richardson et al., 1999, 2000; Cabrita et al., 2001; Paniagua-Chavez and Tiersch, 2001), and smaller volume containers such as 0.25-ml straws were used for species with limited sperm volume (e.g., Huang et al., 2004a, b). Higher post-thaw motility or fertility was obtained with smaller volume straws in sperm cryopreservation of rainbow trout (among 0.5, 1.8 and 5-ml straws) (Cabrita et al., 2001), yellowtail flounder *Pleuronectes ferrugineus* (0.25-ml vs. 1.7-ml straws) (Richardson et al., 1999), and channel catfish (0.25-ml vs. 0.5-ml straws) (Christensen and Tiersch, 1997). In the present study, straw sizes of 0.25-ml and 0.5-ml did not show significant differences in retaining post-thaw motility although higher post-thaw motility was obtained with 0.25-ml straws. In the eastern oyster *C. virginica*, 5-ml straws were found to be especially effective in preserving larvae (Paniagua-Chavez and Tiersch, 2001). The discrepancy of the effects of straw size may due to interactions of cooling rates, cryoprotectants, and thawing methods as indicated above. The better results associated with smaller straw volumes in general may result from their higher surface area-to-volume ratio, and a consequent enhanced uniformity of heat transfer during freezing and thawing processes (Pickett and Berndtson, 1974).

Optimal equilibration time before freezing is necessary to allow permeating cryoprotectants to penetrate the sperm while minimizing toxicity. In general, equilibration times of 10 to 20 min or shorter are considered to be better for oyster and fish semen cryopreservation (e.g., Iwata et al., 1989; Billard and Zhang, 2001; Dong et al., 2005a). However, semen of sea bass,

Decentrarchus labrax, equilibrated for 6 h with 10% ethylene glycol at 0-2 ^{o}C yielded motility when thawed comparable to that of fresh semen (Sansone et al., 2002), and sperm of channel catfish, equilibrated for 10 d with 5% methanol at 4 ^{o}C showed increased motility compared to that of fresh sperm (Christensen and Tiersch, 1996). The present study showed higher post-thaw motility with longer equilibration time (30 - 60 min). Although long equilibration (60 min) was suspected to have detrimental effect on larval development of Pacific oysters (Iwata et al., 1989), it could be beneficial when the cryoprotectant concentration is low and solution is added in a step-wise fashion at low temperatures. Post-thaw motility of 70% with sperm from diploid Pacific oysters was obtained when equilibration time was extended to 1 h with step-wise addition at 5 °C and cooling using commercial dairy protocols (Dong, unpublished data). Indeed, a wide range of equilibration times (30 min to 6 h) with stepwise addition of 7 to 11% glycerol at 5 °C are practiced in routine commercial-scale cryopreservation of dairy bull sperm (Chandler, 2000).

Differences in post-thaw sperm quality (e.g., motility or percent fertilization) among individual males were evident in this study. Some males were found to have high percent fertilization (e.g., CG04M87 in Table 5.6), regardless of the choice of cryoprotectants, while others (e.g., CG04M95 in Table 5.7) were found to have poor fertilization (< 5%), which suggested a difference in the tolerance to cryopreservation processes of sperm from individual males. The term “tolerance” used here is defined as the relative capacity of spermatozoa to fertilize eggs when subjected to unfavorable environmental factors. For example, the fertility of fresh sperm from different males could be same (e.g., > 90% for all controls in this study), but varied when thawed sperm from those same males were compared, which were confirmed with findings in the eastern oyster where the fertility of fresh and thawed sperm from five males were compared (Paniagua-Chavez and Tiersch, 2001). To illustrate this phenomenon, a generalized

classification is introduced here: males can be identified as individuals with broad, intermediate, or narrow tolerances to cryopreservation. Individuals with broad tolerance can accommodate various protocols available in practice and yield good results. On the contrary, individuals with a narrow tolerance can accommodate a more narrow range across protocols, and thus considerable research efforts are required before success could be observed. Those individuals with intermediate tolerance, however, can accommodate a moderate range of practical protocols, and good results will be obtained with some protocols but not others. This same concept could be equally applied to strain or species differences in tolerances of the cryopreservation process. For protocol optimization, the problems associated with individuals either of broad (in a sense of superior males) or narrow tolerance is the difficulty to differentiate treatment effects because the post-cryopreservation sperm quality for treatments would be all good (superior males) or all poor (narrow tolerance).

Factors that affect sperm tolerance can be biotic (e.g., genetic, physiological), abiotic (e.g., environmental factors), or arise from interactions among these. Variation in tolerance among males could be due to difference in their genetic tolerance to cryopreservation, genetic tolerance to stress (e.g., shipping), or genetic tolerance to the combinations of cryopreservation and stress. Findings in a recent study on boar sperm cryopreservation supported the hypothesis that there is a genetic basis for variation in post-thaw sperm quality among individuals (Thurston et al., 2002). In that study, analogous to the classification proposed in this study, boars were grouped into poor, average, and good based on post-thaw recovery assessed from a variety of sperm quality evaluation techniques such as motility, membrane integrity, acrosome integrity, and active motility (CASA: computer-assisted sperm analysis). Subsequent genomic analysis using amplified restriction fragment length polymorphism (AFLP) technology revealed sixteen

candidate molecular markers linked to genes controlling semen response to cryopreservation (Thurston et al., 2002). Successful sperm cryopreservation involves many steps, not only those steps involved in the freezing and thawing processes, but can also include sample shipping (Dong et al., 2005a), broodstock rearing and handling, egg quality, and fertilization methods (Rurangwa et al., 2004). Abiotic factors involved in activities prior to cryopreservation could possibly alter the tolerances of sperm from individual males, such as nutrient availability (Labbe et al., 1995), rearing temperature (Labbe and Maisse, 1996), confinement (Kubokawa et al., 1999), transportation (Allyn et al., 2001), and invasive gonad sampling for sex or ploidy identification (Dong et al., 2005a). In the present study, sperm samples or intact oysters were shipped from the hatchery to the cryopreservation laboratory. The low post-thaw motility (< 5%) reported in some treatments of this study could be due to the effects of shipping stress on sperm samples. Future research is necessary to differentiate these factors and their interactions.

Difference in sperm tolerance could have a profound impact on cryopreservation outcome (Figure 5.8). For example, for a male or a species with a narrow tolerance, either due to biotic (genetic) or abiotic (stress) factors, sperm cryopreservation could be less effective when non-optimized or sub-optimized procedures are used. It is reasonable to conclude that male, strain, or species variation can be important factors responsible for the inconsistency observed in sperm cryopreservation of oysters and aquatic species in general. Besides protocol standardization, optimization of cryopreservation protocols and recognition of male (strain or species) variation in experiments would facilitate the process of reducing inconsistency and controversy. Although this study classified sperm tolerance in theory into three categories (broad, intermediate, and narrow), male oysters in reality displayed tolerance to cryopreservation procedures in a continuous fashion. However, this categorical classification of sperm tolerance may help to

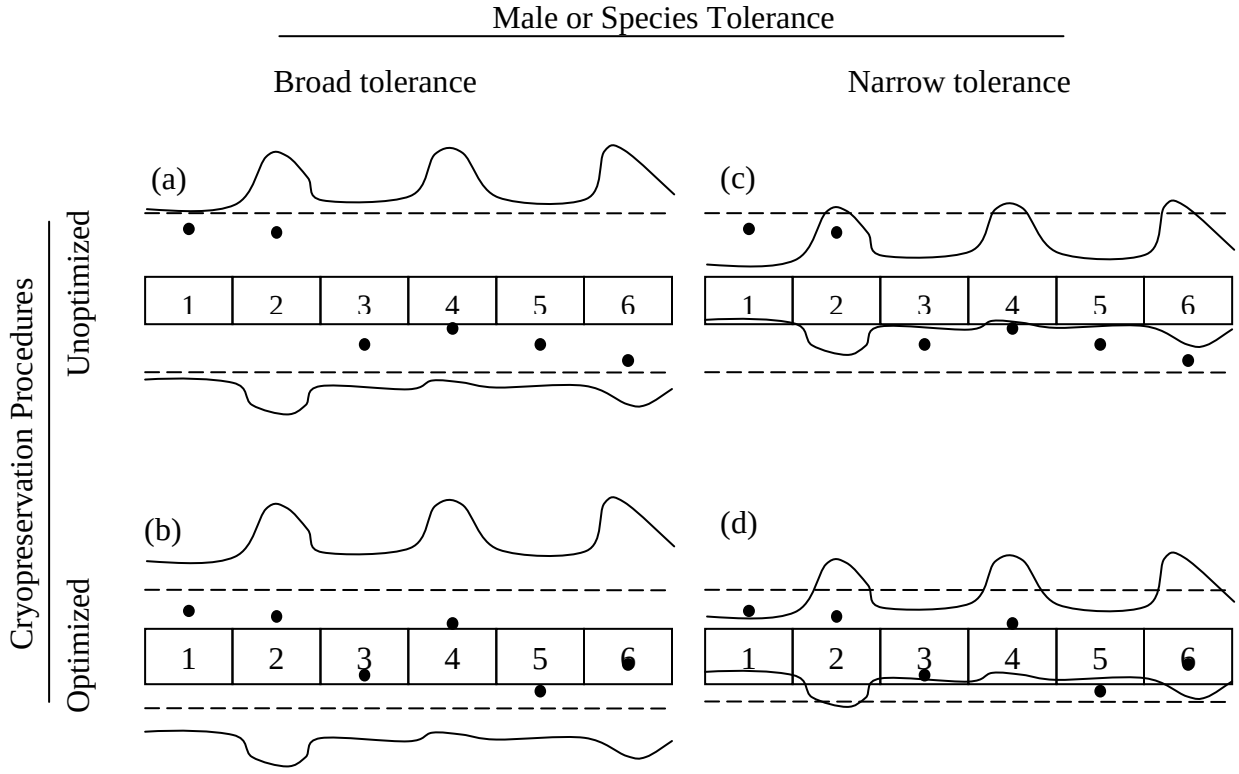

Figure 5.8 Diagramatic representation of interactions between male or species tolerance and cryopreservation procedures. The rectangles identified with the numbers 1 to 6 represent six presumptive critical factors involved in cryopreservation at a central facility (e.g. shipping, cryoprotectant toxicity, cooling rate). Dotted lines indicate the range within which the practical protocols were included. Solid lines indicate the range that the individual males can tolerate, and black dots represent a protocol used in practice. Males with broad tolerance (e.g., genetically more tolerant to cryopreservation or stress, or males that were not subjected to stress) will survive cryopreservation well because all protocols are within the solid lines (a, b). Males with a narrow tolerance (e.g., genetically less tolerant to cryopreservation or stress, or males have been stressed by unfavorable conditions) can accommodate a narrower range and some protocols fall outside the solid lines (c). However, the number of protocols falling outside the solid lines can be reduced when optimized cryopreservation procedures are used (d).

establish standardized cryopreservation technologies. For example, cryopreservation protocols could be developed and optimized separately for males with broad, intermediate, and narrow tolerances. As a result, assays that can differentiate sperm tolerance among different males prior to freezing are necessary in future studies including quantitative assessment tools.

Previous studies of sperm cryopreservation from oysters have rarely involved shipping (for review see Chapter 2). The present study demonstrated that oyster sperm could be collected and shipped chilled to another facility for cryopreservation, and that it can also be shipped back to the hatchery (Figure 2.1) for fertilization performed at a production scale yielding live larvae with > 90% fertilization. Given the existence of facilities for commercial-scale cryopreservation of dairy bull sperm, the methods developed in the present study for oysters provide a template for the potential commercialization of cryopreserved sperm in aquatic species.

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Chapter 6
Optimization of Sperm Cryopreservation for Tetraploid Pacific Oysters

The induction of triploidy is useful for bivalve aquaculture because reduced gametic investment yields improved meat quality and growth. Reduced gamete output can yield other benefits such as minimizing risk of introduction of exotic species, and protection of intellectual property for breeding lines or strains with commercial profits. In oysters, the commercial benefits of triploidy have been evaluated in various species. However, currently this technique has only been commercialized for Pacific oysters, *Crassostrea gigas* (Nell, 2002), and eastern oysters, *C. virginica* (S. Allen, Virginia Institute of Marine Science, personal communication). In the past 15 years, triploids have become an important part of Pacific oyster aquaculture. For example, shellfish hatcheries in Washington and Oregon are producing about 37.5 billion ready-to-set or "eyed" larvae each year, of which 12 billion or about 1/3 are triploid (data based on 1999-2000, Nell, 2002). The main advantage of farming triploid oysters in North America is that they do not spawn during summer and thus are marketable throughout the year.

Until the advent of tetraploid oysters 10 years ago (Guo and Allen, 1994), triploidy induction in oysters was primarily achieved by blocking the release of the second polar body with cytochalasin B (Allen et al., 1989). However, the use of chemical methods causes several problems. First, these techniques typically produce less than 100% triploidy in batches, and larval populations containing a low percentage of triploids are a problem for hatchery management and a waste of production space and money (Eudeline et al., 2000). Second, cytochalasin B is toxic and health concerns may limit its use in food production (Guo et al., 1994b), although other induction methods are available. Thirdly, blocking the second polar body may negatively affect the survival and growth of induced triploids (Chourrout et al., 1986; Downing and Allen, 1987; Guo et al., 1992). Instead, the crossing of gametes from tetraploids

and normal diploids offers reliable triploidy production and high survival rates (Eudeline et al., 2000, 2002; Guo et al., 1994, 1996; Wang et al., 2002), and is likely to continue and increase. This can be facilitated by the use of tetraploid breeding lines rather than the continued chemical production of tetraploids (Nell, 2002). 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. Cryopreservation of sperm from tetraploid oysters has been studied. However, these sperm were found to be more sensitive to cryopreservation than were the sperm of diploids (Dong et al., 2005a). The present study continued efforts to improve methods to cryopreserve sperm from tetraploid Pacific oysters through use of a systematic approach.

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).

Materials and Methods

Sperm Collection and Motility Estimation

Tetraploid Pacific oysters were obtained in June and July, 2004, from Taylor Resources Quilcene Shellfish Hatchery (TRQSH; www.taylorshellfish.com) in Quilcene, Washington (47°

49' 133" N, 122° 49' 523" W). For Experiments 1 and 2, intact oysters were shipped and sperm were collected by dry stripping of the gonad upon arrival (SOP-3, Appendix A). For other experiments, sperm samples were collected with the same method and placed separately in one 15-mL centrifuge tube per male, and each week 8 to 10 samples (undiluted) were shipped in a foam shipper (SOP-4, Appendix A) 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 Chapter 5), the ploidy level of individual oysters was verified by flow cytometry (Allen, 1983; Dong et al., 2005a). 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 mOsm/kg (Dong et al., 2002) and suspensions were filtered through a 40-µm cell strainer (BD Biosciences Discovery Labware, Bedford, Massachusetts). 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, New York) and derived standard curves (Dong et al., 2005b; Chapter 4). A total of 29 males were used in this study, and each oyster or sperm sample was identified by a code (SOP-1, Appendix A). Sperm motility was estimated visually at 200-× magnification using the method described in SOP-5 (Appendix A).

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 5.1, Chapter 5). All solutions were

prepared within 2 h of use with C-F HBSS at 1000 mOsm/kg as the diluent and were stored at 4 °C. All chemicals used for preparation of solutions were of reagent grade (Sigma Chemical Corporation, St. Louis, Missouri).

Freezing and Thawing Procedures

Freezing with a controlled-rate freezer (Kryo 10 Series II; Planer Products, Sunbury-on-Thames, UK) was conducted as described in SOP-9 (Appendix A), and straws were filled manually by hand (SOP-8, Appendix A). Existing commercial freezing methods developed for dairy bulls (SOP-10, Appendix A) were also evaluated in this study, and straws were filled with an automated straw filler (model MRS 1, IMV Int. Corp., Minneapolis, Minnesota). Thawing for motility estimation and fertilization trials was conducted as described in SOP-12 (Appendix A). General observations of sperm morphology such as broken tails or sperm agglutination (for details see Chapter 7) were also documented for thawed samples.

Fertilization and Larval Evaluation

Ten straws of each treatment were transported in a shipping Dewar (CP35, Taylor-Wharton, Theodore, Alabama) to the TRQSH for percent fertilization and hatch evaluation as described in SOP-13 (Appendix A) within 2 months after freezing.

Experimental Design and Data Analysis

A total of eleven experiments (Figure 6.1) were carried out and the experimental design began with a preliminary evaluation of five cooling rates with four cryoprotectants (Experiment 1). Cooling rates of 5 and 30 °C per min were selected to test on eight cryoprotectants each at two concentrations (Experiment 2). Cooling rate of 5 °C per min was selected for subsequent extensive evaluations of single or combined cryoprotectants at various concentrations. Among eight cryoprotectants tested in Experiment 2, six were selected to form 16 single or combined

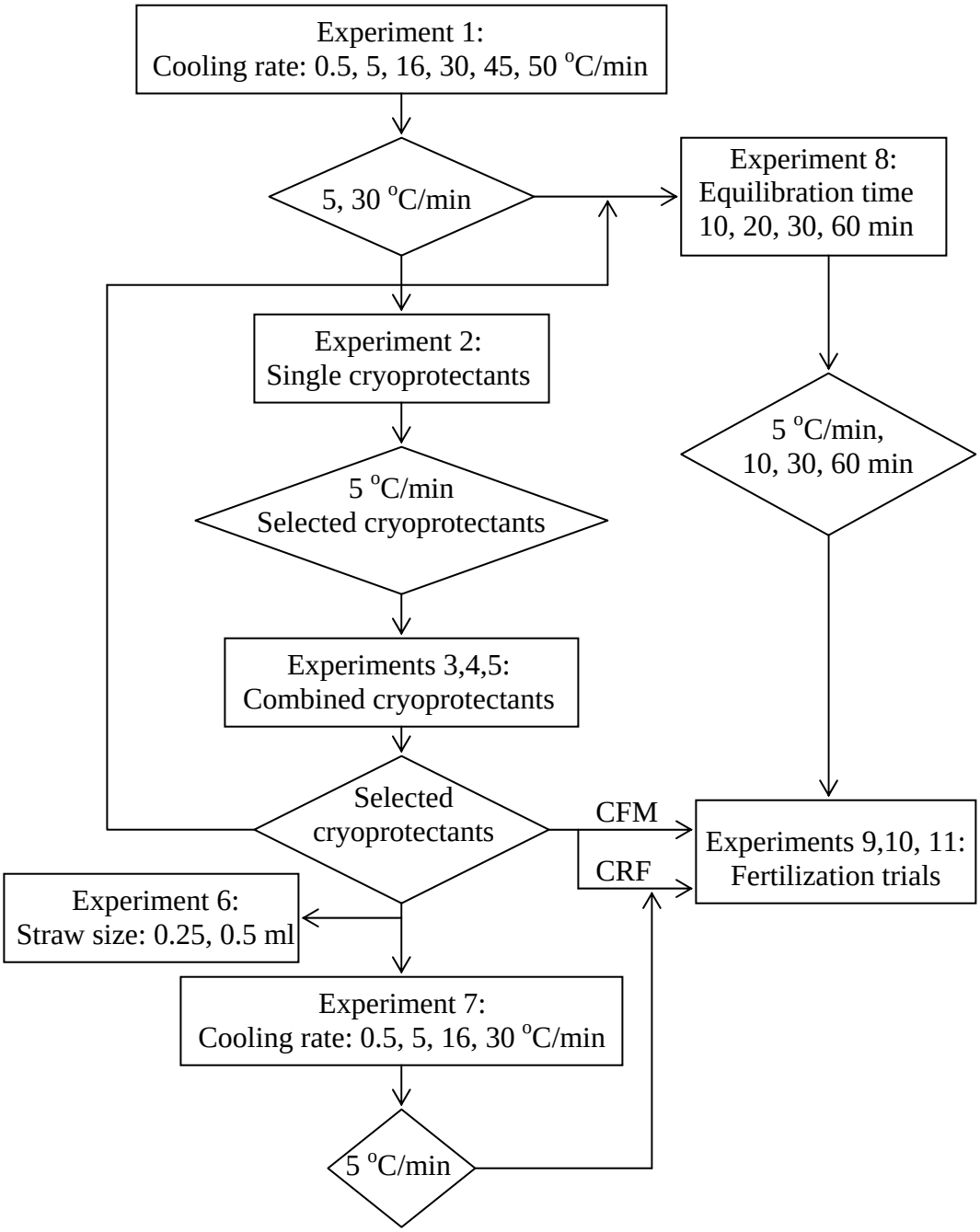

Figure 6.1 Design of experiments for optimization of sperm cryopreservation from tetraploid Pacific oysters. Post-thaw motility was used as the main criterion for procedure optimization and percent fertilization and hatch were used to test the results of optimized procedures. CRF: cooled at 5 °C/min 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, rhomboids indicate decisions made based on experiments.

treatment levels to see possible combinations between different cryoprotectants (Experiment 3). Subsequently, three combination groups (PEG/MeOH, PEG/P-glycol, 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 per treatment per 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 that shown consistently higher post-thaw motility from Experiments 2, 3, 4 and 5 were selected to evaluate straw size (Experiment 6), equilibration time (Experiment 8), and re-evaluate cooling rate (Experiment 7). For all 8 experiments, samples were cooled with the controlled-rate freezer and the criterion used for selection was post-thaw motility. Subsequently, in addition to post-thaw motility, percent fertilization 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 (June 10 to July 7). Due to the large number of treatment factors and levels to be evaluated in initial range-finding experiments within this time constraint, most experiments were replicated only with two males (Table 6.1). However, the experimental design outlined above (Figure 6.1) actually allowed factors such as cooling rate, cryoprotectant, and equilibration time to be repeatedly evaluated in successive experiments (Table 6.1), and in some cases the same treatment (e.g., 6% PEG/4% P-glycol) was tested on as many as 13 males.

Table 6.1 Experimental design and model statement for the eleven experiments.

Experiment	Factor (treatment level)	Male code*	ANOVA	Model statement**
1	Cooling rate (6); Cryoprotectant (4)	35, 36, 37, 38	Two way	$y = \mu + CR + cpa + cr \times cpa + male + \varepsilon$
	Cryoprotectant (8); Concentration (2)			
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	$y = \mu + CR + CPA + CR \times CPA + male + \varepsilon$
	Equilibration time (4); Cryoprotectant (2);			$y = \mu + TIME + cpa + cr + time \times cpa + time \times cr$
8	Cooling rate (2)	101, 102	Three way	$+ cpa \times cr + time \times cpa \times cr + male + \varepsilon$
	Equilibration time (3); combined			
9	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[1]	89, 90, 91	One way	$y = \mu + CPA + male + \varepsilon$
11	Selected cryoprotectant (5) with CFM[2]	93, 94	One way	$y = \mu + CPA + male + \varepsilon$

[1]Cooled at 5 °C/min using a controlled-rate freezer; [2]Cooled using a commercial freezing method developed for dairy bulls
*The full coding would include the designation "CG04M" preceding each number; ***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, lower case letters indicate random factors.

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 6.1). 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, North Carolina). When a significant difference ($\alpha = 0.05$) was observed among treatments, Tukey's Honestly Significant Difference Procedure was used for pair-wise comparisons. Results were presented as means ± SD, and probability values of $P < 0.05$ were considered to be significant. Data for sperm motility, percent fertilization and percent hatch were arcsine-square root transformed prior to analysis (Sokal and Rohlf, 1995).

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 per 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.

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

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 6.2). Sperm suspensions were placed in 0.5-ml straws and cooled at 5 °C per min. Motilities were estimated after suspension and after thawing.

Experiment 4: Effect of Combined Cryoprotectants (Second Selection)

Based on the results of Experiment 3, PEG200 at 0, 2, 4, 6, and 8% was chosen as the non-permeating agent for use in combination with the permeating cryoprotectants MeOH, P-glycol, and DMSO each at 0, 4, 6, or 8% for a total of 48 combinations (Table 6.3). 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, cooled at 5 °C per min, and thawed in a 40 °C water bath for 6 s. Motilities were estimated after suspension and after thawing.

Experiment 5: Effect of Combined Cryoprotectants (Final Selection)

Single or combined cryoprotectants that yielded the highest post-thaw motility at the lowest concentration combinations in Experiment 4 were chose for use in this experiment (Table 6.4). Sperm from two males (CG04M85, 86) were used and suspensions were placed in 0.25-ml straws, cooled at 5 °C per min, and thawed in a 40 °C water bath for 6 s. Motilities were estimated after suspension and after thawing.

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 per min and thawed in a 40 °C water bath 6 s for 0.25-ml straws and 10 s for 0.5-ml straws. Motilities were estimated after suspension and after thawing.

Experiment 7: Effect of Interactions between Cooling Rate and Cryoprotectant

Sperm from two males (CG04M99, 100) were used to re-evaluate the cooling rates of 0.5, 5, 16, and 30 °C per 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 thawed in a 40 °C water bath for 6 s. Motilities were estimated after suspension and after thawing.

Experiment 8: Effect of Equilibration Time

Sperm from two males (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 per min, and thawed in a 40 °C water bath for 6 s. Motilities were estimated after suspension and after thawing.

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 per min. Motilities were estimated after suspension and after thawing, and percent fertilization were estimated as described in SOP-13 (Appendix A).

Experiment10: 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 7 selected single or combined cryoprotectants at a cooling rate of 5 °C per 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 in SOP-13 (Appendix A).

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 in SOP-13 (Appendix A).

Results

Initial Sperm Motility after Shipment

A total of 29 Pacific oysters were transported in 6 shipments from June 10 to July 7, 2004. For the first shipment, intact oysters (n = 7) were shipped and sperm were collected upon arrival. For the other shipments (n = 22), undiluted sperm samples were shipped and the samples were diluted in C-F HBSS immediately prior to freezing. The initial motility ranged from 5 to 95% with the average of 64 ± 25%. The median value was 70%, and the mode was 90% (Figure 6.2). The initial motility of sperm samples collected from shipped oysters (44 ± 25%) had significantly (P = 0.009) lower initial motility than did shipped sperm samples (70 ± 22%).

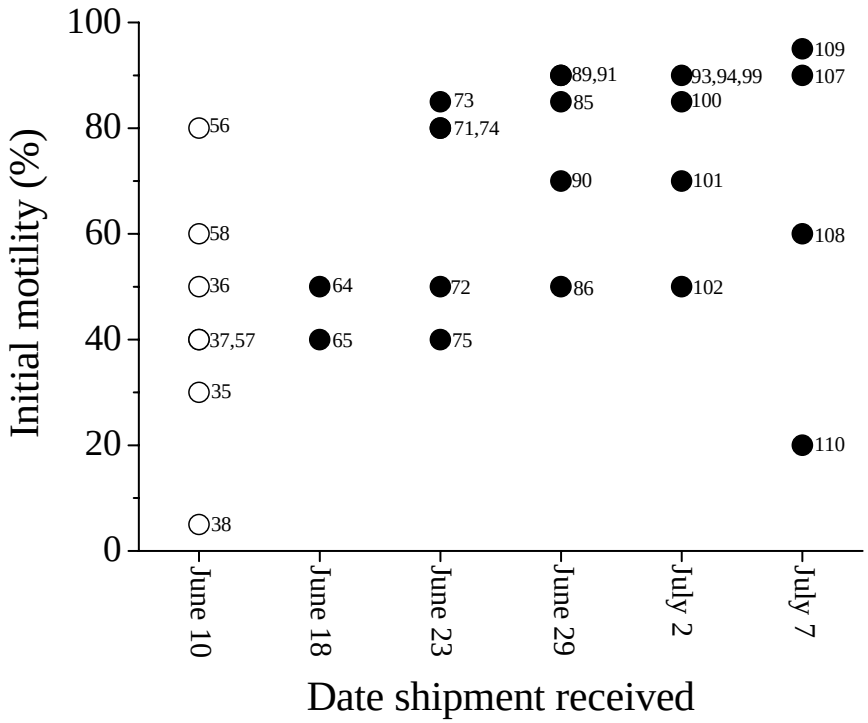

Figure 6.2 The motility of sperm from 29 tetraploid oysters transported in 6 shipments from June 10 to July 7, 2004. Intact oysters (open circles) were transported in the first shipment (n = 7), and undiluted sperm (filled circles) were transported in the other shipments (n = 22). The numbers identified each individual oyster at the order of their usage in experiments (the full coding would include the designation "CG04M" preceding each number).

Experiment 1: Effect of Cooling Rate

Post-thaw motility of sperm cooled at rates ranging from 0.5 to 50 °C per min were significantly different from one another ($P = 0.032$), with the highest post-thaw motility obtained with the rates between 0.5 and 30 °C per min (Figure 6.3). 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 significantly different among males (P=0.032) with the lowest post-thaw motility found for male CG04M36 (< 5% for all treatments). After thawing, the highest motility (8 ± 10%) was found for samples suspended in 10% DMSO and cooled at 5 °C per min. Cooling rates of 5 and 30 °C per min were chosen for subsequent experiments.

Experiment 2: Effect of Single Cryoprotectant

For samples cooled at 5 °C per min (Figure 6.4), significant differences were observed among the eight cryoprotectants ($P = 0.015$), and post-thaw motility of samples suspended in DMSO was found to be significantly higher than the motility of those suspended with glycerol and PEG600. There were no significant 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 significant 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 per min (Figure 6.4), no significant differences were observed in post-thaw motility among the eight cryoprotectants ($P = 0.231$). However, there was a

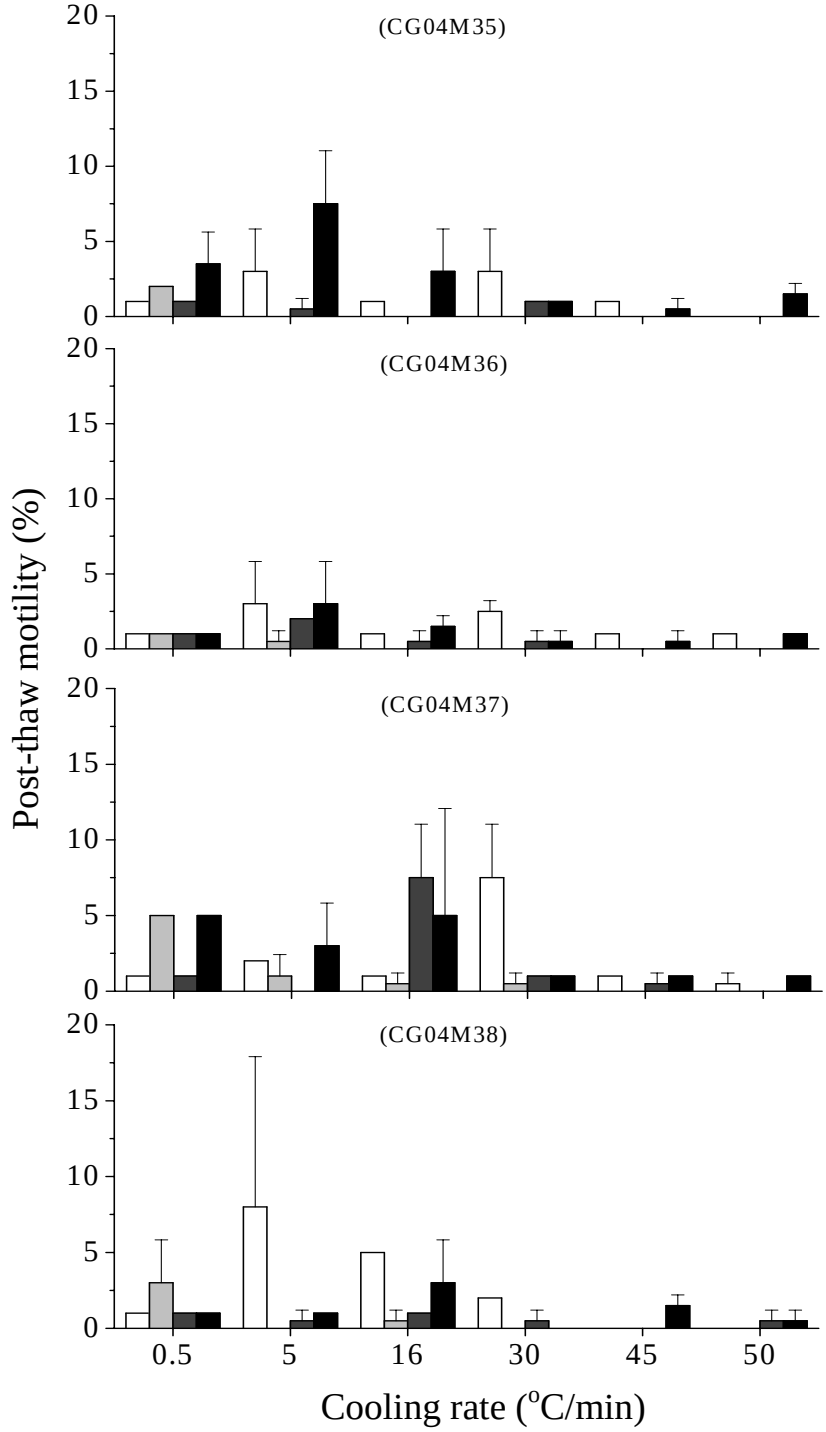

Figure 6.3 Post-thaw motility (mean ± SD) of sperm samples suspended in 10% DMSO (white bars), 10% E-glycol (light gray bars), 5% MeOH (dark gray bars), and 10% P-glycol (black bars), and cooled at 0.5, 5, 16, 30, 45, and 50 °C per min. Four males were used (CG04M35, CG04M36, CG04M37, and CG04M38).

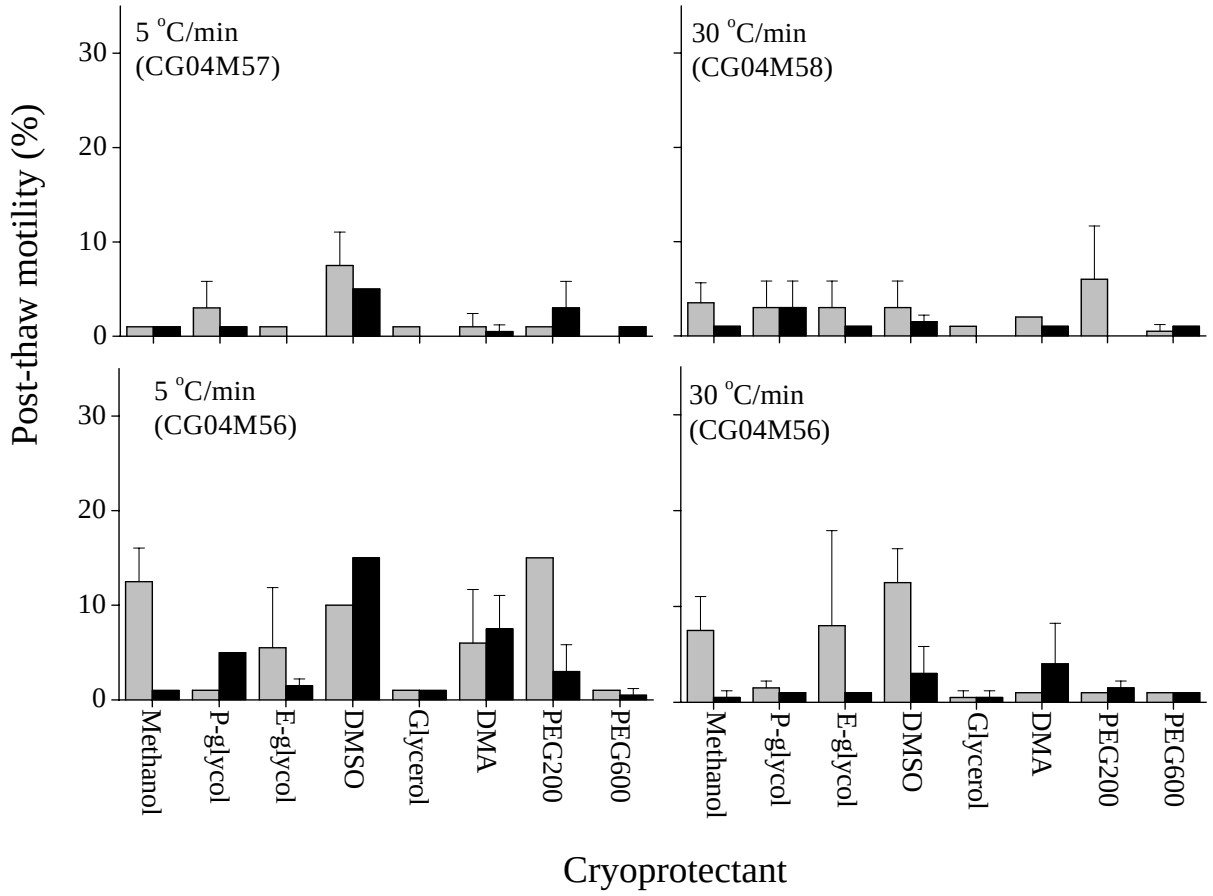

Figure 6.4 Post-thaw motility (mean ± SD) of sperm samples suspended in eight cryoprotectants: MeOH, P-glycol, DMA, DMSO, E-glycol, glycerol, PEG200 and PEG600 each at 5 (light gray bars) and 10% (black bars), and cooled at 5 and 30 °C per min. Three males were used (CG04M56, CG04M57, and CG04M58).

significant difference between 5 and 10% ($P = 0.037$) with 5% yielding higher motility. The interaction between cryoprotectant and concentration ($P = 0.930$) was not significant and post-thaw motility of sperm from the two males ($P = 0.250$) was not significantly different. The highest post-thaw motility (13 ± 4%) was found 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) observed 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.

Experiment 3: Effect of Combined Cryoprotectants (First Selection)

Significant differences ($P < 0.001$) were observed among the 16 single and combined cryoprotectants, with the combined cryoprotectants of PEG/P-glycol (23 ± 10%), PEG/MeOH (20 ± 15%), and PEG/DMSO (15 ± 10%) having the highest post-thaw motility (Table 6.2). post-thaw motility of sperm from the two males were significantly 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.

Experiment 4: Effect of Combined Cryoprotectants (Second Selection)

Significant differences ($P < 0.001$) were found for various concentration combinations within each group of combined cryoprotectants (Table 6.3). 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 did not show significant variation ($P = 0.877$). Of the single cryoprotectants and cryoprotectant combinations

Table 6.2 Post-thaw motility (mean ± SD) of sperm samples suspended in 16 single or combined cryoprotectants and cooled at 5 °C per 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}

Table 6.3 Post-thaw motility (mean ± SD) of sperm samples suspended in single or combined cryoprotectants, and cooled at 5 °C per min.

		Second cryoprotectant (CPA)		
	Second	**MeOH**	**P-glycol**	**DMSO**
PEG 200 (%)	**CPA (%)**	(CG04M74, 75)*	(CG04M71, 72)	(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 significantly different at $P = 0.05$.

*Male numbers.

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 significantly different from one another ($P > 0.050$). Post-thaw motility between two males were significant different ($P < 0.001$). Of the single cryoprotectants and cryoprotectant combinations of PEG200 and DMSO, the highest post-thaw motility was found in samples suspended in 6% PEG/4% DMSO (21 ± 14%), followed by samples suspended in 2% PEG/8% DMSO (18 ± 11%), and 4% PEG/8% DMSO (16 ± 7%). Post-thaw motility between the two males were significant different ($P < 0.001$). Five concentration combinations from each of the three combined cryoprotectants yielded high post-thaw motility at low total concentrations and were chosen to further compare their effectiveness with the same males in the subsequent experiment. PEG200 at 8% was also included as a single cryoprotectant comparison.

Experiment 5: Effect of Combined Cryoprotectants (Final Selection)

Significant differences ($P < 0.001$) were observed among the re-grouped 16 single or combined cryoprotectants with the highest post-thaw motility observed in samples 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 6.4). The two males used in this experiment showed significant variation ($P = 0.004$). Based on the combined results of Experiments 3 through 5, 6% PEG/4% MeOH, 6% PEG/4% P-glycol, 6% PEG/4% DMSO were selected for subsequent experiments, and 6% MeOH, 8% P-glycol, 8% DMSO, and 8% PEG200 were also included as single cryoprotectant comparison.

Table 6.4 Post-thaw motility (mean ± SD) of sperm samples suspended in 16 single or combined cryoprotectants, and cooled at 5 °C per 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 significantly different at $P = 0.05$.

Experiment 6: Effect of Straw Size (0.25-ml versus 0.5-ml)

To evaluate possible effects from straw size differences, 0.25-ml and 0.5-ml straws were compared in this experiment (Figure 6.5). A significant difference in post-thaw motility was not observed for straw sizes ($P = 0.281$) and there was no significant interaction between straw size and cryoprotectant ($P = 0.104$). Significant differences were observed among the five cryoprotectants ($P = 0.002$) and the two males ($P < 0.001$) used in this experiment. 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).

Experiment 7: Effect of Interactions between Cooling Rate and Cryoprotectant

Samples cooled at 5 °C per min yielded the highest post-thaw motility, which was significantly different from that observed for cooling at 0.5, 16, and 30 °C per min ($P < 0.001$) (Figure 6.6). Samples cooled at 16 and 30 °C per min were not significantly different from one another ($P = 0.936$), but these cooling rates yielded higher post-thaw motility than did samples cooled at 0.5 °C per min ($P < 0.001$). Significant effects were also observed 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 significantly 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 per min. Consequently, a cooling rate of 5 °C per min was chosen for subsequent experiments.

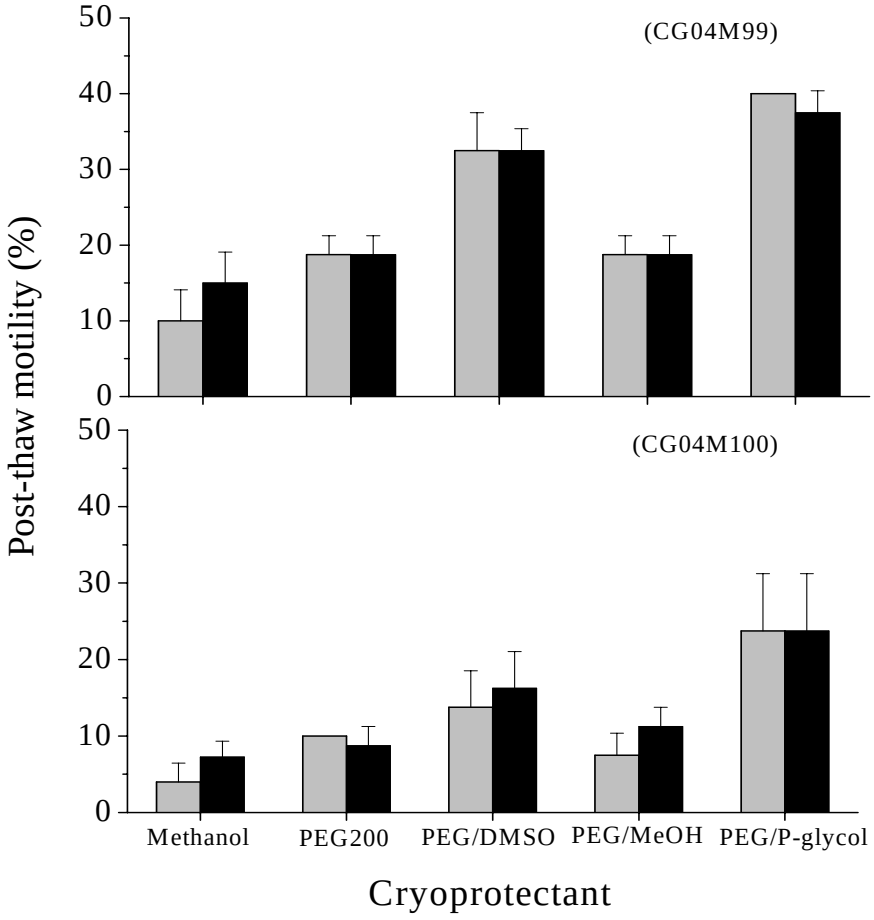

Figure 6.5 Post-thaw motility (mean ± SD) of sperm samples suspended in 6% MeOH, 8% PEG200, 6% PEG/4% DMSO, 6% PEG/4% MeOH, and 6% PEG/4% P-glycol, and equilibrated for 12 min in 0.25-ml (light gray bars) or 0.5-ml straws (black bars). Samples were cooled at 5 °C per min and thawed in a water bath at 40 °C (6 s for 0.25-ml straws, and 10 s for 0.5-ml straws). Two males were used (CG04M99 and CG04M100).

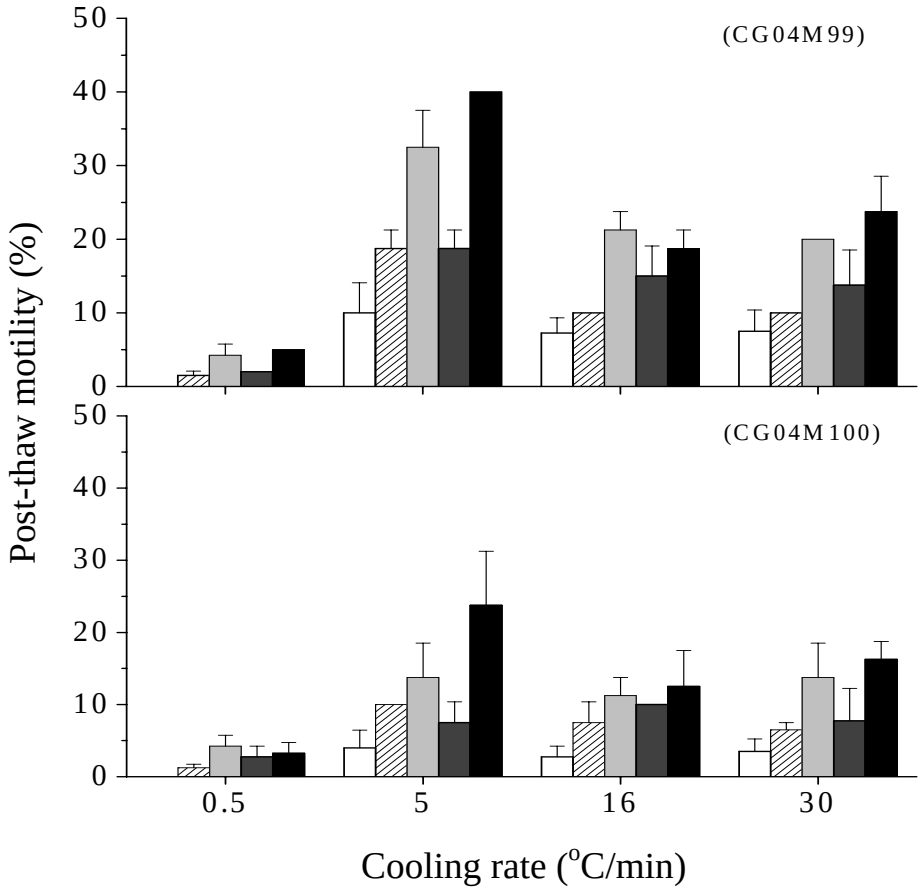

Figure 6.6 Post-thaw motility (mean ± SD) of sperm samples suspended in 6% MeOH (white bars), 8% PEG200 (hatched bars), 6% PEG/4% DMSO (light gray bars), 6% PEG/4% MeOH (dark gray bars), and 6% PEG/4% P-glycol (black bars), and equilibrated for 12 min in 0.25-ml straws. Samples were cooled at 0.5, 5, 16, and 30 °C per min, and thawed in a 40 °C water bath for 6 s. Two males were used (CG04M99 and CG04M100).

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) (Figure 6.7). All interactions among equilibration time, cooling rate, and cryoprotectant were found to be non-significant ($P > 0.100$). Cooling rates of 5 and 30 °C per min were not significantly 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).

Experiment 9: Evaluation of Equilibration Time on Percent Fertilization

Equilibration time of 30 and 60 min were found to not affect post-thaw motility in the previous experiment. To evaluate whether longer equilibration might affect percent fertilization, this experiments compared 10, 30 and 60 min with different cryoprotectants (Table 6.5). For three combined cryoprotectants, no significant differences were observed among equilibration times for post-thaw motility (P = 0.121) or for percent fertilization (P = 0.229). However, higher percent fertilization was observed with longer equilibration time with the highest (30%) found in samples suspended with 6% PEG/4% DMSO with 60 min equilibration. Within cryoprotectants, significant differences were found for post-thaw motility (P = 0.007), but not for percent fertilization (P = 0.608). The interaction between equilibration time and cryoprotectants were not significant for post-thaw motility (P = 0.864) and percent fertilization (P = 0.792). For three single cryoprotectants, although there were no significant differences among the equilibration times for post-thaw motility (P = 0.112), the percentage of fertilization were significantly higher (P = 0.033) in samples with longer equilibration with the highest value (48%) found in samples suspended in 6% MeOH for 60 min (Table 6.5). The interaction between equilibration time and

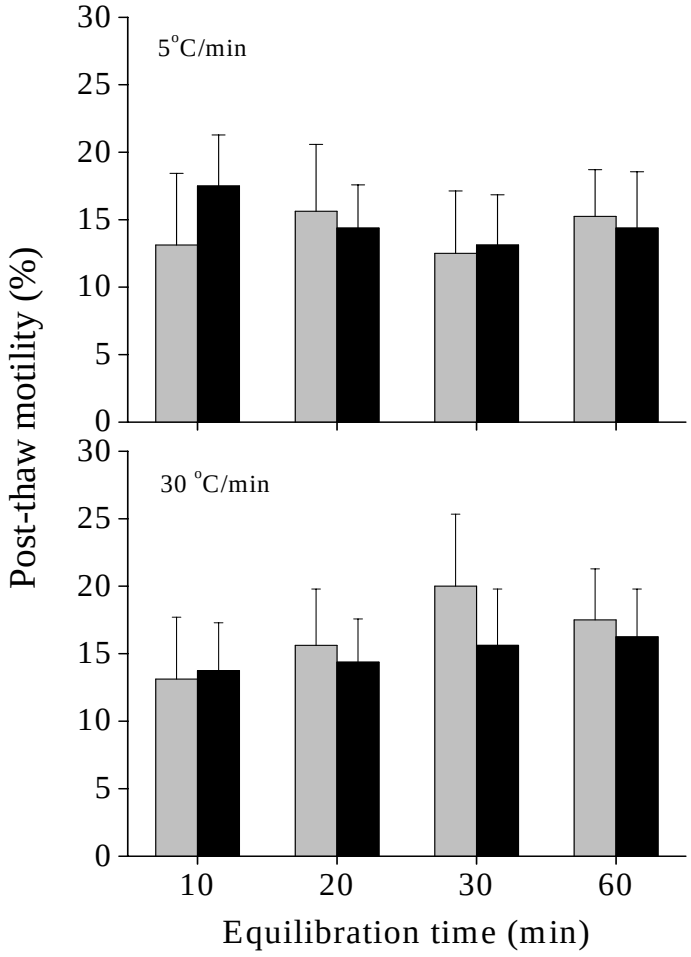

Figure 6.7 Post-thaw motility (mean ± SD) of sperm samples suspended 6% PEG/4% DMSO (light gray bars) and 6% PEG/4% P-glycol (black bars), equilibrated for 10, 20, 30 and 60 min, and cooled at 5 and 30 °C per min. Two males were used (CG04M101 and CG04M102).

Table 6.5 Post-thaw motility (mean ± SD) and percent fertilization of sperm samples suspended in various cryoprotectants, equilibration for 10, 30, and 60 min, and cooled at 5 °C/min using a controlled-rate freezer.

Male		6% PEG/4% MeOH			6% PEG/4% P-glycol			6% PEG/4% DMSO		
	Criterion	**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 significantly different at $P = 0.05$.

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 6.5). Fresh eggs without any sperm addition yielded 0% fertilization.

Experiment 10: Evaluation of Selected Cryoprotectants on Percent Fertilization

For a cooling rate of 5 °C per min with the controlled-rate freezer (Table 6.6), 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 significantly higher than those of the other cryoprotectants although significant ($P < 0.001$) variation was observed 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 significant difference in percent fertilization among all cryoprotectants ($P = 0.810$).

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

Table 6.6 Post-thaw motility (mean ± SD), and percent fertilization and hatch of sperm samples 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 per min with a controlled-rate freezer.

Male	**Criterion**	**PEG/MeOH**	**MeOH**	**PEG/P-glycol**	**P-glycol**	**PEG/DMSO**	**DMSO**	**PEG200**
Control*	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 significantly different at $P = 0.05$
*Fresh sperm expose to the same treatments as the thawed sperm (same cryoprotectants, concentration, equilibration time and batch of eggs).

motility and fertilization (Table 6.7). The treatment effects of different cryoprotectants were found to be significant for post-thaw motility ($P < 0.001$) despite the low values, but were found to be non-significant for percent fertilization ($P = 0.504$), which may be due to the significant male variation ($P = 0.002$) observed for the two males used in this experiment.

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 6.6), 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.

Table 6.7 Post-thaw motility (mean ± SD), percent fertilization of sperm samples 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 significantly different at $P = 0.05$.

Discussion

Similar to previous findings (Dong et al., 2005a), 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 time (Chapter 5). For the limited number of fertilization trials tested, percent fertilization was low in general and the highest value was 48%. However, post-thaw motility was improved compared to earlier studies (Dong et al., 2005a), 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 5-50% 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 (Chapter 3). 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, which 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 (Chapter 3), 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 (He et al., 2004), 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, 50 °C per min indicated an optimal range of 0.5 to 30 °C per min, and subsequent attempts with optimized cryoprotectants and concentrations showed that 5 °C per min was significantly better than other rates in retaining post-thaw motility, which agreed with the findings for sperm from diploid oysters (Chapter 5). 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. Various optimal cooling rates were reported in previous studies

of sperm from diploid oysters (Chapter 2). However, as stated in Chapter 5, 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.

Sperm from tetraploids were found to be more negatively affected by the cryopreservation process than were sperm from diploids (Dong et al., 2005a), 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 (Meryman, 1971). 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 were found to yield 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. 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.

Despite the fact that sperm from a large oyster (e.g., with a shell height ~ 100 mm) could produce more than 200 0.5-ml straws with a sperm concentration of 1×10^9 cells mL^{-1}, straws of small volume such as 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). *Crassostrea gigas* is a protandric species, in which at first maturation, they 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 10 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 (Dong et al., 2005a). Similar to findings for sperm from diploid oysters, straw sizes of 0.25-ml and 0.5-ml did not show significant differences in retaining post-thaw motility.

Long equilibration times of 30 and 60 min were found to yield higher post-thaw motility for sperm of diploid oysters (Chapter 5). 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 showed 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 straw filling if done manually. In addition, long equilibration times (30 min to 6 h) with stepwise addition of cryoprotectants is a routine practice in freezing facilities for sperm of dairy bulls (Chandler, 2000). 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 6.2). Observations of male-to-male variation are not limited to sperm of Pacific oysters (this study and Chapter 5) and eastern oysters *C. virginica* (Paniagua-Chavez and Tiersch, 2001), but has been reported in other aquatic species such as zebrafish *Danio rerio* (Harvey et al., 1982), rainbow trout *Oncorhynchus mykiss* (Stoss and Holtz, 1983), Atlantic halibut *Hippoglossus hippoglossus* (Bolla et al. 1987), and sea urchin *Evechinus chloroticus* (Adams et al., 2004). It is important to note that male-to-male variation is also well recognized in mammalian species such as dogs, bulls, boars, stallions, and humans (Leibo and Bradley, 1999; Holt, 2000). 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 (Thurston et al., 2002). Individual variations in fish sperm cryopreservation have been attributed to genetic variability, membrane quality, collection techniques (thus contamination with urine), and seasonality (for reviews see Rana, 1995; Maisse, 1996; Suquet et al. 2000). As a result, pooling of milt from males has been practiced routinely in studies of fish semen cryopreservation to reduce individual variation (Ciereszko et al., 2000). 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 (SOP-3, Appendix A) 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 confirm hypotheses that apply to oyster sperm or sperm from other aquatic species in general.

Compared to sperm from diploid oysters (Chapter 5), 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 (Dong et al., 2005a). It appears that sperm from tetraploids have a higher sensitivity to the 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. Based on the classification of sperm tolerance proposed in Chapter 5, sperm from tetraploids could be considered as having a narrow tolerance 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.

Studies with mouse sperm cryopreservation showed that 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 (Songsasen and Leibo, 1997). 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 (Gao et al., 1995). 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 (Lahnsteiner et al., 1996; Warnecke and Pluta, 2003; He and Woods, 2004). In diploid Pacific oysters, cryopreserved sperm are thought to be 30- to 100- fold less fertile than fresh sperm, and a sperm-to-egg ratio ranging from 1,600 to 5,000 was required to produce 50% fertilization for cryopreserved sperm (Adams et al., 2004). In this project with sperm of diploid (Chapter 5) 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 (Dong et al., 2005b). 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, 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 to 100% sperm availability after thawing, Chapter 7) and the percentage of post-thaw motility for sperm from diploid oysters (2 to 70% with the average of 29%, Chapter 5), the actual sperm-to-egg ratio would be in a range of 60-7,000 sperm per egg. Within this range, percentage fertilization as high as 98% were obtained with thawed samples in sperm from diploid oysters (Chapter 5). 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 found to be more negatively affected by cryopreservation. The fact that combinations of non-permeating and permeating cryoprotectants were found to be more effective in retaining post-thaw motility than were permeating compounds alone provides presumptive evidence for the interpretation that plasma membrane properties plays 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. Difference 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 ration is still required and may help to increase the percentage of fertilization.

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Chapter 7
Agglutination of Sperm from Diploid and Tetraploid Pacific Oysters upon Cryopreservation

In previous studies, cryopreserved sperm from diploid and tetraploid oysters were found to agglutinate after thawing with the formation of elongated noodle-like structures in extreme cases (Dong et al., 2005a). Sperm agglutination in thawed samples has been observed in fish species such as common carp *Cyprinus carpio* (Sneed and Clemens, 1956; Miskolczi et al., 2005) and Atlantic croaker *Micropogonias undulates* (Gwo and Arnold, 1992). In oysters, this phenomenon was mentioned in studies of the Pacific oyster *Crossostrea gigas* (Bougrier and Rabenomanana, 1986; Kurokura et al., 1990; Adams et al., 2004) and eastern oyster *C. virginica* (Hughes, 1973). 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 cases 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 for various species, no attempts have been made to explain this phenomenon. In addition, agglutination is generally considered as a negative outcome, and often thought to be an indicator of failure in cryopreservation (Bougrier and Rabenomanana, 1986; Kurokura et al., 1990). 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 (Dong et al., 2005a). The fact that sperm agglutination did not necessarily indicate a reduction in fertility was also reported in common carp (Miskolczi et al., 2005).

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 in a qualitative scale), and to evaluate the effect on sperm agglutination scale of: (2) interaction among cooling, cryoprotectant (type and concentration), and thawing; (3) thawing method versus dimethyl sulfoxide (DMSO) concentration; (4) sperm concentration versus DMSO concentration, and compare (5) the morphology of sperm agglutination from samples frozen with and without addition of cryoprotectant.

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 (Allen, 1983; Dong et al., 2005a). Sperm were collected by dry stripping of the gonad (SOP-3, Appendix A) and suspended in calcium-free Hanks' balanced salt solution (C-F HBSS) at 1000 mOsm/kg (Dong et al., 2002), 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 from a spectrophotometer (Genesys™ 20, Thermo Spectronic, Rochester, New York) and derived standard curves (Dong et al., 2005b). Sperm motility was estimated visually at 200-× magnification using darkfield microscopy (Optiphot 2,

Nikon Inc., Garden City, New York) and was expressed as the percentage of cells actively moving in a forward direction (SOP-5, Appendix A).

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) was conducted as described in SOP-9 (Appendix A), and straws were filled manually by hand (SOP-8, Appendix A). For cooling rates of 1, 5, and 16 ^{o}C per min, samples were cooled in two steps, initially to -30 ^{o}C at these rates, followed by cooling at 45 ^{o}C per min from –30 ^{o}C to –80 ^{o}C. For cooling rates of 45 and 50 ^{o}C per min, samples were cooled in a single step from 5 ^{o}C to –80 ^{o}C directly at the specified rate. All straws were held at –80 ^{o}C for 5 min before being plunged into liquid nitrogen in a storage Dewar. Existing commercial freezing methods developed for dairy bulls (SOP-10, Appendix A) were used in the present study, and straws were filled with an automated straw filler (model MRS 1, IMV Int. Corp., Minneapolis, Minnesota).

Thawing procedures was described in SOP-12 (Appendix A). 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, Illinois) was performed at various temperatures for different time periods: 13 s at 20 ^{o}C, 7 s at 40 ^{o}C, 6 s at 60 ^{o}C, and 5 s at 80 ^{o}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 tissue culture dishes (35 x 10 mm) (Falcon, Becton Dickinson, Franklin Lakes, New Jersey), and the degree of sperm agglutination was classified by visual observation into six levels based on the descriptive criteria for each level (Table 7.1). Examples of each level were photographed using a digital camera (Coolpix 5700, Nikon).

Table 7.1 Description for sperm agglutination at 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

Sperm Counts after Thawing

For each agglutination classification, the number of sperm that were not agglutinated (e.g., groups of two or more agglutinated sperm were not counted) was estimated with a hemacytometer (Hausser Scientific, Horsham, Pennsylvania) and the average of duplicate counts were used (SOP-6, Appendix A). 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 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 5 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. All treatment combinations were evaluated with sperm samples from diploid (one male) and tetraploid (milt pooled from 5 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 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.

Effect of Thawing and DMSO Concentration

Sperm samples in this experiment were equilibrated with DMSO at 2, 5, 8, 10, 12, 15, 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 2 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.

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 (Chapter 5) or without any addition of cryoprotectant. Samples were thawed at RT and fixed with 4% glutaraldehyde in 0.1M sodium cacodylate buffer (CB, pH 7.4) with the adjusted osmolality of 1000 mOsmol/kg. The subsequent steps followed those described for sample preparation for transmission electron microscopy in Chapter 3. Cross sections (0.5 μm) were cut with a DuPont Sorvall microtome (MT-2, Ivan Sorvall Inc., Norwalk, Connecticut), and 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, Missouri), covered with cover slides, and sealed with Permount (Fisher Scientific, Fairlawn, New Jersey). Slides were examined at 200 and 800-× magnification with light microscopy. Digital images were captured through a diagnostic instrument CCD camera (SPOT RT Slider, SpectraCore, Inc. Webster, New York). The oyster used in this experiment was received on August 13, 2004.

Effect of Sperm Agglutination on Percent Fertilization

Fertilization data collected during July 2004 were used to evaluate the effect of sperm agglutination on percent fertilization. For fertilization trials (SOP-13, Appendix A), agglutinated samples were crushed immediately after thawing and poured onto eggs. A total of 87 data points

from 22 diploid oysters and 99 data points from 19 tetraploid oysters were sorted into the 6 agglutination levels defined in this study based on the sample appearance after thawing.

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 agglutination scale was reported as discrete numbers, they were ordinal variables (categorical variables having ordered scales). They 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, North Carolina). 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 (Sokal and Rohlf, 1995).

Results

Agglutination Scale

Sperm samples after thawing showed various degree of agglutination (Table 7.1), ranging from homogenous suspensions (Figure 7.1 A) to the formation of elongated “noodles” in extreme cases (Figure 7.1 B).

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 sperm available after thawing with only 8% available for samples with a level of 5 (Table 7.1). For agglutination levels of 4 and 5, sperm counts were increased after crushing the aggregates, especially for samples with an agglutination level of 5, in which a 25% increase in sperm number was observed after crushing (Table 7.2).

Table 7.2 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%	--*	--
1	74%	--	--
2	75%	--	--
3	55%	--	--
4	40%	45%	5%
5	8%	33%	25%

*Not evaluated (only evaluated elongated clumps).

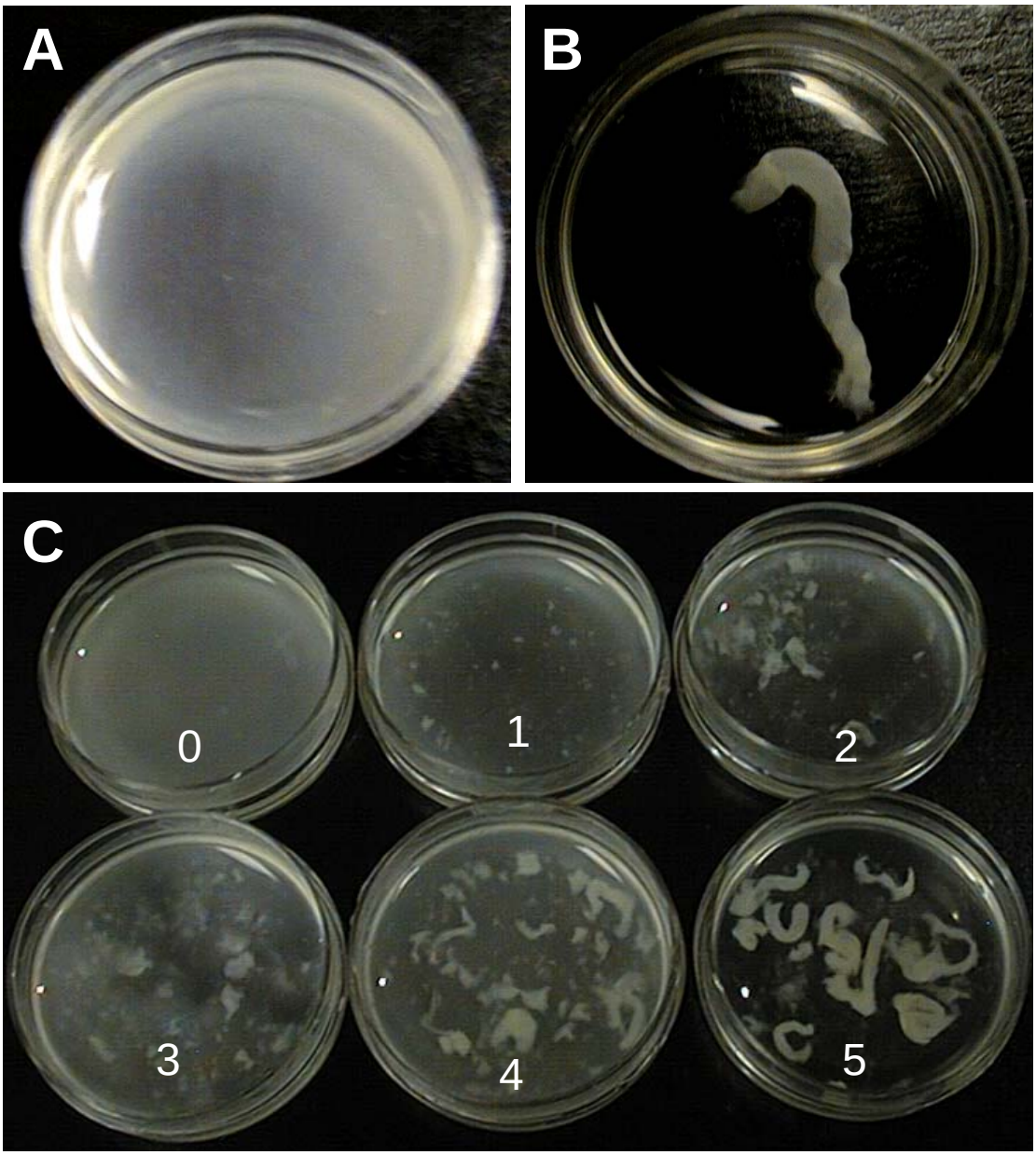

Figure 7.1 The appearance of sperm samples after thawing. A: homogenous suspension; B: elongated “noodle”; C: six levels of sperm agglutination: 0, homogeneous suspension; 1, few clumps discernable; 2, many clumps evident; 3, aggregation of clumps; 4, formation of elongated clumping ("noodles"); 5, formation of well-developed noodles. (All tissue culture dishes were the same size with a diameter of 35 mm).

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 7.3). 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 (Figures 7.2 and 7.3). Cryoprotectants at a concentration of 5% (Figure 7.2) were also found to yield higher levels of sperm agglutination than at 10% (Figure 7.3). 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 and 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 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 (Figure 7.4). 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

Table 7.3 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 x Cryoprotectant	12	0.0001
Cooling rate x Concentration	4	0.0148
Cooling rate x Thawing	4	< 0.0001
Cryoprotectant x Concentration	3	< 0.0001
Cryoprotectant x Thawing	3	< 0.0001
Concentration x Thawing	1	< 0.0001
Cooling rate x Cryoprotectant x Concentration	12	0.2743
Cooling rate x Cryoprotectant x Thawing	12	0.0005
Cooling rate x Concentration x Thawing	4	< 0.0001
Cryoprotectant x Concentration x Thawing	3	< 0.0001
Cooling rate x Cryoprotectant x Concentration x Thawing	12	0.1223
Ploidy	1	0.0616
Error	159	

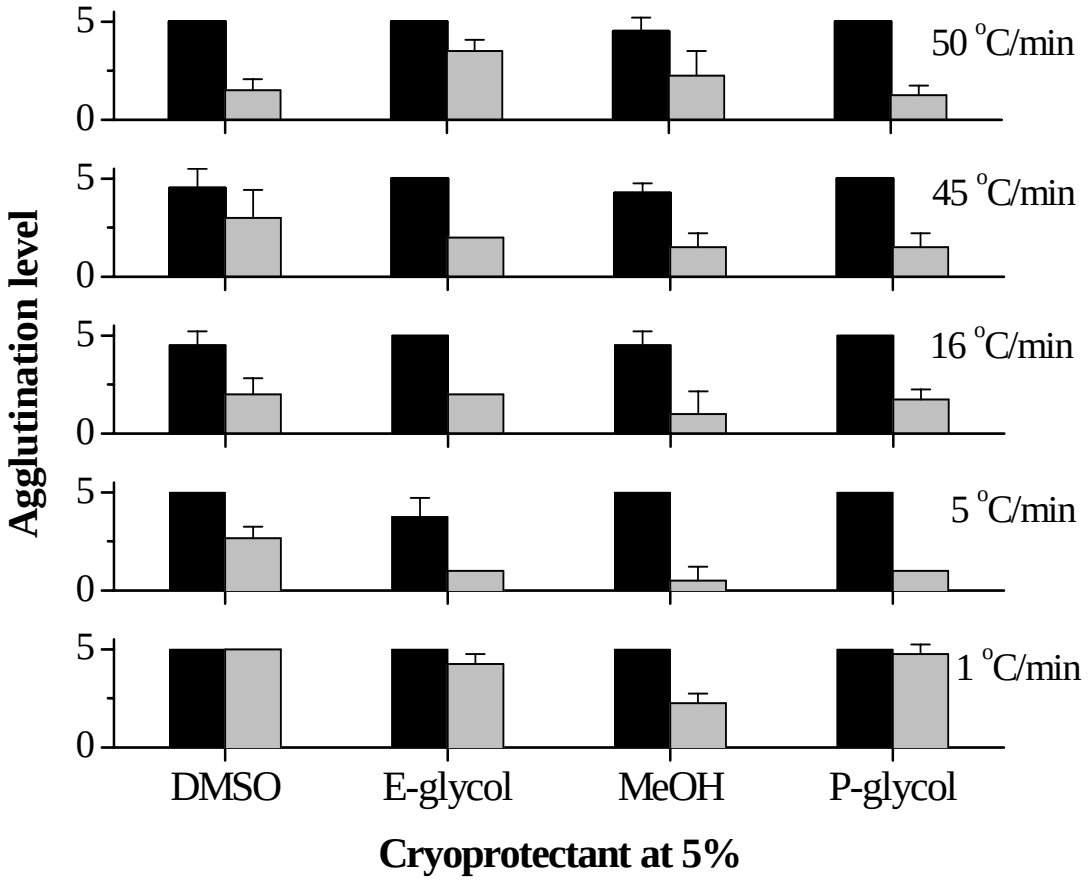

Figure 7.2 Agglutination level of sperm samples equilibrated with dimethyl sulfoxide (DMSO), ethylene glycol (E-glycol), methanol (MeOH), and propylene glycol (P-glycol) at 5%, cooled at 1, 5, 16, 45, and 50 °C per min with a controlled-rate freezer, thawed in the air at room temperature for 4 min (black bars) or in a water bath of 40 °C for 7s (light gray bars).

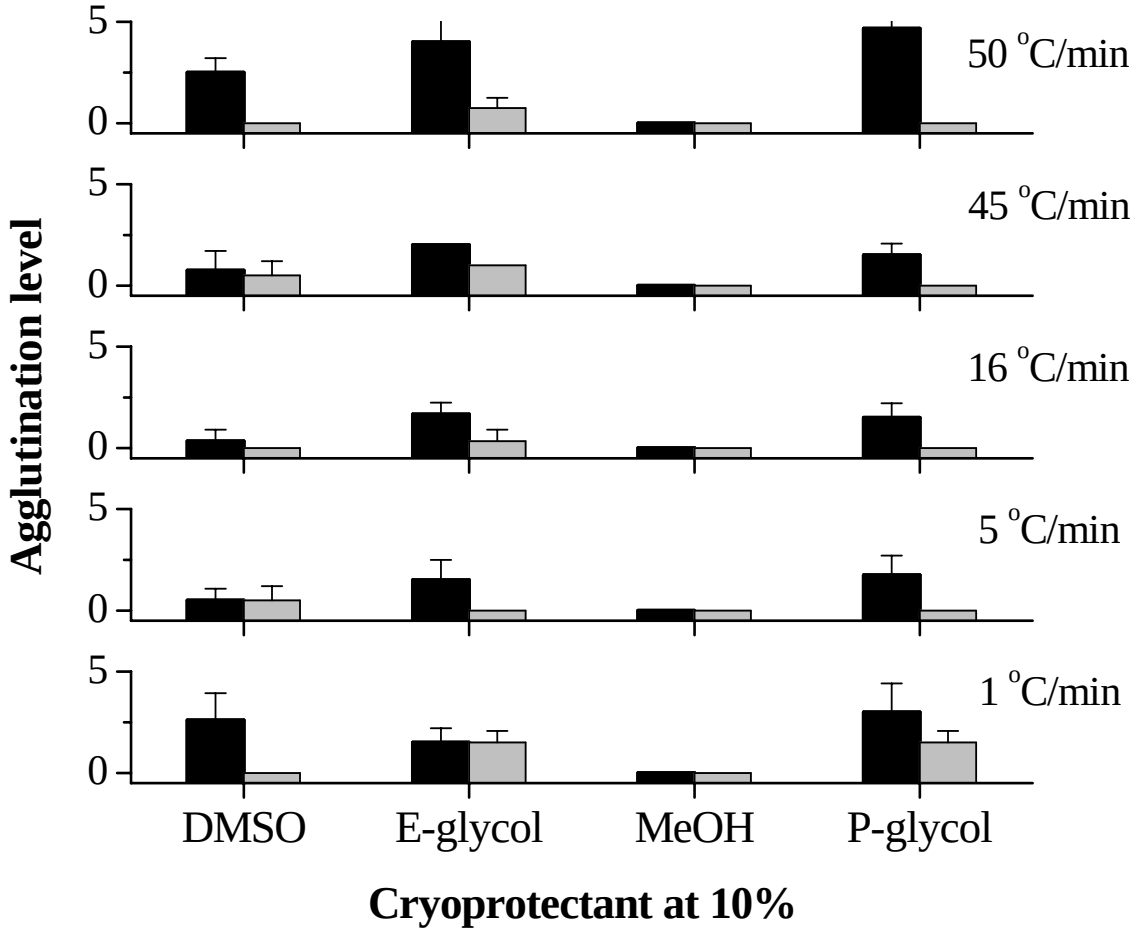

Figure 7.3 Agglutination level of sperm samples equilibrated with dimethyl sulfoxide (DMSO), ethylene glycol (E-glycol), methanol (MeOH), and propylene glycol (P-glycol) at 10%, cooled at 1, 5, 16, 45, and 50 °C per min with a controlled-rate freezer, thawed in the air at room temperature for 4 min (black bars) or in a water bath of 40 °C for 7s (light gray bars).

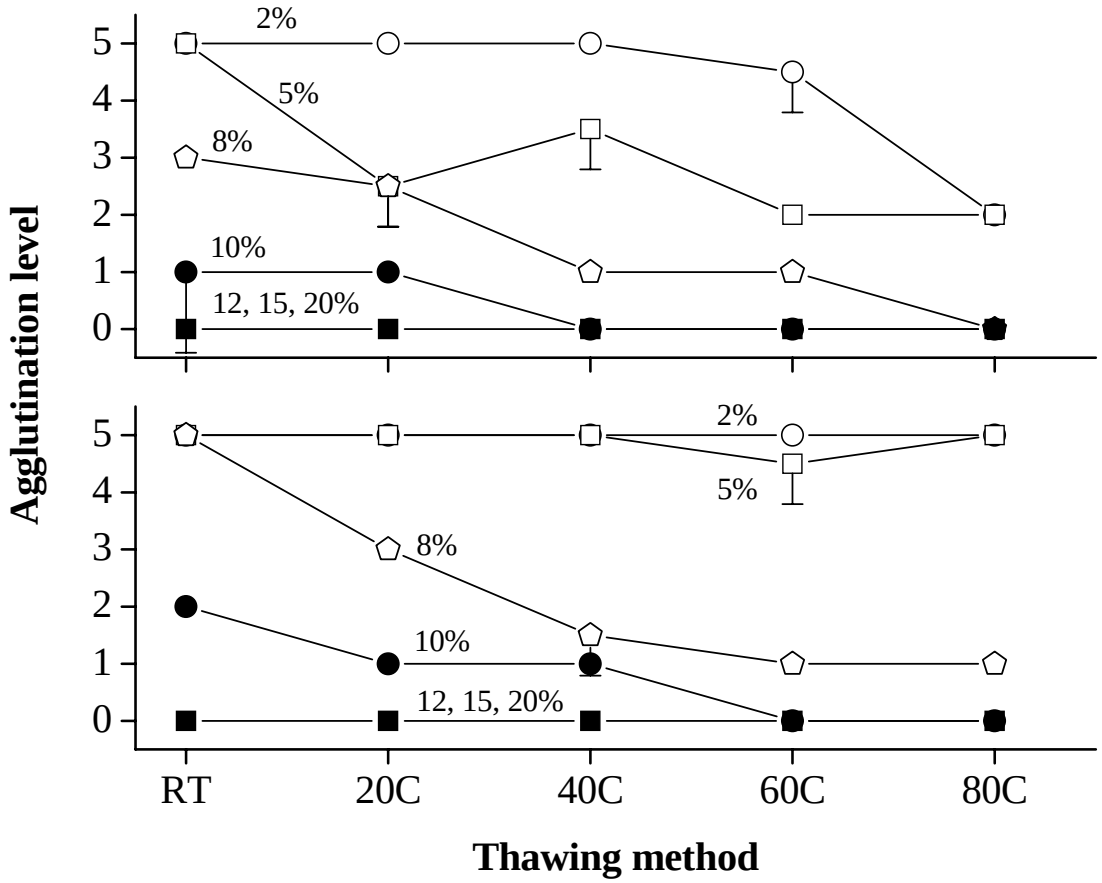

Figure 7.4 Agglutination level (mean ± SD) of sperm samples cryopreserved with dimethyl sulfoxide at 2, 5, 8, 10, 12, 15 and 20%, thawed in the air at room temperature for 4 min (RT), or in a water bath at 20 °C for 13 s (20C), 40 °C for 7 s (40C), 60 °C for 6 s (60C), and 80 °C for 5 s (80C). The upper panel is diploid samples; the lower panel is tetraploid.

($P = 0.0035$), and samples from tetraploid oysters equilibrated with 2% DMSO consistently showed levels of 5 regardless of thawing rate (Figure 7.4).

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 (Figure 7.5). Agglutination levels of samples with sperm 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 (Figure 7.5). 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 (Figure 7.6). 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

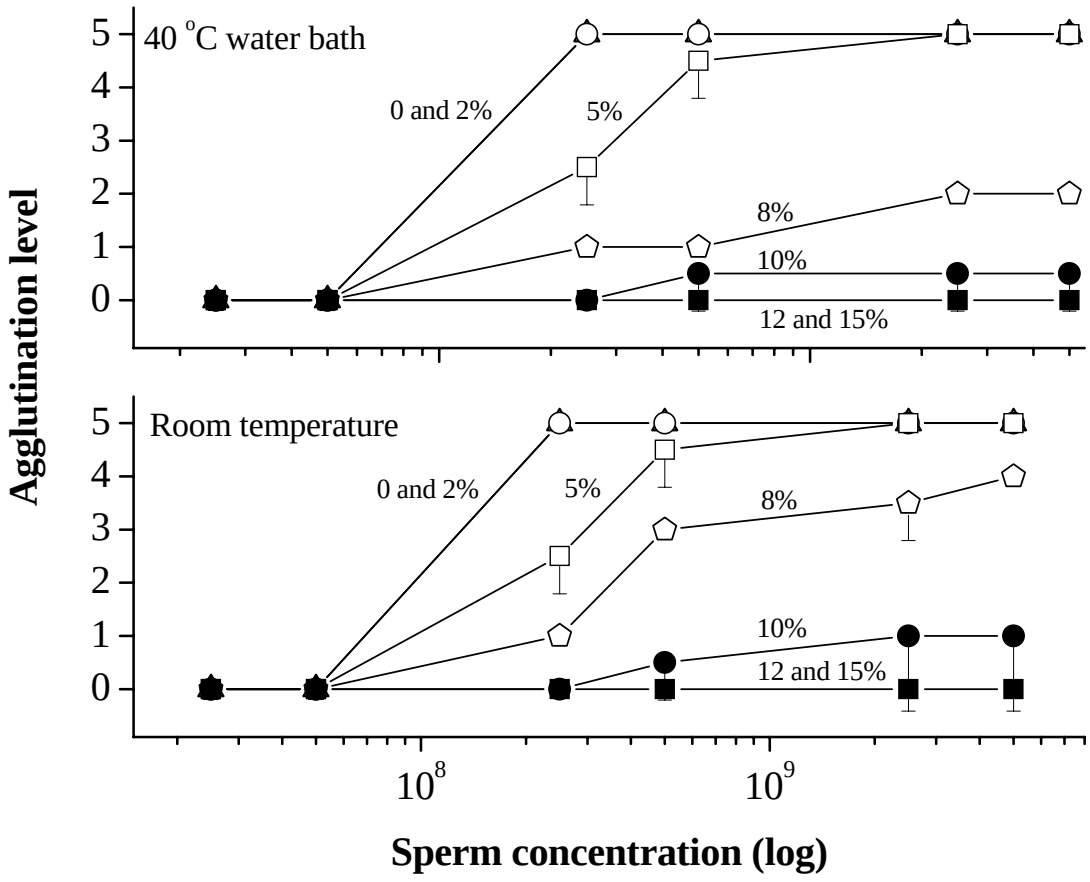

Figure 7.5 Agglutination level (mean ± SD) of sperm samples from diploid oysters at sperm concentrations of 5 x 10^9, 2.5 x 10^9, 5 x 10^8, 2.5 x 10^8, 5 x 10^7, 2.5 x 10^7 cells mL^{-1}, cryopreserved with dimethyl sulfoxide at 0, 2, 5, 8, 10, 12, and 15%, thawed in the air at room temperature for 4 min, or in a water bath at 40 °C for 7 s.

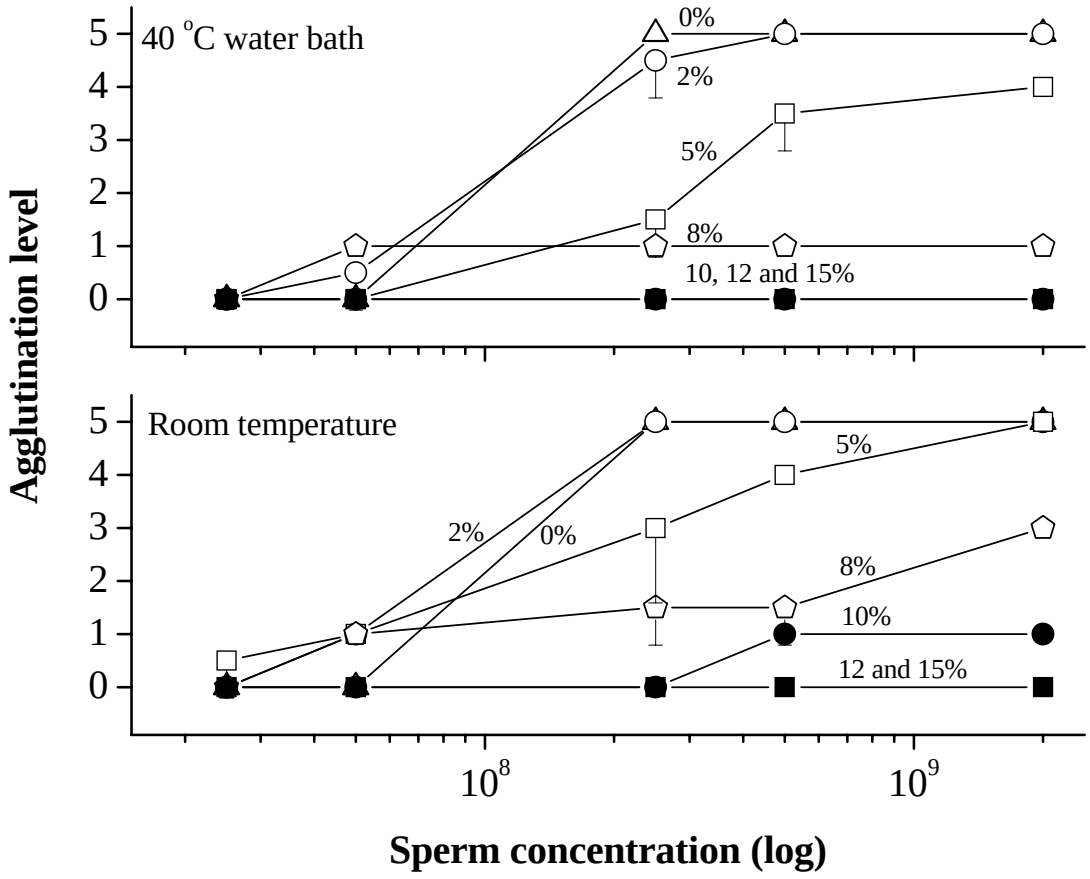

Figure 7.6 Agglutination level (mean ± SD) of sperm samples from tetraploid oysters at sperm concentrations of 2 x 10^9, 5 x 10^8, 2.5 x 10^8, 5 x 10^7, 2.5 x 10^7 cells mL^{-1}, cryopreserved with dimethyl sulfoxide at 0, 2, 5, 8, 10, 12, and 15%, thawed in the air at room temperature for 4 min or in a water bath at 40 °C for 7 s.

level of zero were observed for samples with 2.5 x 10^7 cells mL^{-1} and which was significantly lower than for those with 5 x 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 fixed. The initial sperm motility was 50% (diploid) in this experiment. Morphological differences of the cross sections of "noodles" formed with (Figure 7.7 A, B) and without (Figure 7.7 C, 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 Figures 7.7 A, B), and the majority of sperm heads retained their round shapes and appeared distributed randomly (Figure 7.7 B). On the contrary, "noodles" formed without the addition of cryoprotectant were characterized with large empty areas formation (arrows in Figures 7.7 C, D) and lysed sperm with few discernable sperm heads (Figure 7.7 D). The empty areas observed in the cross sections (arrows in Figure 7.7) could be due to formation 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 7.4). For sperm from diploid oysters, there was no significant difference in percent fertilization among the 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 the difference among the six levels.

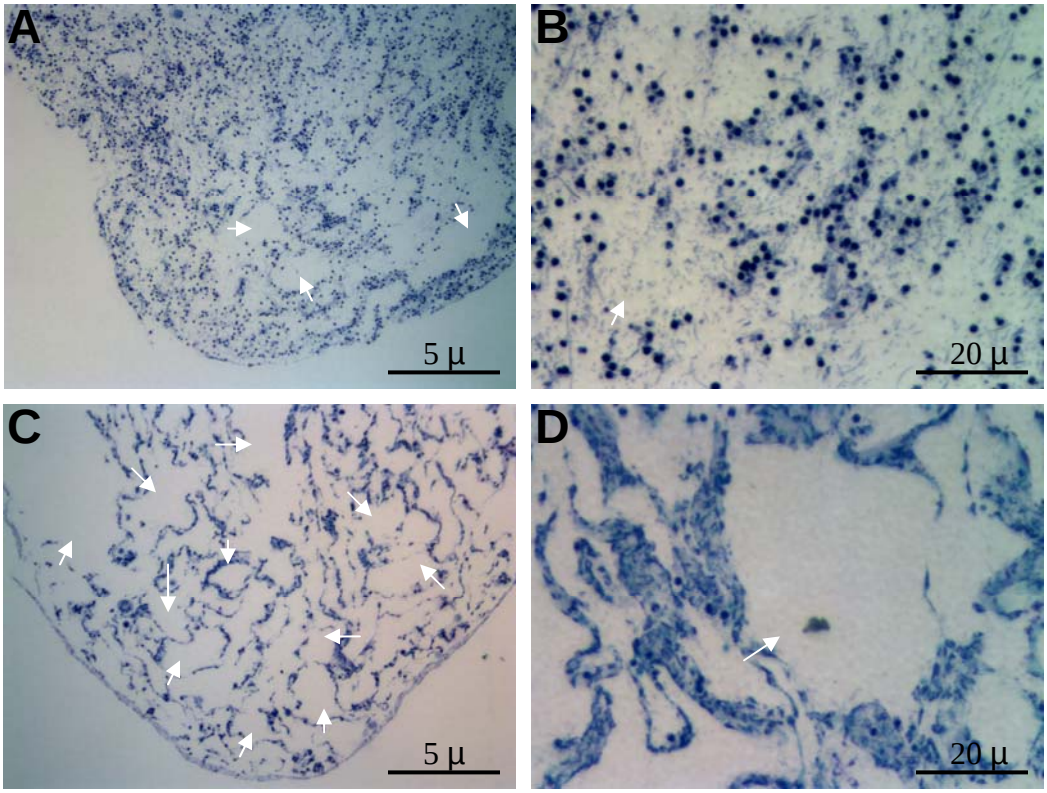

Figure 7.7 Microscopic examination of diploid oyster sperm samples with "noodle" formation (agglutination level of 5) after thawing. Cross sections of "noodles" formed in samples with the combination of cryoprotectants (4% methanol and 2% polyethylene glycol) at 200-× magnification (A) and at 800-× (B), 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 the freezing process.

Table 7.4 Percentage fertilization of samples from diploid and tetraploid oysters with different agglutination levels after thawing. There was no significant difference in percent fertilization among the six agglutination levels for sperm from diploids ($P = 0.1659$), but significant differences were found in sperm from tetraploids ($P = 0.0385$).

	Diploid			Tetraploid		
Level	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

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 6 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, 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 (Gwo and Arnold, 1992).

The effects of cryoprotecant (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 sperm (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 (Bougrier and Rabenomanana, 1986) and common carp (Sneed and Clemens, 1956). 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 (Figure 7.4). 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 the 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 (Gwo and Arnold, 1992). The fact that sperm agglutination level varied with different treatment factors or their combinations confirmed the importance of protocol optimization for sperm cryopreservation with multiple factors involved.

For specific species, inconsistency among various studies is often related to cryoprotectant concentration rather than the type of cryoprotectant, which 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 (Adams et al., 2004; for review see Chapter 2). 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 was reflected by the fact that the majority of studies in oysters do not standardize sperm concentration prior to freezing, instead, using dilution ratios based on volume (Chapter 2). In two studies of *C. gigas* comparing DMSO concentrations among 5, 10, and 15% (Smith et al., 2001) and 5, 8, and 10% (Dong, unpublished data) each suggested the optimal DMSO concentration to be 5% when the same sperm concentration (1×10^9 cells mL^{-1}) was used for freezing.

Samples frozen at high sperm concentration (e.g., $> 1 \times 10^8$ cells mL^{-1}) with low cryoprotectant concentrations (e.g., 2% DMSO) or without addition of any cryoprotectant were both found to form the highest level of sperm agglutination (noodles) when thawed at sub-optimized conditions such as thawed in the air at room temperature. However, microscopic

examination revealed that these two types of noodles were different. Agglutination formed without addition of cryoprotectant was found to be associated with the formation of large open areas and sperm that exhibited morphological damages. On the contrary, cross sections of agglutination formed with addition of cryoprotectant at low concentrations showed sperm heads that retained their round shapes, and few large open areas present. This observation provides a direct evidence of cryoprotective properties of the chemicals for the sperm morphology during freezing and thawing. Micrographic differences of the cross sections from these two types noodles (treatment with vs. without cryoprotectant) also suggested that variations in other treatment factors such as cooling rate, different types of cryoprotectants may also could be detected by using the same technique, although 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 erythrocytes (http://www.asymptote.co.uk/gallery/erythrocytecryopreservation), in which a cross fracture of the straw (0.25-ml) followed by deep etching reveals the structure of ice (etched areas) and of the freeze-concentrated materials including erythrocytes (non-etched material). The etched areas would correspond to the open areas in this study, suggesting the presence of ice crystal formation during the freezing or thawing processes. Freeze fracture is the only technique that enables the visual observation of samples within the micro-environment of the straw, and samples observed are in the frozen state due to this technique. Sperm agglutination, specifically noodle formation that helps to retain the spatial structure of samples (Figure 7.1 B) even after thawing, would provide another tool to observe samples within 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 difference using image analysis. Sperm agglutination could thus be an important tool to understand the cryopreservation process within the micro-environment of the straw.

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. Agglutination in oyster sperm after thawing is likely due at least in part to progression of the acrosomal reaction upon thawing. However, similar agglutination has been observed for non-acrosomal fish sperm, for example, the Atlantic croaker (Gwo and Arnold, 1992). It is possible that the physical processes of freezing and thawing cause the sperm membrane to rupture, especially when there is no cryoprotectant or a low concentration of cryoprotectant, and the ruptured sperm release their cytoplasm, which contains agents (e.g. peptides, proteins or enzymes) that agglutinate sperm. Future studies are required to uncover the mechanism of sperm agglutination in aquatic species. In addition, the classification for sperm agglutination presented in this study is on a macroscopic scale with visual observation. Homogeneous suspensions, i.e., presumed to have no agglutination, might reveal some level of sperm agglutination if systematic observations were made microscopically.

It is noteworthy that 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 (Miskolczi et al., 2005). In the present study, high post-thaw motility (> 30%) was observed for 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 (Sneed and Clemens, 1956). Apparently, sperm viability was relatively high in samples with high agglutination scales considering their 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 (Adams et al., 2004).

Results presented in this study call attention to the requirement for researchers 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 process underlying sperm agglutination during cryopreservation observed in samples from diploid and tetraploid oysters is the same as that observed in other aquatic species. The formation of sperm agglutination in oyster sperm is perhaps enhanced by the acrosome reaction, and could provide an important tool to understand the cryopreservation process within the micro-environment of the straw. 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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Chapter 8
Summary and Conclusions

This dissertation is part of a larger project with the overall goal to develop protocols for commercial-scale cryopreservation of sperm from diploid and tetraploid Pacific oysters, *Crassostrea gigas*, especially for development of an industry for production of triploid seedstocks using cryopreserved sperm from tetraploids. This dissertation for the first time addressed comparative studies of sperm cryopreservation of diploid and tetraploid aquatic organisms, with the emphasis on development of standardized and optimized protocols. Specifically, this dissertation: 1) provided a review of current research of sperm cryopreservation in oysters; 2) examined ultrastructural differences between sperm from diploid and tetraploid oysters; 3) developed methods for rapid estimation of sperm concentration; 4) optimized various components in sperm cryopreservation protocols of diploid oysters; 5) optimized various components in sperm cryopreservation protocols of tetraploid oysters, and 6) evaluated the mechanism for sperm agglutination in thawed samples.

Review of the previous research of sperm cryopreservation in oysters (Chapter 2) indicated a systematic lack of standardization in various component procedures. In addition, most studies were limited to research settings. Due to the applied goal of using cryopreserved sperm from tetraploid Pacific oysters to produce triploids, this research project involved collaborations among a university research laboratory (LSUAC-Aquaculture Research Station), a commercial oyster hatchery (Taylor Resources, Inc. Quilcene Shellfish Hatchery, Quilcene, Washington) and a central freezing facility for commercial dairy bull sperm production (Genex Cooperative, Inc. Custom Collection at the LSU T. E. Patrick Dairy Improvement Center, Baton Rouge, Louisiana). Thus, the approaches taken were oriented to commercial settings. The use of shipped sperm translated into the use of samples 26 h after collection (due to the addition of 24 h

shipment time), further distinguishing this work from previous studies in oyster sperm cryopreservation. Problems identified here are applicable to sperm cryopreservation in other aquatic species and may help to explain why sperm cryopreservation has not yet found application in aquaculture on a commercial scale.

Ultrastructural studies (Chapter 3) revealed that sperm from tetraploids were compositionally similar to diploids except for overall size and the number of mitochondria. 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, which may partially explain the low initial motility associated with sperm from tetraploids (i.e., insufficient compensation in mitochondrial capacity for the doubled volume of sperm from tetraploids). The linear dimensions of sperm components such as acrosome height and width, sperm head length and width, mitochondrial height, length of the main piece and end piece of flagellum, and total length of flagellum in tetraploids were approximately 1.25 times of the corresponding measurements in diploids, which corresponds to the doubled volume in sperm of tetraploids compared to that of diploids.

Standardization of sperm concentration prior to freezing is important for future application in commercial-scale production, but also for optimization of freezing protocols for research and practical purposes. The concentration of dimethyl sulfoxide (DMSO) considered to be optimal varies among previous studies and likely could be due to the lack of standardization in initial sperm concentration at freezing. In the future, a sperm concentration of 1×10^9 cells mL^{-1} could be used as a standardized freezing concentration for *C. gigas* because it has been found to yield the highest percent fertilization in various studies (Staeger, 1974; Smith et al., 2001; Dong et al., 2005). Estimation of sperm concentration for diploid and tetraploid Pacific oysters with a

spectrophotometer was obtained at the wavelengths of 550 and 581 nm based on separate standard curves (Chapter 4).

For sperm from diploid oysters, evaluation of cooling rates revealed an optimal rate of 5 °C per min to -30 °C followed by cooling at -45 °C per min to - 80 °C before plunging into liquid nitrogen. Screening of single or combined cryoprotectants at various concentrations suggested that a low concentration (2%) of polyethylene glycol (FW 200) was effective in retaining post-thaw motility and fertilizing capability when combined with permeating cryoprotetcants such as DMSO, methanol (MeOH), and propylene glycol (P-glycol). However, polyethylene glycol alone was not as effective as MeOH, DMSO and P-glycol when using the same methods. The highest post-thaw motility (70%) and percent fertilization (98%) were obtained for samples cryopreserved with 6% MeOH. However, this does not exclude other cryoprotectants such as DMSO or P-glycol as effective agents in other studies. There was no significant difference in post-thaw motility between straw sizes of 0.25-ml and 0.5-ml. Equilibration time (exposure to cryoprotectant) of 60 min could be beneficial when the cryoprotectant concentration is low and solution is added in a step-wise fashion at low temperature. Differences in post-thaw sperm quality (e.g., motility or percent fertilization) among individual males were evident in this research. As a consequence, a generalized classification describing males with different tolerances (broad, intermediate, and narrow) to cryopreservation was developed. This classification could be applied to strain or species differences in tolerances to the cryopreservation process.

For sperm from tetraploid oysters, initial motility (64 ± 25%) after shipment was low compared with sperm samples from diploid oysters (82 ± 22%) collected and shipped at the same time (Table 8.1). Despite low post-thaw fertilization (< 10%) in general for sperm from

Table 8.1 Summary of the studies of sperm cryopreservation from diploid and tetraploid Pacific oysters in Chapters 5 and 6.

Parameter	**Diploid**	**Tetraploid**
Oyster received dates (year 2004)	June 4 to July 7	June 10 to July 7
Number of males used	27	29
Initial motility		
Range	5 - 95%	5 - 95%
Average (mean ± SD)	82 ± 22%	64 ± 25%
Cooling rate[1] (°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;	6% PEG/4% P-glycol;
	2% PEG/4% MeOH	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 vs. 0.5 ml)	No significant difference	No significant difference
Cooling methods (CRF[2] vs. CFM[3])	Better with CRF	No direct comparison
Highest post-thaw motility	70%	50%
Highest percent fertilization	98%	48%
Highest percent hatch	67%	28%

[1]For the cooling rates of 0.5, 5, 16, and 30 ^{o}C per min, samples were cooled in two steps, initially to -30 ^{o}C at these rates, followed by cooling at 45 ^{o}C per min from –30 ^{o}C to –80 ^{o}C. For cooling rates of 45 and 50 ^{o}C per min, samples were cooled in a single step from 5 ^{o}C to –80 ^{o}C directly at the specified rate.
[2]Cooled at 5 °C/min using a controlled-rate freezer.
[3]Cooled using a commercial freezing method developed for dairy bulls.

tetraploids, the post-thaw motility was improved considerably compared to earlier studies (Dong et al., 2005), 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. Optimal cooling rate was the same as for sperm from diploids. Screening of single or combined cryoprotectants at various concentrations showed that combined cryoprotectants of 6% PEG/4% P-glycol and 6% PEG/4% DMSO yielded consistently high post-thaw motility in various trials. However, more fertilization trials are required to confirm their effectiveness in retaining fertilization capability. Similar to the study with diploids, there was no significant difference in post-thaw motility between straw sizes of 0.25-ml and 0.5-ml. A long equilibration (60 min) was found to yield higher percent fertilization, and confirmed that long equilibration could be beneficial when low concentrations of cryoprotectant are used.

Results of this project showed 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 were found to improve the post-thaw motility in sperm from tetraploids provides presumptive evidence for this interpretation. Therefore, techniques used to minimize osmotic injury (membrane damage) such as serial addition and removal of

cryoprotectant merit attention of future studies. Sperm from tetraploids have different numbers of mitochondria (Chapter 3), and even those with the highest number of mitochondria (6) may be deficient in comparison to the mitochondrial number (4) and capacity of sperm from diploids, which may partially explain their consistently relatively low initial motility. This difference could also relate to the freezing sensitivity in sperm from tetraploids, for example, spermatozoa having four mitochondria and those with five or six may have different susceptibility to cryopreservation damage. Overall, based on the classification of sperm tolerance proposed in Chapter 5, sperm from tetraploids could be considered as having a narrow tolerance in comparison to sperm from diploids, thus continued optimization of current methods is necessary for further improve post-thaw sperm quality in tetraploid oysters. In addition, other factors related to tetraploid broodstock in a more general sense such as their fecundity, sex ratio of progeny, growth rate, and gamegenesis should also be evaluated to improve understanding about their biology.

Viability assays used in this project included motility, percent fertilization and hatch. 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 fishes and mammals (Kerby, 1983; Billard, 1988; Graham, 2001; Warnecke and Pluta, 2003; He and Woods, 2004). This study showed weak correlations between post-thaw motility and fertilization. For example, sperm samples that were non-motile after thawing yielded high fertilization in diploids (45%) and tetraploids (28%). One explanation is that motility is only one of many attributes that a spermatozoon must possess to fertilize an egg and thus data from multiple sperm assays would provide better prediction of fertility (Graham, 2001). Another explanation is that fertility is a composite measure of sperm quality, egg quality, and the fertilization method (Figure 8.1).

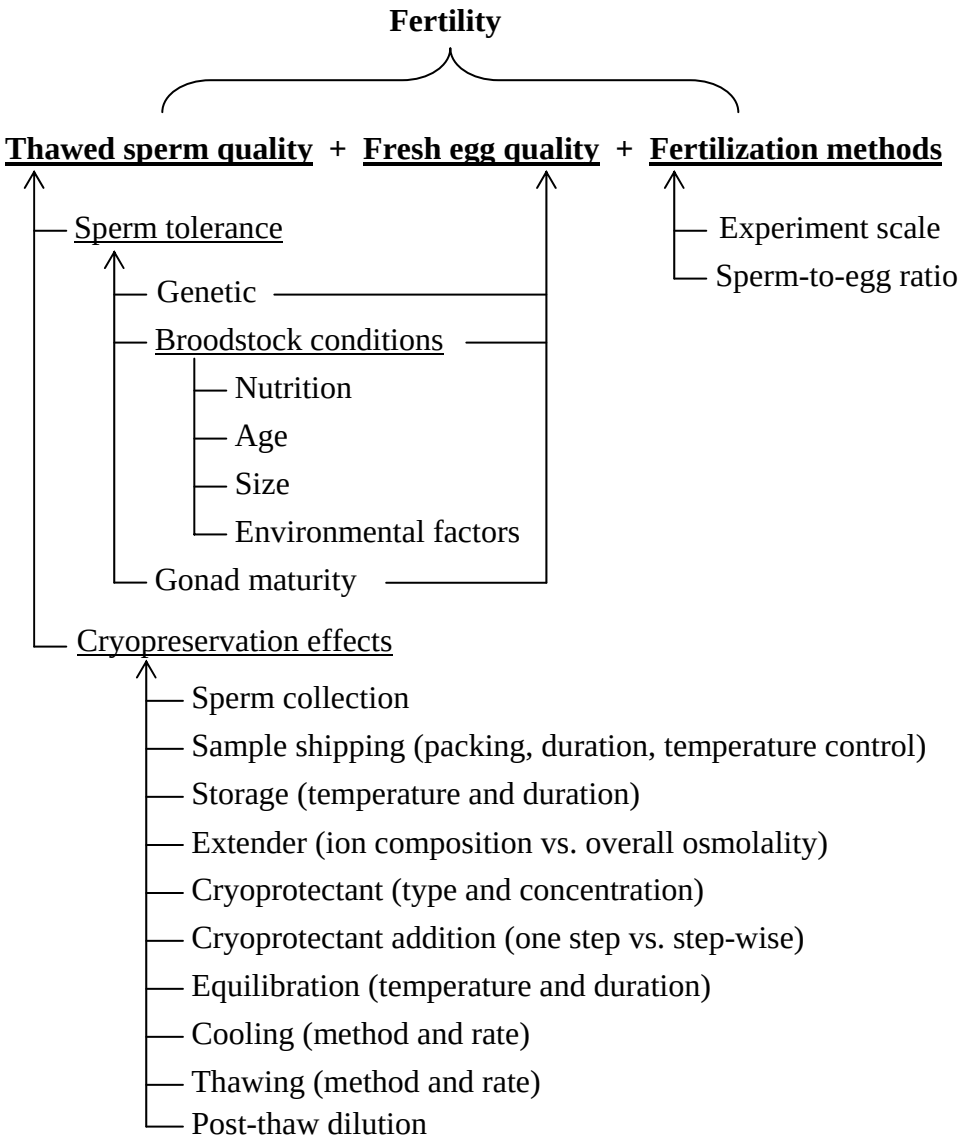

Figure 8.1 Diagram of factors affecting fertility (percent fertilization) of sperm samples after thawing.

Egg quality (or female effect) has been shown to limit measurable fertility of cryopreserved sperm in eastern oysters, *Crassostrea virginica* (Zell et al., 1979), rainbow trout, *Oncorhnchus mykiss* (Stoss and Holtz, 1981; Baynes and Scott, 1987), Atlantic salmon, *Salmo solar* (Stoss and Refstie, 1983), and brown trout, *Salmo trutta*, and Arctic charr, *Salvelinus alpinus* (Piironen, 1993). In practice, fertilization trials could be limited, such as in projects that collaborate with commercial hatcheries (e.g. this study), in which space, money, and effort for research are constrained due to the primary focus on production. Using motility as a general indicator of cell damage due to treatment effects will help to facilitate the process of range-finding experiments and protocol optimization, thus reduce the necessary number of fertilization trials.

Other than motility, various parameters considered to be related to fertilization ability of sperm were also evaluated for fish semen, for example, spermatocrit, sperm density, chemical composition of seminal plasma, enzymatic activity, and computer-assisted sperm analysis (CASA) (for review see Rurangwa et al., 2004). One major limitation of the parameter measurements described above is that they are descriptions of the milt characteristics of fresh samples. Fertility of fresh sperm from different males can be same, but fertility of these males can vary after cryopreservation (Paniagua-Chavez and Tiersch, 2001; Thurston et al., 2002). Thawed sperm quality is dictated by sperm tolerance (Chapter 5) and the cryopreservation effects (Figure 8.1). Therefore, it is the sperm tolerance rather than descriptive milt characteristics that mainly affect the outcome of sperm cryopreservation. As defined in Chapter 5, tolerance could be detected when spermatozoa were subjected to unfavorable environmental factors. In oyster sperm, future studies could address assays such as those to evaluate the ability of fresh sperm to fertilize eggs after: (1) exposure to hypotonic or hypertonic solutions; (2)

acrosome reaction when treated with membrane destabilizing compounds; (3) tails were mechanically damaged, and (4) refrigerated storage for various durations.

In this project, high fertilization generally leads to high percent hatch for thawed samples from diploid and tetraploid oysters. Fertility could be considered to be an end point for evaluation of cryopreserved sperm samples in oysters, especially when low concentrations of cryoprotectants are used. Studies of larval development and subsequent metamorphosis and settling did not reveal any significant difference between thawed sperm and non-frozen controls (Zell et al., 1979; Yankson and Moyse, 1991; Usuki et al., 1997; Smith et al., 2001; Ieropoli et al., 2004), and in some cases better performance with thawed sperm samples was observed (Usuki et al., 1997; Adams et al., 2004).

Sperm agglutination was systematically evaluated for the first time in this project, and was classified into 6 levels with a scale ranging from 0 (homogenous suspension) to 5 (well-developed "noodles"). It was found that sperm agglutination in thawed samples was mainly due to the relationship between the concentration of cryoprotectant and sperm number. 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 agglutination. The exact mechanism of sperm agglutination remains unclear. However, morphological examination of the cross sections of the noodles (agglutination level 5) could be used as an important tool to understand the cryopreservation process within the micro-environment of the straw. In addition, the fact that the level of sperm agglutination was determined by sperm concentration, the type of cryoprotectant, cryoprotectant concentration, and cooling and thawing methods emphasized the importance of procedure standardization and systematic optimization of protocols involving multiple factors that was emphasized earlier.

Sperm cryopreservation involves a sequence of steps, and deficits in single steps can lead to a cumulative total loss of viable cells (Leibo, 2000). Cumulative effects rather than optimization of single steps determine the ultimate success, which was illustrated clearly in this research with males having different sperm tolerances (Chapters 5 and 6). A literature review in oyster sperm cryopreservation revealed that most studies have been devoted to optimizing specific components of the cryopreservation procedure, particularly extender, cryoprotectant, or cooling and thawing rates. Evaluation of the cumulative effects arising from all activities between broodstock condition and fertilization is lacking for aquatic species in general. For example, for processes as simple as gamete collection, various methods have been applied and no attempts have been made to compare the effects on cryopreservation outcomes. In this research, the possible effect of shipping was emphasized repeatedly, and places in protocols that were different from others were detailed explicitly, with the purpose of emphasizing cumulative effects rather than cryopreservation technique in a narrow sense (cryoprotectant, cooling, and thawing). The ultimate success of cryopreservation relies on identification and quantification of the cumulative effects from the broodstock condition to fertilization, which will be facilitated if standardized procedures were applied in various studies.

Considering the already established market for triploid seedstocks (Chapter 1), the potential for cryopreserved sperm from tetraploids will be high. Although that the protocols outlined in this dissertation for sperm from tetraploid oysters require further improvement, an average market-sized diploid female oyster (3 inches in height) can produce 50-100 million eggs in a single spawning (Quayle, 1969), a 10% fertilization rate on average would result in at least 5 million eggs being fertilized. In addition, the highest post-thaw motility of sperm from those tetraploid males used for fertilization trials in this project was 20%, while as high as 50% post-

thaw motility was obtained with males used for range-finding and procedure optimization experiments. Higher percent fertilization would be expected when using males with high post-thaw motility, as in the case with sperm from diploid oysters where percent fertilization was always greater than 90% when post-thaw motility was greater than 40%. Thus, the methods and results presented here indicate a potential for application in commercial hatcheries. For marketing of all-triploid seedstocks, pooled males (thus reducing male-to-male variation) could be used to obtain consistent larval quality. Pooling of commercially relevant numbers of males (e.g., 50-100) should be explored in future cryopreservation research, as this would enable bulk processing and would simplify quality control and assessment of gamete quality. For breeding purposes such as developing new lines, sperm from individual males should be used. For sperm from diploid oysters, given males with intermediate or broad tolerances, the protocols developed in this dissertation will yield fair success (e.g., > 50% fertilization) to assist various breeding programs. In addition to those mentioned above, future studies should also evaluate economic feasibility for the marketing of cryopreserved sperm in various aquatic species as well as the production costs for the commercial hatchery.

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Appendix A
Standard Operating Procedures

SOP-1. Identification codes for oysters

Each oyster used in experiments received a unique identification code; see Example 1 for the code used during year 2001 to 2003. To facilitate the sample searching, the information of ploidy level was added in the code in year 2004 (see Example 2).

Example 1:

CG03M01

CG: *Crassostrea gigas*
03: year 2003
M: male
01: sample number

Example 2:

2CG04M18		**4CG04M23**	
2:	**Diploid**	**4:**	**Tetraploid**
CG:	*Crassostrea gigas*	**CG:**	*Crassostrea gigas*
04:	year 2004	**04:**	year 2004
M:	male	**M:**	male
18:	sample number	**23:**	sample number

SOP-2. Preparation of Calcium-free Hanks' balanced salt solution (C-F HBSS) at 1000 mOsmol/kg

Materials needed:

Chemicals as listed below — Flask
Top-loading Balance — Spatula
Stir plate (Barnstead/Thermolyne, Dubuque, Iowa) — Stir bar
0.45-µm CA (cellulose acetate) filter (Corning Incorporated, Corning, New York)
Osmometer (model 5500, Wescor Inc., Logan Utah)

Procedure:

1. Fill less than 1 L (~ 800 mL) of distilled water into the flask
2. Add the amount of chemicals in this order: NaCl 26.32 g; KCl 1.32 g; $MgSO_4 \cdot 7H_2O$ 0.65g; $Na_2HPO_4 \cdot 7H_2O$ 0.18g; KH_2PO_4 0.18g; $NaHCO_3$ 1.15g; $C_6H_{12}O_6$ (glucose) 3.30g.
3. Bring ingredients to 1 L by addition of sufficient distilled water
4. Stir for 30 min
5. Adjust pH to 7.0-7.4
6. Verify osmolality of 1000 mOsmol/kg using a vapor pressure osmometer
7. Filter through a 0.45-µm filter
8. Label the container (e.g., C-F HBSS 1000 mOsmol/kg, initials of technician, date)
9. Store at 4 °C prior to use

SOP-3. Sperm collection

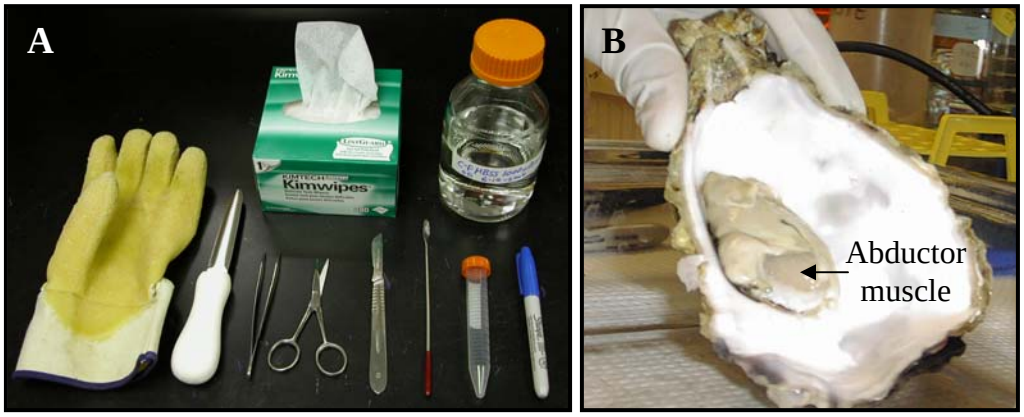
Materials needed (Figure A):

Gloves	Oyster knife
Tweezers	Scissors
Scalpel	Spatula
15-mL centrifuge tubes	Permanent marker
Kimwipes	C-F HBSS (1000 mOsm/kg) or filtered sea water (32 ppt)

40-µm cell strainer (BD Biosciences Discovery Labware, Bedford, Massachusetts)

Procedure:

1. Carefully remove the top shell using oyster knife (wear gloves, at least on the hand holding the oyster)
2. Use tweezers and scissors to carefully peel off the mantle, gills, labial palps and other tissues, but leave gonad intact and attached to the abductor muscle (Figure B)
3. Use tweezers to remove the heart and associated tissues
4. Use filtered sea water or C-F HBSS to rinse the gonad 3 times
5. Use Kimwipes to dry the gonad and clean the inside shell
6. Use the scalpel to cut openings on the gonad
7. Use the spatula to collect the gonad tissue into the 15-mL centrifuge tube. Avoid contamination with digestive gland (yellowish-green material)
8. Label the tubes
9. Store the undiluted sperm samples at 4 ºC before shipping or use
10. After suspension with C-F HBSS, filter samples through a 40-µm cell strainer prior to adjusting cell concentration



SOP-4. Shipping live oysters and undiluted sperm samples

Materials needed:
Two ice jelly packs
Foam shipper (interior L x W x H: 19 x 19 x 19 cm; wall thickness: 1.2 cm)
Cardboard carton
Cardboard
Paper towel
15-mL centrifuge tubes
Temperature data logger (e.g. HOBO, Onset computer, Bourne, Massachusetts)
Ziploc® bag
Newspaper
Tape

Procedure:
1. Have ice jelly packs frozen in -20 °C freezer prior to use
2. Place two ice jelly packs in the foam shipper
3. Place the cardboard on top of the ice jelly pack as the divider between ice pack and samples (Tiersch, 2000)
4. Place samples on top of the cardboard divider. In the case of live oysters, wrap oysters with paper towels soaked with seawater, and in case of sperm samples, wrap the 15-mL centrifuge tubes with paper towels
5. Place the temperature data logger into a Ziploc® bag with the samples
6. Stuff the extra space in the shipper with newspaper
7. Seal the openings with tape
8. Place the foam shipper inside the cardboard carton
9. Seal the cardboard carton with tape
10. Send the package by overnight delivery service, and notify recipient
11. Send out information about samples and shipment to the recipient
12. Transfer samples to 4 ºC refrigerator immediately upon receiving

Reference:

Tiersch, T. R. 2000. Shipping of refrigerated samples. In: *Cryopreservation in Aquatic Species*. Tiersch, T. R. and P. M. Mazik, Editors. World Aquaculture Society, Baton Rouge, Louisiana. Page 281.

SOP-5. Motility estimation

Materials needed:
Micropipette and tips (10 and 100 µL)
Two-well glass microscope slide
Microscope with darkfield and 20-× objectives
C-F HBSS (1000 mOsmol/kg)

Procedure:

1. Take 10 ml C-F HBSS from a stock solution stored at -20 °C and allow it warm to room temperature*
2. Evaluate the C-F HBSS with microscopy for presence of bacterial contamination
3. Place 30 µL of C-F HBSS inside the wells on the two-well glass microscope slide
4. Determine initial motility by adding undiluted non-motile sperm (Dong et al. 2005) or 1µL of sperm suspensions (after suspending in C-F HBSS at 1000 mOsm/kg) and mixing gently with the tip of the pipette.
5. Evaluate post-thaw motility by adding 2 µL of sperm suspension and mixing gently with the tip of the pipette
6. Equilibrate sperm suspensions inside the wells on the glass slide at 23 °C for 2 min before motility estimation
7. Estimate the percent of sperm that are actively moving in a forward direction at 200-× magnification using darkfield microscopy
8. Estimate the percent motility in increments of 5%. Samples with motility below 5%, but still having motile sperm are considered as 1%
9. Record the readings in a notebook
10. Identify the treatments**

*It is recommended to use the same activation solution throughout a single working season. C-F HBSS can be aliquotted into small volumes such as 10 mL and stored at -20 °C.
**To avoid the bias associated with observer, it is especially important to estimate sperm motility without knowledge of the treatment of the straw.

Reference:

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

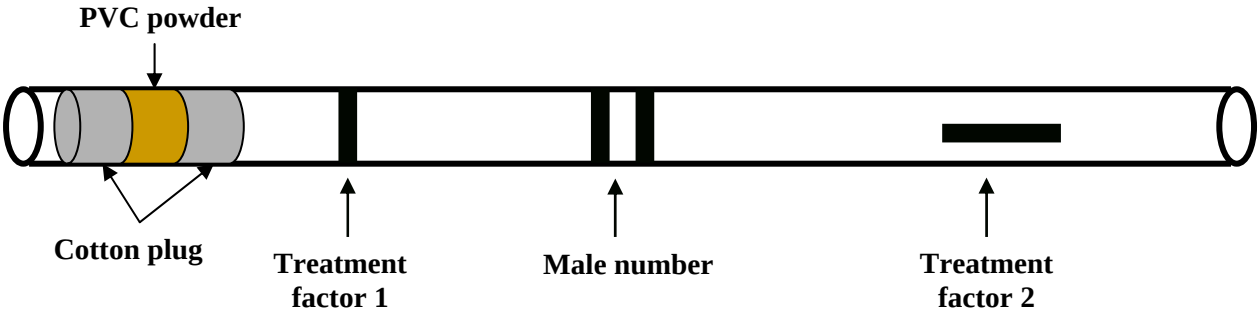
SOP-6. Determining sperm concentration with a Hemocytometer

Materials needed:
Hemacytometer and cover slip (Hausser Scientific, Horsham, Pennsylvania)
Micropipettes and tips (10, 200, and 1000 µL)
1.5-ml microcentrifuge tubes
Microscope

Procedure:
1. Mix the sperm sample to be counted by gentle inversion the tubes
2. Dilute sperm with extender (C-F HBSS) at a low osmolality of 200 mOsmol/kg to immobilize the sperm, normally 10 uL sperm suspension into 1400 uL of extender
3. Mix for 2 min using a pipette
4. Place a coverslip on the hemocytometer
5. Place 10 uL of the diluted sperm under the cover slip on each side of the hemocytometer. Avoid bubbles and over filling of the chamber
6. Place the hemocytometer on a microscope. Focus with a 10-× objective, and switch to 20-× for counting. Be careful not to break the coverslip by focusing onto it
7. Make sure that the sperm separate individually, otherwise prepare a new sample
8. Wait for 5 min to allow the sperm precipitate to the bottom before counting
9. Count the sperm in 5 of the 25 squares. Be sure to count 5 squares on each side of the hemocytometer
10. The center line of each square is the edge of the counting area. For sperm that lie exactly on the edge of the counting area (the center line), count only the top and right sides
11. Record two counts and make calculation based on the formula below:
    Cells/mL = (average of two counts in 5 squares) x (dilution factor) x 5 x 10^4

SOP-7. Labeling of straws

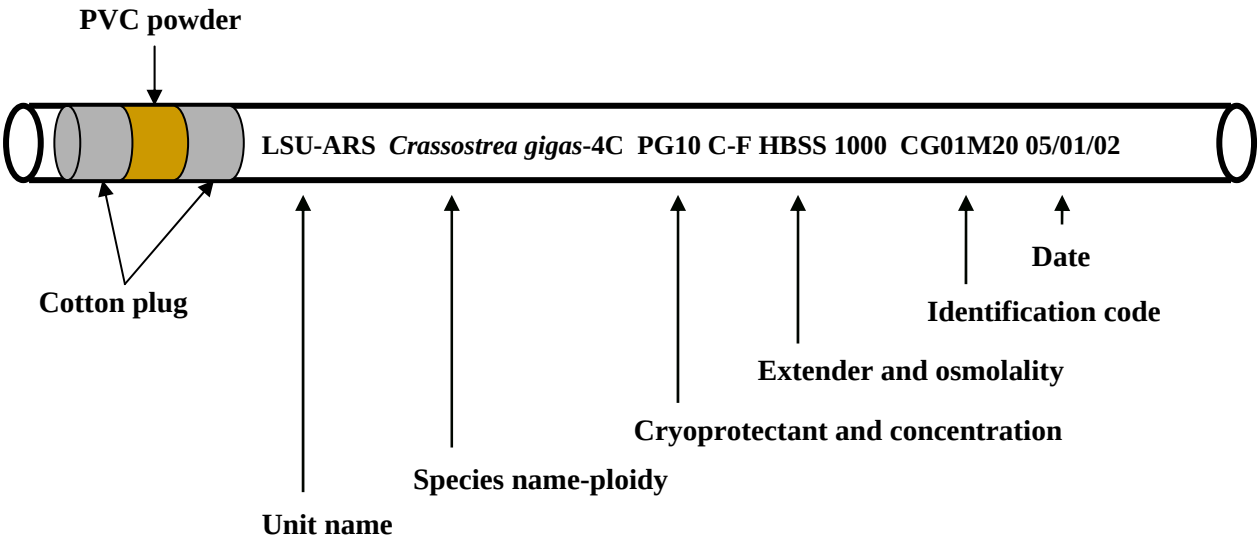
Hand labeling



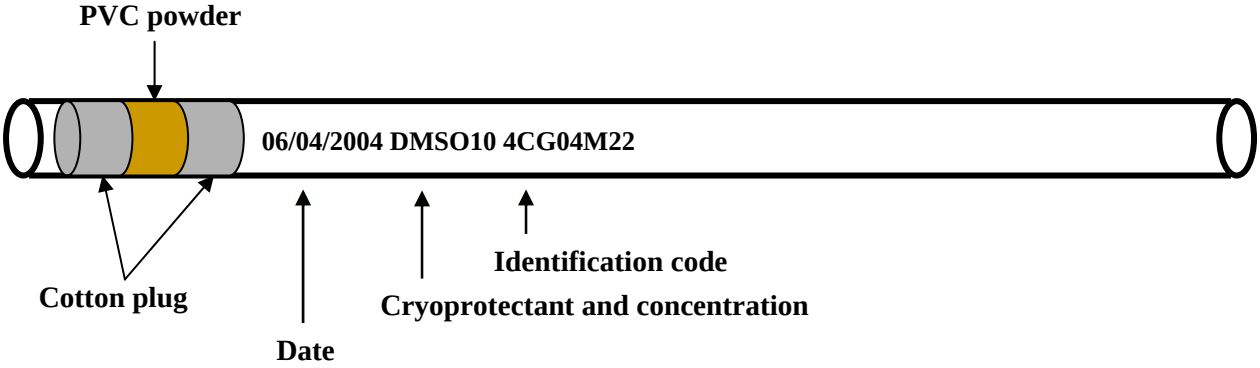
Lines perpendicular to the straw indicate a value of one; bars parallel to the straw indicate a value of five.

Automated labeling

Years 2001-2003



Year 2004



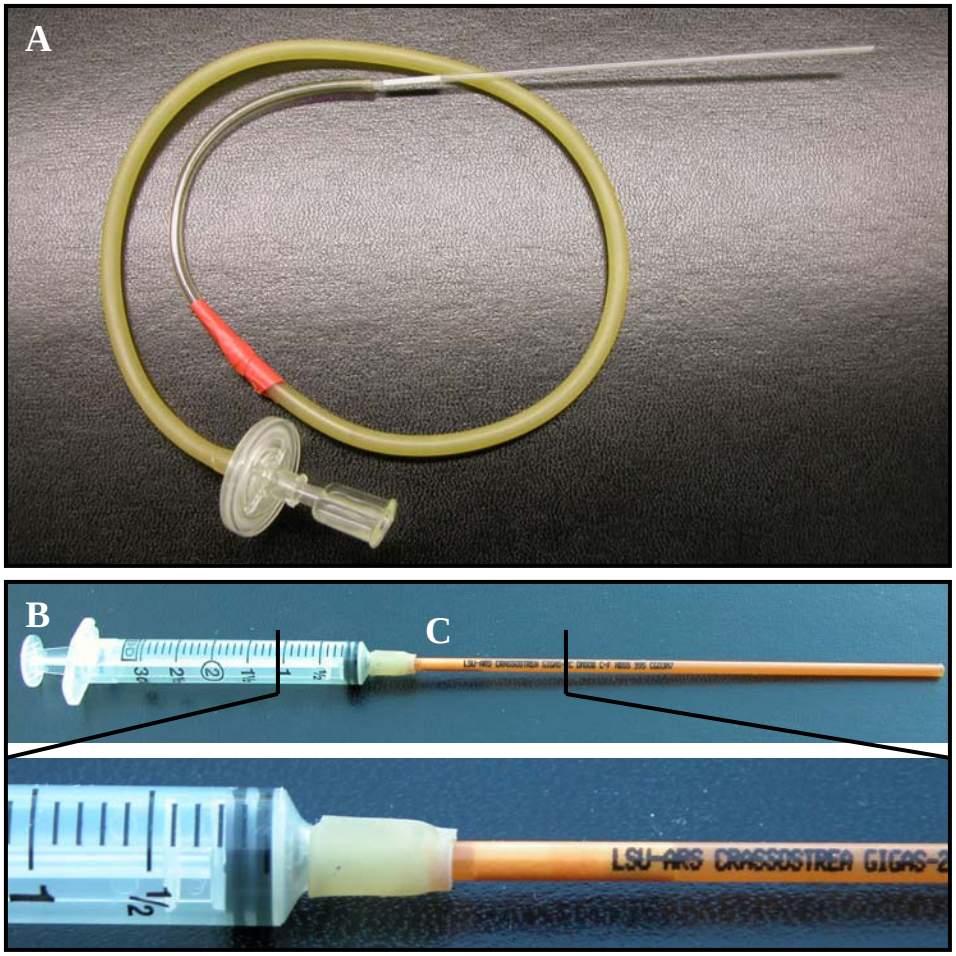
SOP-8. Hand filling of 0.25-ml and 0.5-ml straws

Materials needed:

Mouthpiece
Rubber tube (interior diameter of 0.2 and 0.3 cm)
Plastic tube (interior diameter of 0.15 cm)
PVC powder
Acrodiscs (0.22 µm)
Tape
3-cc syringe without needle
Paper towel

Procedure:

1. Connect the cotton plug end of the straw to the plastic tube adaptor (0.25-ml straws, Figure A) or the rubber tube adaptor (0.5-ml straws, Figures B and C)
2. Fill the straw by the amount of suction as regulated by mouth (0.25-ml straws) or simply by drawing on the syringe plunger (0.5-ml straws)
3. Remove the straws from the solution when the sperm suspension is 1 cm from the bottom of the cotton plug,
4. Continue the suction until the PVC powder within the cotton plug is wet
5. Seal the straws by tamping the open end of straws into PVC powder and dip this end into C-F HBSS to complete the sealing. Wipe the straws with the paper towel before placing into 10-mm goblets

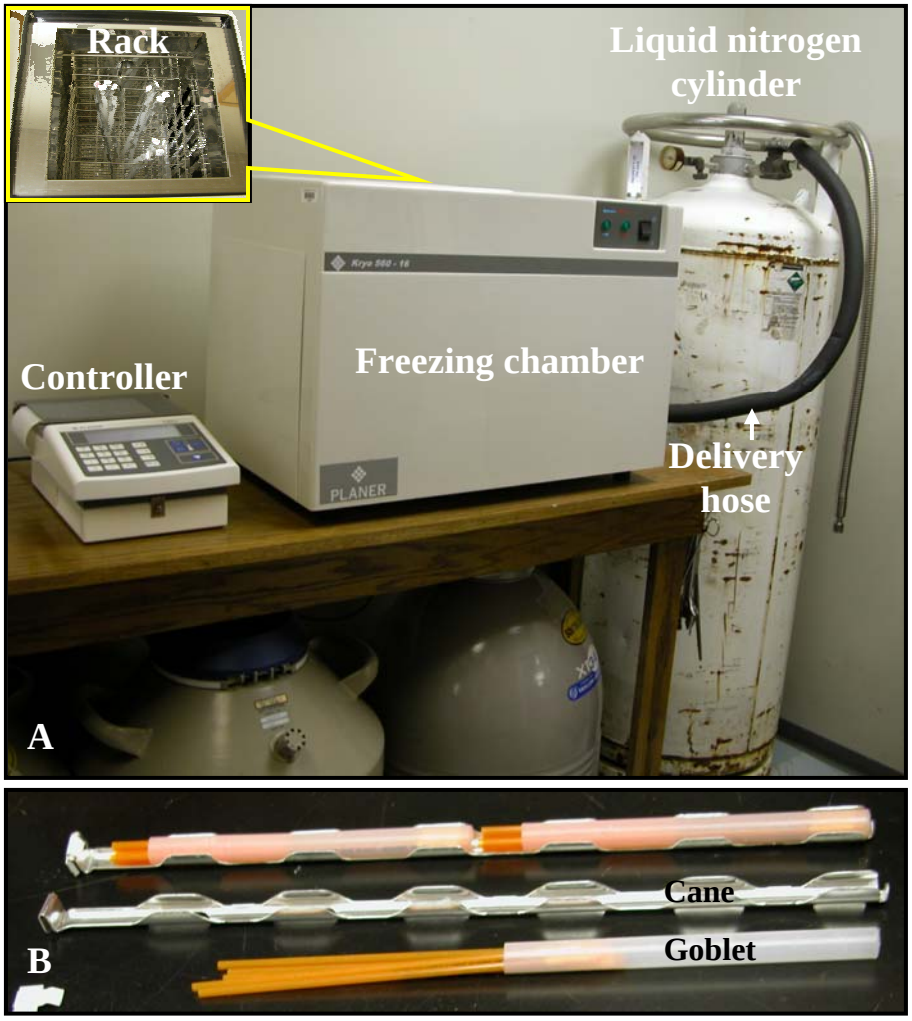


SOP-9. Controlled-rate freezing

Freezer: Kryo 560-16; Planer plc., Sunbury-on-Thames, UK
Low-pressure liquid nitrogen cylinder (22 psi)

Procedure:

1. Connect the delivery hose from the freezer to the liquid nitrogen cylinder, and tighten it with a wrench (Figure A)
2. Completely open the "liquid" valve on the cylinder in a counter-clockwise direction
3. Turn on the power switch on the controlled-rate freezing chamber (Figure A)
4. Follow the instructions on the controller (Figure A)
5. Program the cooling protocols or select cooling protocol
6. Load sperm suspension into 0.25-ml or 0.5-ml French straws (IMV International, Minneapolis) as described above
7. Place 8 0.25-ml straws or 5 0.5-ml straws into a 10-mm plastic goblet (Figure B)
8. Attach two goblets to a 10-mm aluminum cane (Figure B)
9. Equilibration time starts at the mixing of sperm suspension with cryoprotectant solution and includes the straw filling time, and time inside the freezing chamber before initiation of the protocol
10. Insert the canes into the rack inside the freezing chamber (Figure A)
11. Equilibrate samples at 5 °C for 5 min before cooling at the desired rate
12. For the cooling rates of 0.5, 5, 16, and 30 ^{o}C per min, cool samples in two steps, initially to -30 ^{o}C, followed by cooling at 45 ^{o}C per min from –30 ^{o}C to –80 ^{o}C.
13. For cooling rates of 45 and 50 ^{o}C per min, cool samples in a single step from 5 ^{o}C to –80 ^{o}C at the specified rate.
14. Hold straws at –80 ^{o}C for 5 min
15. Remove samples swiftly from the freezing chamber and immediately plunge them into liquid nitrogen in a storage Dewar.
16. Retain the printout of the cooling curves for the specific run from the controller
17. Allow the chamber to warm to room temperature before turning off the power switch.
18. Close the liquid nitrogen valve in a clockwise direction
19. Disconnect the delivery hose from the liquid nitrogen cylinder after defrosting
20. Dry the freezing chamber with a paper towel


Rack
Liquid nitrogen cylinder
Controller
Freezing chamber
Delivery hose
PLANER
A
Cane
Goblet
B

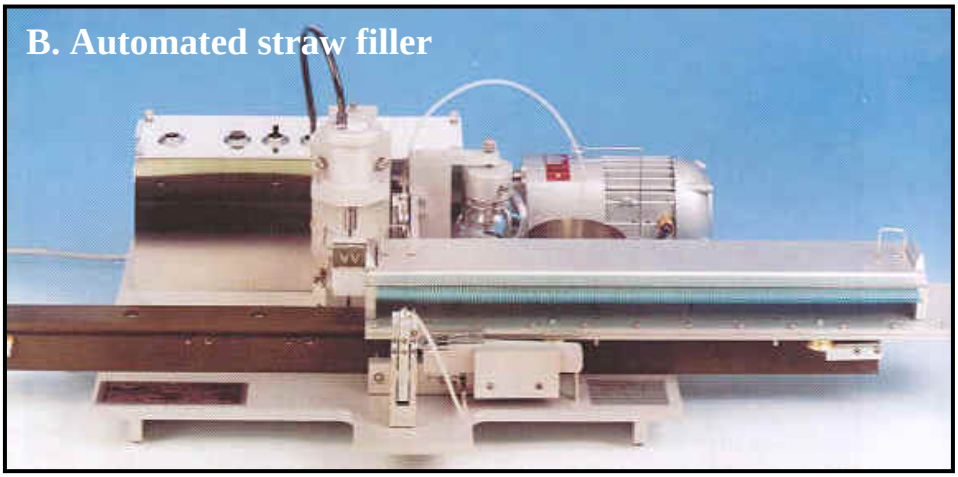
SOP-10. Commercial-scale freezing procedure
(Performed at Genex Custom Collection, Inc. in Baton Rouge, Louisiana)

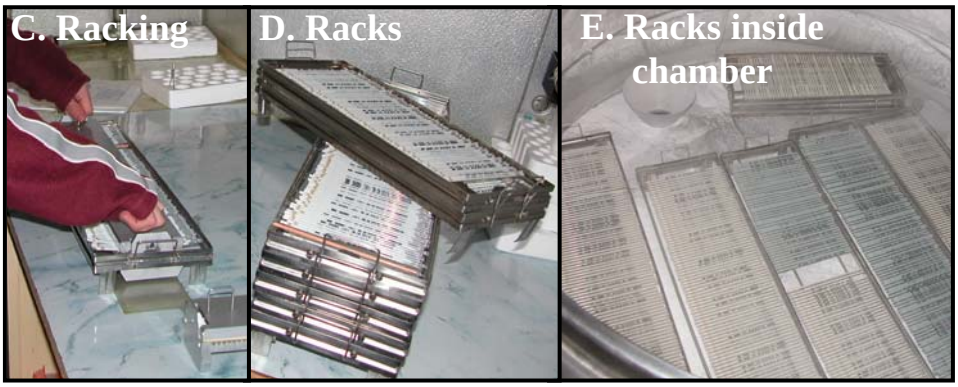
1. Select samples for freezing and estimate initial motility
2. Have straws labeled by personnel at Genex Custom Collection, Inc. (Genex) by use of the specialized laser printer (Domino Codebox 2, Domino Amjet, Inc. Gurnee, Illinois) (Figure A) after providing them with sample information
3. Transport samples to Genex at T.E. Patrick Dairy Improvement Center (10 min driving distance from the laboratory at LSU Aquaculture Research Station) in a styrofoam box with ice jelly packs to keep samples from warming
4. Place samples in walk-in cooler (5 °C)
5. Verify the straw labeling
6. Add cryoprotectant and start equilibration time
7. Fill straws using automated straw filler (model MRS 1, IMV Int. Corp., Minneapolis, Minnesota) (Figure B)
8. Place straws on racks and add enough milk-filled straws to make 660 total straws in case insufficient straws contain oyster sperm samples (Figures C, D, and E)
9. Allow Genex personnel to freeze samples (Figures F and G)
10. Place the racks in freezing chamber held at –140°C
11. During the first 3 min of freezing, the chamber will warm from –140°C to –60°C as a result of the heat load of the samples.
12. In the next 5 min, liquid nitrogen is added to the chamber to cool at a rate of 16°C/min returning the chamber to –140°C (Chandler et al. 1984).
13. At the end of the freezing process, plunge straws into liquid nitrogen
14. Label canes with the oyster identification codes (Figure H)
15. Transfer straws into 10-mm goblets (5 straws per goblet), and place goblets on canes (two goblets on one cane)
16. Place canes into long-term storage Dewars at the T.E. Patrick Dairy Improvement Center (Figure H) or transfer to a shipping Dewar for transport to LSU Aquaculture Research Station for post-thaw motility estimation, or Taylor Shellfish Hatchery at Quilcene, Washington for fertilization and percent hatch estimation.

Reference:

Chandler, J. E., C. F. Ruiz, R. W. Adkinson, and K. L. Koonce. 1984. Relationship between final temperature, thaw rate, and quality of bovine semen. Journal of Dairy Science 67:1806-1812.


A. Laser printer


B. Automated straw filler


C. Racking
D. Racks
E. Racks inside chamber


F. Freezing apparatus


G. Start freezing


H. Storage

SOP-11. Shipment of frozen samples

Materials needed:
Shipping Dewar (Figure A) (CP35, Taylor-Wharton, Theodore, Alabama)
Dewar case (Figure C)
Liquid nitrogen
Protective gloves
Plastic cable tie wraps
Tape

Procedure:
1. Fill the shipping Dewar with liquid nitrogen at least three times before use
2. Check the saturation level of liquid nitrogen by examining the calm surface of the liquid (no bubbling)
3. Load sperm samples (canes or goblets)
4. Secure the Dewar top and the body with a plastic cable tie wrap (Figure B)
5. Empty the liquid nitrogen by inverting the Dewar
6. Put the Dewar into the Dewar case
7. Close the Dewar case
8. Seal the openings with transparent tape
9. Be sure Dewar case is labeled with LSU Agricultural Center logo
10. Ship by overnight delivery
11. Samples stored in liquid nitrogen vapor in a shipping Dewar generally last for less than a week after shipping. However, it is always recommended to transfer samples to a liquid nitrogen storage Dewar immediately upon receiving the shipping Dewar, or top the shipping Dewar with liquid nitrogen to extend the storage time in cases where no storage Dewar is available.

SOP-12. Thawing methods

Materials needed:
Safety glasses*
Water bath with temperature controls (Model 1141, VWR Scientific, Niles, Illinois)
Long tweezers
Timer
Scissors
Paper towel
1.5-mL microcentrifuge tubes

Procedures:

For motility estimation:

1. Thaw samples after a minimum of 12 h of storage in liquid nitrogen
2. Remove individual straws from the 10-mm goblets with the long tweezers
3. Thaw at room temperature (23-25 °C) by placing the straws on paper towels on the laboratory bench, and allowing the straws to warm in the air for approximately 4 min.
4. Thaw in a water bath at various temperatures for different time periods: 13 s at 20 ^{o}C, 7 s at 40 ^{o}C, 6 s at 60 ^{o}C, and 5 s at 80 ^{o}C.
5. In the case of using 0.25-ml straws, thaw for 6 s at 40 ^{o}C, 5 s at 60 ^{o}C
6. Thawing duration is controlled with a timer or by counting "one thousand one" as one second
7. Wipe straws with paper towel and cut the PVC powder sealing end
8. Empty the straw into 1.5-mL microcentrifuge tubes by cutting the cotton plug end to release the content into the tubes
9. In cases with sperm agglutination, record agglutination scale (see Chapter 8) and disrupt sperm agglutination before motility estimation

For fertilization:

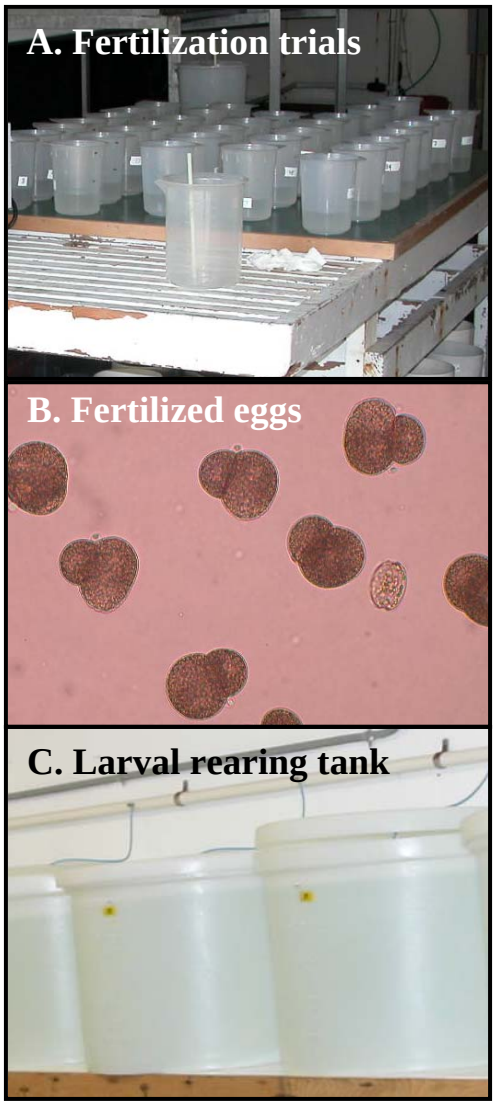
1. Thaw samples after a storage time ranging from 1 week to 1 year, and generally thaw 10 0.5-ml straws from each treatment
2. Thaw five straws from the 10-mm goblets simultaneously by dumping them into the water bath at 40 °C for 7 s (in cases with unsealed straws, seawater is used to replace with fresh water for the water bath)
3. Wipe straws with paper towel, and cut both ends to release the contents into egg solutions. Disrupt sperm agglutinations with the straw

*It is always recommended to wear safety glasses for sample thawing. Straws can explode for various reasons during this process.

SOP-13. Assessment of fertilization and percent straight-hinge larvae
(Performed at Taylor Shellfish Hatchery in Quilcene, Washington)

Materials needed:
Water bath with temperature controls
Constant supply of filtered seawater at 34 ppt and 25 °C
25-μm and 60-μm meshes
1-L beakers
100-L tanks

Procedures:
1. Use diploid females for fertilization trials. Eggs from individual females are obtained by dissection, and are sieved, washed through 60-μm mesh, retained on 25-μm mesh, and suspended in filtered seawater (34 ppt) at 25°C
2. Pool unfertilized eggs (fresh) from three females
3. Determine the number of eggs per ml by Coulter Counter
4. Hold the eggs in seawater at 25°C for at least 30 min to observe germinal vesicle breakdown at 100-× magnification
5. Separate eggs into 1-L beakers with each beaker containing 500,000 eggs (fresh) in 250 ml of seawater (Figure A)
6. Thaw ten straws of each treatment as described above (SOP-12)
7. Conduct fertilization trials by mixing 5 ml of thawed sperm suspension (the pooled contents of ten 0.5-ml straws) with 500,000 eggs in 250 mL of seawater
8. Incubate the gametes at 25°C and calculate percent fertilization by counting developing embryos at 2 h after insemination (n = 100) (Figure B)
9. Hold treatments for further evaluation of percent hatch by transferring to 100-L tanks filled with fresh seawater (Figure C)
10. Drain the tanks 24 h after fertilization through a 45-μm mesh and concentrate larvae into 1 L of seawater
11. After mixing, fix 5 1-mL subsamples with the Lugol's solution (Mix 100 ml acetic acid with 1 liter of Lugol solution, Sigma Chemical Corporation., St. Louis, Missouri), and calculate percent hatch by counting normal straight-hinge larvae with a dissecting scope
12. For a negative control, monitor eggs after treatment as described above without addition of sperm
13. For the evaluation of egg quality, collect fresh (non-frozen) sperm from diploid males using the techniques described above, and wash the sperm through a 70-μm mesh and add to fresh eggs to obtain about 100 spermatozoa per egg. Perform sperm counts with a spectrophotometer using standard curves (DR/2000, Hach Company, Loveland, Colorado) (see Chapter 4 for detailed methods)
14. For the evaluation of cryoprotectant toxicity, expose fresh sperm at the same concentration as thawed sperm samples to the same treatments (concentration, equilibration time, batch of eggs), and estimate percent fertilization
15. To avoid contamination of gametes among individuals, handle the animals with care and wash all surfaces with 0.01% bleach. Hold the sexes separately in different containers to avoid unintended fertilization


A. Fertilization trials
B. Fertilized eggs
C. Larval rearing tank

Appendix B
Unanalyzed Data in Chapters

Chapter 3

Table B.1 Size (μm) of spermatozoal components of diploid and tetraploid Pacific oysters (fixed samples).

Tetraploid (CG03M32, 34)					Diploid (CG03M31, 33)				
Head		Flagellum			Head		Flagellum		
Length	Width	Main piece	End piece	Total length	Length	Width	Main piece	End piece	Total length
3.12	2.98	45.73	8.09	53.82	2.76	2.32	34.14	7.40	41.540
3.48	2.79	42.41	6.75	49.16	2.76	2.11	35.39	7.41	42.800
3.34	2.91	43.37	11.40	54.77	2.83	2.4	34.06	7.56	41.620
3.36	2.77	43.22	10.93	54.15	2.40	2.18	34.45	7.46	41.910
3.27	2.83	44.18	11.51	55.69	2.40	2.33	34.74	7.10	41.840
3.41	2.98	42.41	11.14	53.55	2.40	2.33	34.34	7.92	42.260
3.49	2.98	43.53	5.67	49.20	2.88	2.42	32.75	8.73	41.480
3.41	2.98	45.83	7.82	53.65	2.47	2.38	34.40	7.90	42.300
3.29	2.88	42.48	8.48	50.96	2.47	2.32	32.32	8.41	40.730
3.29	2.88	42.12	6.73	48.85	2.62	2.32	34.57	7.86	42.430
3.05	2.83	44.18	10.49	54.67	2.57	2.39	34.38	8.48	42.860
3.33	2.98	41.10	8.67	49.77	2.67	2.36	33.37	9.48	42.850
3.49	2.83	44.05	11.42	55.47	2.61	2.40	34.20	8.01	42.210
3.27	2.83	41.32	9.80	51.12	2.47	2.25	32.63	8.59	41.220
3.08	2.83	46.01	12.38	58.39	2.40	2.32	33.06	7.47	40.530
3.26	2.85	43.31	9.73	53.04	2.70	2.24	33.65	8.48	42.130
3.41	2.83	45.04	12.00	57.04	2.61	2.32	32.92	7.83	40.750
3.26	2.68	43.72	12.34	56.06	2.62	2.34	31.88	5.82	37.700
3.56	2.98	44.98	10.59	55.57	2.47	2.25	32.84	7.77	40.610
3.48	2.89	45.15	11.66	56.81	2.58	2.37	33.53	8.12	41.650
3.49	3.12	40.65	7.20	47.85	2.47	2.27	33.10	8.03	41.130
3.49	3.34	42.91	6.65	49.56	2.69	2.47	34.58	7.83	42.410
3.63	3.41	43.07	10.63	53.70	2.69	2.25	32.44	7.45	39.890
3.49	3.20	42.78	10.62	53.40	2.69	2.47	33.57	8.00	41.570
3.49	3.20	41.51	10.81	52.32	2.76	2.32	31.89	7.95	39.840
3.34	3.27	44.35	10.09	54.44	2.61	2.32	33.04	6.13	39.170
3.63	3.34	42.15	10.41	52.56	2.68	2.34	33.64	8.17	41.810
3.63	3.12	40.94	10.89	51.83	2.69	2.32	31.95	7.95	39.900
3.31	3.17	46.40	10.84	57.24	2.61	2.25	30.86	8.52	39.380
3.49	3.12	42.91	5.24	48.15	2.65	2.36	34.00	7.45	41.450
3.63	3.22	42.52	11.07	53.59	2.63	2.31	32.93	8.88	41.810
3.78	3.27	45.61	10.36	55.97	2.63	2.31	31.57	7.41	38.980
3.65	3.12	45.84	10.82	56.66	2.67	2.31	31.69	8.65	40.340
3.63	3.05	43.46	10.80	54.26	2.54	2.32	32.68	6.41	39.090
3.44	2.99	42.99	9.44	52.43	2.64	2.31	32.75	8.05	40.800
3.58	3.09	45.61	10.36	55.97	2.61	2.32	32.27	8.12	40.390
3.43	3.06	43.83	6.56	50.39	2.63	2.32	32.84	7.88	40.720
3.19	3.31	40.88	9.90	50.78	2.83	2.40	34.61	8.82	43.430
3.63	3.41	41.73	11.12	52.85	2.62	2.34	33.37	8.68	42.050
3.29	3.21	40.75	6.11	46.86	2.47	2.32	30.44	8.84	39.280

Table B.2 Size (μm) of spermatozoal components of diploid Pacific oysters (live samples)

Oyster Identification	Acrosome		Head		Mitochondria	Flagellum
	Height	Width	Length	Width	Height	Length
CG03M13	0.564	1.032	2.554	2.404	0.741	37.098
CG03M13	0.741	1.159	2.267	2.307	0.629	35.430
CG03M13	0.637	1.215	2.546	2.315	0.611	34.964
CG03M13	0.637	1.274	2.326	2.228	0.895	34.888
CG03M13	0.597	1.279	2.514	2.516	0.787	32.285
CG03M13	0.798	1.319	2.399	2.301	0.801	38.735
CG03M13	0.699	1.159	2.354	2.087	0.683	38.213
CG03M13	0.815	1.294	2.267	2.049	0.744	34.716
CG03M13	0.593	1.335	2.426	2.370	0.564	38.319
CG03M13	0.741	1.335	2.239	2.098	0.715	40.268
CG03M14	0.555	1.346	2.415	2.203	0.813	38.744
CG03M14	0.615	1.233	2.386	2.078	0.697	33.932
CG03M14	0.601	1.144	2.443	2.177	0.756	37.017
CG03M14	0.709	1.305	2.612	2.445	0.713	39.299
CG03M14	0.761	1.109	2.506	2.537	0.606	38.333
CG03M14	0.538	1.080	2.101	2.000	0.693	35.655
CG03M14	0.758	1.093	2.128	2.353	0.841	37.165
CG03M14	0.688	1.243	2.523	2.309	0.744	36.602
CG03M14	0.769	1.310	2.435	2.139	0.810	37.810
CG03M14	0.792	1.376	2.527	2.253	0.587	37.119
CG03M15	0.709	1.063	2.49	2.360	0.810	38.291
CG03M15	0.785	1.215	2.097	2.065	0.642	40.474
CG03M15	0.713	1.189	2.593	2.283	0.849	37.015
CG03M15	0.606	1.203	2.374	2.139	0.693	36.377
CG03M15	0.677	1.203	2.482	2.332	0.681	40.663
CG03M15	0.693	1.128	2.393	1.968	0.693	37.121
CG03M15	0.672	1.189	2.283	2.243	0.693	38.855
CG03M15	0.647	1.189	2.399	2.425	0.606	37.444
CG03M15	0.693	1.280	2.292	2.137	0.693	36.737
CG03M15	0.799	1.382	2.503	2.606	0.672	37.915

Table B.3 Size (μm) of spermatozoal components of tetraploid Pacific oysters (live samples).

Oyster	Acrosome		Head		Mitochondria	Flagellum
Identification	Height	Width	Length	Width	Height	Length
CG03P01	0.630	1.484	3.124	3.437	0.940	51.702
CG03P01	0.667	1.564	3.172	3.005	0.859	49.100
CG03P01	0.735	1.335	3.004	2.937	0.870	52.215
CG03P01	0.804	1.329	3.148	2.816	0.704	51.329
CG03P01	0.895	1.345	3.297	3.134	0.824	51.808
CG03P01	0.633	1.215	3.008	3.012	0.704	53.419
CG03P01	0.899	1.492	2.985	2.986	0.676	45.005
CG03P01	0.793	1.388	3.135	2.982	0.642	47.954
CG03P01	0.768	1.305	3.312	2.805	0.804	50.751
CG03P01	0.873	1.215	3.352	3.045	0.935	50.334
CG03P01	0.752	1.280	3.562	2.797	0.752	53.578
CG03P01	0.799	1.305	3.218	2.765	0.799	50.078
CG03P01	0.827	1.215	3.410	3.234	0.915	51.993
CG03P01	0.841	1.427	3.269	2.807	0.813	52.243
CG03P01	0.827	1.429	3.241	2.727	0.767	49.391
CG03P01	0.774	1.355	3.309	2.644	0.810	55.058
CG03P01	0.908	1.345	3.298	2.695	0.908	50.471
CG03P01	0.709	1.294	3.126	2.902	0.813	51.624
CG03P01	0.759	1.313	3.357	2.846	0.830	45.138
CG03P01	0.681	1.382	3.083	2.925	0.810	50.320
CG03P02	0.894	1.579	3.301	3.089	0.817	51.126
CG03P02	0.767	1.570	3.321	3.085	0.801	49.847
CG03P02	0.736	1.420	3.342	2.779	0.909	50.771
CG03P02	0.727	1.495	3.240	2.598	0.619	50.319
CG03P02	0.699	1.499	3.221	2.703	0.813	51.111
CG03P02	0.695	1.597	3.096	2.847	0.849	50.591
CG03P02	0.750	1.495	3.480	3.060	0.750	51.179
CG03P02	0.727	1.570	3.265	2.959	0.837	51.037
CG03P02	0.727	1.454	3.317	2.983	0.771	49.966
CG03P02	0.750	1.448	3.292	2.914	0.760	51.500
CG03P02	0.736	1.547	3.334	3.008	0.894	50.133
CG03P02	0.655	1.466	3.492	2.938	0.817	50.299
CG03P02	0.690	1.438	3.123	2.877	0.750	50.878
CG03P02	0.736	1.547	3.333	2.847	0.817	51.301
CG03P02	0.732	1.465	3.277	2.902	0.894	49.013
CG03P02	0.736	1.463	3.415	2.982	0.732	49.436
CG03P02	0.767	1.492	3.518	2.775	0.879	51.806
CG03P02	0.736	1.466	3.496	2.848	0.898	50.863
CG03P02	0.699	1.495	3.427	2.887	0.801	50.555
CG03P02	0.736	1.463	3.462	2.927	0.894	51.279
CG03P03	0.691	1.224	3.435	2.444	0.767	52.982
CG03P03	0.620	1.387	3.231	2.846	0.850	52.802
CG03P03	0.662	1.354	3.271	2.691	0.809	49.613
CG03P03	0.651	1.334	3.312	3.016	0.798	51.161
CG03P03	0.715	1.380	3.039	2.633	0.731	50.431
CG03P03	0.658	1.316	3.070	2.667	0.824	52.393

Oyster Identification	Acrosome		Head		Mitochondria	Flagellum
	Height	Width	Length	Width	Height	Length
CG03P03	0.693	1.290	3.260	2.741	0.775	51.541
CG03P03	0.667	1.318	3.238	2.688	0.823	50.556
CG03P03	0.705	1.271	3.196	2.64	0.815	51.231
CG03P03	0.693	1.363	3.502	3.027	0.835	49.983
CG03P03	0.675	1.262	3.148	2.582	0.877	50.786
CG03P03	0.635	1.327	3.212	2.577	0.745	50.893
CG03P03	0.620	1.354	3.366	2.714	0.773	51.693
CG03P03	0.648	1.380	3.289	2.496	0.884	51.402
CG03P03	0.712	1.402	3.377	2.868	0.813	52.008
CG03P03	0.677	1.369	3.402	2.923	0.863	51.714
CG03P03	0.693	1.356	3.516	3.021	0.877	49.938
CG03P03	0.639	1.234	3.121	2.708	0.791	51.092
CG03P03	0.705	1.310	3.154	2.741	0.835	51.753
CG03P03	0.698	1.393	3.343	2.853	0.814	50.770
CG03P04	0.902	1.429	3.158	2.858	0.677	45.987
CG03P04	1.023	1.620	3.644	3.022	1.076	49.347
CG03P04	0.752	1.436	3.035	2.554	0.647	50.477
CG03P04	0.677	1.506	3.084	2.556	0.905	52.762
CG03P04	0.774	1.481	3.340	2.658	0.813	47.590
CG03P04	0.756	1.533	3.424	2.707	0.830	48.828
CG03P04	0.785	1.533	3.246	2.574	0.827	47.676
CG03P04	0.709	1.339	2.920	2.526	0.752	48.725
CG03P04	0.713	1.313	2.794	2.473	0.813	51.753
CG03P04	0.606	1.355	3.045	2.557	0.810	45.646
CG03P05	0.763	1.259	2.938	2.404	0.845	47.194
CG03P05	0.741	1.333	3.038	2.593	0.963	46.511
CG03P05	0.818	1.335	2.889	2.627	0.828	46.712
CG03P05	0.963	1.335	3.116	2.554	0.845	49.256
CG03P05	0.818	1.483	2.877	2.587	0.892	45.688
CG03P05	0.741	1.311	3.038	2.621	0.787	50.785
CG03P05	0.741	1.319	3.009	2.676	0.787	47.617
CG03P05	0.683	1.378	2.777	2.621	0.787	46.773
CG03P05	0.818	1.425	3.126	2.804	0.937	46.723
CG03P05	0.683	1.279	2.982	2.520	0.744	49.051
CG03P06	0.625	1.243	2.792	2.559	0.625	53.851
CG03P06	1.077	1.290	3.087	2.704	0.877	48.989
CG03P06	0.943	1.320	3.234	3.196	1.043	52.230
CG03P06	1.008	1.299	3.048	3.116	1.020	52.001
CG03P06	0.993	1.318	2.918	2.770	0.907	50.808
CG03P06	0.907	1.165	2.485	2.253	0.639	50.490
CG03P06	1.008	1.226	2.651	2.971	0.867	52.805
CG03P06	0.884	1.318	2.673	2.433	0.775	53.724
CG03P06	0.933	1.299	3.086	2.843	0.907	51.906
CG03P06	0.907	1.250	2.872	2.808	0.853	50.540

Table B.4 Basic parameters of oysters received and used in experiments (bold in identification) during June and August 2002.

Received date	Oyster identification	Ploidy	Body wet weight (g)	Shell height (mm)	Gonad net wet weight (g)
6/4/2002	**CG02M27**	2C	105.87	108.1	11.54
6/4/2002	**CG02M29**	2C	87.77	84.8	5.68
6/4/2002	CG02M31	2C	54.61	72.2	4.15
6/4/2002	CG02M33	2C	34.70	78.2	1.87
6/11/2002	CG02M43	2C	29.40	87.1	1.34
6/11/2002	**CG02M44**	2C	33.50	74.1	2.82
6/11/2002	CG02M45	2C	52.20	90.7	2.83
6/11/2002	**CG02M46**	2C	54.10	82.9	4.55
6/19/2002	CG02M50	2C	28.50	84.7	1.76
6/19/2002	CG02M51	2C	56.40	108.9	3.84
6/19/2002	CG02M52	2C	53.30	93.0	3.60
6/19/2002	CG02M53	2C	44.30	113.0	2.58
6/26/2002	CG02M57	2C	48.10	82.2	2.87
6/26/2002	CG02M58	2C	25.80	92.5	1.35
6/26/2002	CG02M59	2C	49.30	92.8	5.25
7/3/2002	**CG02M63**	2C	29.20	82.5	1.76
7/3/2002	**CG02M64**	2C	79.00	100.0	5.88
7/3/2002	**CG02M65**	2C	37.40	100.0	2.57
7/3/2002	**CG02M66**	2C	27.40	79.9	2.02
7/10/2002	**CG02M71**	2C	36.50	79.2	3.73
7/10/2002	**CG02M72**	2C	30.00	84.2	2.19
7/10/2002	**CG02M73**	2C	52.50	80.6	2.20
7/10/2002	**CG02M74**	2C	33.40	84.0	2.42
7/16/2002	**CG02M80**	2C	63.70	122.1	3.75
7/16/2002	**CG02M81**	2C	47.80	110.0	2.51
7/16/2002	**CG02M82**	2C	69.30	113.0	6.15
7/23/2002	**CG02M89**	2C	38.90	82.0	2.56
7/23/2002	**CG02M90**	2C	102.90	92.5	7.23
7/23/2002	**CG02M91**	2C	77.00	115.8	4.44
7/23/2002	**CG02M92**	2C	64.50	114.9	3.74
7/30/2002	CG02M99	2C	28.30	65.0	1.45
7/30/2002	**CG02M100**	2C	63.20	99.8	4.42
7/30/2002	**CG02M101**	2C	74.20	115.5	3.69
7/30/2002	**CG02M102**	2C	68.20	98.2	4.25
6/4/2002	**CG02M35**	4C	249.45	132.0	13.46
6/4/2002	CG02M36	4C	243.14	137.0	6.06
6/4/2002	**CG02M37**	4C	269.88	143.0	13.59
6/4/2002	CG02M38	4C	209.62	132.0	7.29
6/4/2002	CG02M39	4C	152.63	108.1	6.69
6/11/2002	**CG02M40**	4C	324.70	146.0	12.75
6/11/2002	CG02M41	4C	308.10	142.9	11.69
6/11/2002	CG02M42	4C	183.60	122.0	5.28

Received date	Oyster identification	Ploidy	Body wet weight (g)	Shell height (mm)	Gonad net wet weight (g)
6/19/2002	CG02M47	4C	310.30	136.7	9.74
6/19/2002	CG02M48	4C	347.60	125.5	13.91
6/19/2002	CG02M49	4C	271.90	144.0	7.20
6/26/2002	CG02M54	4C	266.30	129.6	12.11
6/26/2002	CG02M55	4C	149.20	98.9	8.64
6/26/2002	CG02M56	4C	234.40	112.0	6.82
7/3/2002	**CG02M60**	4C	116.90	94.0	3.19
7/3/2002	**CG02M61**	4C	152.40	93.2	3.37
7/3/2002	**CG02M62**	4C	342.60	130.0	5.19
7/10/2002	**CG02M67**	4C	65.20	87.2	4.49
7/10/2002	**CG02M68**	4C	54.00	91.0	3.06
7/10/2002	**CG02M69**	4C	66.60	98.2	3.08
7/10/2002	**CG02M70**	4C	43.90	83.2	2.26
7/16/2002	CG02M75	4C	38.50	81.8	1.00
7/16/2002	CG02M76	4C	50.20	85.2	1.62
7/16/2002	**CG02M77**	4C	88.10	94.6	4.95
7/16/2002	**CG02M78**	4C	70.10	100.2	4.47
7/16/2002	**CG02M79**	4C	69.30	97.0	4.00
7/23/2002	**CG02M83**	4C	41.10	93.5	2.32
7/23/2002	**CG02M84**	4C	45.00	80.0	1.87
7/23/2002	**CG02M85**	4C	45.00	77.8	2.08
7/23/2002	**CG02M86**	4C	49.10	95.2	2.56
7/23/2002	**CG02M87**	4C	47.40	85.0	1.20
7/23/2002	**CG02M88**	4C	35.40	79.0	1.07
7/30/2002	**CG02M93**	4C	73.60	100.0	2.95
7/30/2002	**CG02M94**	4C	64.50	100.0	2.30
7/30/2002	**CG02M95**	4C	44.80	89.0	1.90
7/30/2002	**CG02M96**	4C	46.50	86.7	2.31
7/30/2002	**CG02M97**	4C	43.10	85.0	2.52
7/30/2002	**CG02M98**	4C	54.00	95.0	2.75

Table B.5 Absorbance readings for sperm from tetraploid Pacific oysters.

Oyster identification	**Concentration** (wavelength at 380 nm)																
	$1x10^9$	**$8x10^8$**	**$6x10^8$**	**$4x10^8$**	**$2x10^8$**	**$1x10^8$**	**$8x10^7$**	**$6x10^7$**	**$4x10^7$**	**$2x10^7$**	**$1x10^7$**	**$8x10^6$**	**$6x10^6$**	**$4x10^6$**	**$2x10^6$**	**$1x10^6$**	**$1x10^5$**
CG02M40	1.418	1.400	1.393	1.458	1.249	0.860	0.721	0.567	0.354	0.122	0.053	0.041	0.027	0.016	0.006	0.000	0.000
CG02M77	1.548	1.545	1.487	1.427	1.259	1.002	0.903	0.763	0.573	0.289	0.111	0.079	0.046	0.012	0.000	0.000	0.000
CG02M78	1.604	1.573	1.558	1.502	1.393	1.194	1.114	1.011	0.828	0.513	0.265	0.212	0.158	0.103	0.049	0.025	0.004
CG02M79	1.590	1.563	1.543	1.482	1.334	1.114	1.023	0.895	0.705	0.400	0.204	0.160	0.117	0.077	0.038	0.019	0.003
CG02M93	1.601	1.564	1.533	1.460	1.306	1.083	0.979	0.849	0.666	0.372	0.185	0.147	0.109	0.070	0.032	0.019	0.001
CG02M94	1.615	1.596	1.553	1.491	1.334	1.114	1.036	0.887	0.687	0.381	0.190	0.147	0.109	0.072	0.034	0.018	0.001
CG02M97	1.605	1.572	1.535	1.470	1.305	1.053	0.951	0.810	0.605	0.330	0.162	0.127	0.094	0.061	0.031	0.015	0.003
	Wavelength at 550 nm																
CG02M40	2.228	2.096	1.945	1.706	1.273	0.793	0.627	0.457	0.271	0.093	0.042	0.032	0.023	0.013	0.006	0.000	0.000
CG02M77	2.282	2.158	1.990	1.757	1.373	0.964	0.830	0.666	0.471	0.219	0.083	0.058	0.035	0.011	0.000	0.000	0.000
CG02M78	2.461	2.338	2.172	1.937	1.575	1.214	1.099	0.943	0.727	0.406	0.199	0.156	0.115	0.074	0.036	0.018	0.002
CG02M79	2.312	2.195	2.027	1.799	1.445	1.077	0.954	0.795	0.586	0.307	0.152	0.118	0.086	0.056	0.028	0.014	0.002
CG02M93	2.259	2.130	1.970	1.751	1.396	1.021	0.903	0.744	0.546	0.285	0.141	0.110	0.080	0.051	0.024	0.014	0.001
CG02M94	2.295	2.171	2.013	1.792	1.434	1.062	0.939	0.778	0.564	0.290	0.140	0.110	0.081	0.053	0.025	0.014	0.002
CG02M97	2.239	2.113	1.955	1.733	1.367	0.986	0.860	0.698	0.491	0.247	0.118	0.094	0.070	0.046	0.023	0.011	0.002
	Wavelength at 581 nm																
CG02M40	2.071	1.966	1.870	1.652	1.234	0.757	0.604	0.438	0.263	0.090	0.041	0.031	0.022	0.013	0.006	0.000	0.000
CG02M77	2.196	2.051	1.898	1.700	1.330	0.934	0.801	0.643	0.454	0.212	0.081	0.057	0.034	0.011	0.000	0.000	0.000
CG02M78	2.288	2.201	2.070	1.861	1.528	1.178	1.065	0.915	0.703	0.392	0.194	0.152	0.112	0.073	0.035	0.017	0.003
CG02M79	2.186	2.087	1.944	1.739	1.404	1.041	0.923	0.766	0.567	0.296	0.148	0.114	0.084	0.054	0.027	0.013	0.001
CG02M93	2.141	2.034	1.890	1.698	1.358	0.990	0.875	0.721	0.528	0.276	0.135	0.084	0.078	0.050	0.024	0.013	0.001
CG02M94	2.176	2.071	1.930	1.730	1.393	1.032	0.908	0.752	0.544	0.282	0.136	0.107	0.080	0.052	0.025	0.015	0.002
CG02M97	2.121	2.015	1.879	1.678	1.326	0.955	0.830	0.673	0.472	0.240	0.115	0.091	0.068	0.045	0.022	0.010	0.002
CG02M96	2.167	2.064	1.922	1.720	1.375	1.019	0.897	0.746	0.533	0.272	0.131	0.105	0.078	0.054	0.027	0.014	0.005
	Wavelength at 780 nm																
CG02M40	1.930	1.829	1.755	1.538	1.112	0.640	0.506	0.360	0.215	0.076	0.036	0.032	0.027	0.019	0.011	0.006	0.000
CG02M77	2.087	1.940	1.794	1.590	1.210	0.812	0.683	0.537	0.373	0.171	0.067	0.048	0.029	0.010	0.000	0.000	0.000
CG02M78	2.193	2.093	1.960	1.749	1.414	1.051	0.938	0.792	0.585	0.318	0.160	0.126	0.094	0.063	0.031	0.016	0.002
CG02M79	2.079	1.974	1.830	1.621	1.282	0.914	0.794	0.646	0.464	0.240	0.123	0.096	0.072	0.047	0.024	0.012	0.001
CG02M93	2.024	1.920	1.778	1.584	1.240	0.865	0.750	0.604	0.435	0.227	0.116	0.092	0.068	0.045	0.021	0.012	0.001
CG02M94	2.073	1.963	1.815	1.624	1.277	0.907	0.784	0.634	0.447	0.231	0.116	0.092	0.069	0.046	0.022	0.014	0.002
CG02M97	2.014	1.908	1.761	1.559	1.205	0.831	0.706	0.560	0.385	0.196	0.098	0.078	0.059	0.040	0.019	0.010	0.002

Table B.6 Absorbance readings for sperm from diploid Pacific oysters.

Oyster identification	**Concentration** (wavelength at 380 nm)																	
	$2x10^9$	**$1x10^9$**	**$8x10^8$**	**$6x10^8$**	**$4x10^8$**	**$2x10^8$**	**$1x10^8$**	**$8x10^7$**	**$6x10^7$**	**$4x10^7$**	**$2x10^7$**	**$1x10^7$**	**$8x10^6$**	**$6x10^6$**	**$4x10^6$**	**$2x10^6$**	**$1x10^6$**	**$1x10^5$**
CG02M44	1.429	1.528	1.353	1.315	1.237	1.029	0.747	0.649	0.516	0.369	0.185	0.083	0.066	0.046	0.028	0.015	0.001	0.000
CG02M46	1.449	1.425	1.408	1.379	1.318	1.218	0.906	0.802	0.660	0.475	0.242	0.133	0.100	0.075	0.049	0.023	0.010	0.001
CG02M80	1.584	1.523	1.492	1.441	1.348	1.131	0.833	0.724	0.585	0.411	0.203	0.096	0.073	0.054	0.036	0.017	0.007	0.000
CG02M81	1.573	1.546	1.476	1.430	1.342	1.141	0.844	0.745	0.609	0.439	0.226	0.111	0.087	0.063	0.037	0.017	0.007	0.000
CG02M82	1.584	1.503	1.470	1.415	1.314	1.090	0.791	0.689	0.557	0.391	0.198	0.095	0.076	0.054	0.033	0.015	0.007	0.000
CG02M100	1.609	1.565	1.522	1.480	1.395	1.203	0.932	0.815	0.674	0.493	0.257	0.122	0.093	0.071	0.044	0.022	0.011	0.002
CG02M101	1.609	1.551	1.522	1.484	1.373	1.163	0.869	0.760	0.623	0.437	0.225	0.107	0.084	0.063	0.042	0.019	0.010	0.004
CG02M102	1.587	1.513	1.467	1.399	1.291	1.040	0.723	0.615	0.485	0.332	0.163	0.078	0.062	0.045	0.029	0.013	0.006	0.003
Wavelength at 550 nm																		
CG02M44	2.300	1.922	1.806	1.651	1.434	1.025	0.647	0.537	0.410	0.276	0.135	0.066	0.053	0.037	0.022	0.012	0.003	0.000
CG02M46	2.584	2.220	2.110	1.898	1.659	1.198	0.790	0.667	0.515	0.348	0.172	0.094	0.071	0.055	0.036	0.017	0.008	0.002
CG02M80	2.358	1.975	1.855	1.701	1.489	1.103	0.716	0.602	0.462	0.314	0.154	0.074	0.058	0.043	0.029	0.013	0.007	0.001
CG02M81	2.334	1.959	1.836	1.686	1.476	1.112	0.735	0.620	0.488	0.336	0.170	0.085	0.067	0.049	0.031	0.014	0.005	0.000
CG02M82	2.290	1.896	1.779	1.630	1.416	1.048	0.675	0.566	0.438	0.297	0.148	0.073	0.058	0.042	0.026	0.012	0.006	0.000
CG02M100	2.439	2.068	1.935	1.783	1.572	1.202	0.821	0.695	0.547	0.380	0.192	0.095	0.075	0.056	0.036	0.018	0.009	0.002
CG02M101	2.366	1.988	1.864	1.713	1.505	1.136	0.750	0.632	0.496	0.333	0.168	0.083	0.066	0.049	0.033	0.015	0.006	0.003
CG02M102	2.214	1.832	1.710	1.561	1.339	0.955	0.592	0.486	0.371	0.246	0.120	0.059	0.049	0.034	0.023	0.011	0.005	0.002
Wavelength at 581 nm																		
CG02M44	2.125	1.822	1.726	1.586	1.380	0.989	0.620	0.516	0.394	0.265	0.131	0.064	0.051	0.036	0.022	0.011	0.003	0.000
CG02M46	2.316	2.065	1.979	1.802	1.591	1.157	0.756	0.632	0.490	0.331	0.163	0.088	0.067	0.051	0.033	0.016	0.007	0.001
CG02M80	2.216	1.897	1.789	1.649	1.440	1.069	0.688	0.578	0.447	0.303	0.148	0.073	0.056	0.042	0.028	0.013	0.006	0.001
CG02M81	2.203	1.880	1.772	1.628	1.431	1.078	0.708	0.595	0.470	0.323	0.165	0.083	0.065	0.047	0.030	0.013	0.005	0.000
CG02M82	2.165	1.825	1.723	1.579	1.376	1.014	0.650	0.544	0.420	0.286	0.144	0.071	0.056	0.041	0.025	0.012	0.005	0.000
CG02M100	2.287	1.983	1.861	1.735	1.523	1.163	0.789	0.668	0.527	0.366	0.186	0.092	0.072	0.054	0.036	0.017	0.009	0.002
CG02M101	2.237	1.908	1.800	1.660	1.460	1.104	0.723	0.608	0.478	0.322	0.163	0.080	0.064	0.047	0.033	0.015	0.006	0.002
CG02M102	2.097	1.766	1.657	1.513	1.298	0.926	0.570	0.468	0.357	0.238	0.117	0.058	0.047	0.034	0.022	0.010	0.005	0.002
Wavelength at 780 nm																		
CG02M44	1.986	1.688	1.603	1.464	1.259	0.852	0.502	0.412	0.312	0.211	0.106	0.052	0.042	0.029	0.018	0.008	0.002	0.000
CG02M46	2.164	1.911	1.821	1.660	1.440	0.991	0.582	0.478	0.366	0.244	0.120	0.065	0.048	0.041	0.024	0.011	0.005	0.000
CG02M80	2.105	1.789	1.674	1.527	1.316	0.927	0.566	0.463	0.356	0.240	0.119	0.059	0.046	0.034	0.022	0.010	0.005	0.001
CG02M81	2.094	1.768	1.653	1.515	1.312	0.938	0.581	0.482	0.373	0.254	0.131	0.067	0.052	0.040	0.023	0.011	0.004	0.000
CG02M82	2.058	1.715	1.608	1.462	1.255	0.875	0.529	0.434	0.333	0.227	0.115	0.056	0.046	0.034	0.020	0.009	0.004	0.000
CG02M100	2.182	1.863	1.757	1.606	1.404	1.027	0.655	0.545	0.423	0.290	0.149	0.075	0.059	0.045	0.029	0.014	0.009	0.002
CG02M101	2.122	1.793	1.678	1.547	1.339	0.963	0.592	0.490	0.379	0.256	0.129	0.065	0.052	0.039	0.026	0.013	0.005	0.001
CG02M102	1.992	1.653	1.536	1.389	1.168	0.781	0.454	0.369	0.281	0.188	0.094	0.047	0.037	0.027	0.018	0.009	0.004	0.002

Table B.7 Sperm concentration per gram of wet gonad weight of sperm from diploid and tetraploid Pacific oysters.

Tetraploid			Diploid		
Date	Oyster identification	sperm g^{-1} gonad (x 10^{10})	Date	Oyster identification	sperm g^{-1} gonad (x 10^{10})
6/11/2002	CG02M40	2.59	6/11/2002	CG02M44	4.29
6/11/2002	CG02M41	2.11	6/11/2002	CG02M45	4.63
7/3/2002	CG02M60	1.71	6/11/2002	CG02M46	2.29
7/3/2002	CG02M61	2.24	7/3/2002	CG02M63	3.97
7/3/2002	CG02M61	1.67	7/3/2002	CG02M64	5.50
7/3/2002	CG02M62	1.92	7/3/2002	CG02M64	6.40
7/3/2002	CG02M62	2.46	7/3/2002	CG02M65	4.51
7/10/2002	CG02M67	2.08	7/3/2002	CG02M65	5.20
7/10/2002	CG02M67	3.09	7/3/2002	CG02M66	5.01
7/10/2002	CG02M67	2.36	7/10/2002	CG02M71	4.42
7/10/2002	CG02M68	2.66	7/10/2002	CG02M71	5.70
7/10/2002	CG02M68	2.72	7/10/2002	CG02M71	5.42
7/10/2002	CG02M69	2.23	7/10/2002	CG02M72	4.37
7/10/2002	CG02M70	2.54	7/10/2002	CG02M72	5.72
7/16/2002	CG02M77	2.50	7/10/2002	CG02M73	3.77
7/16/2002	CG02M78	1.98	7/10/2002	CG02M74	5.03
7/16/2002	CG02M79	2.44	7/16/2002	CG02M80	6.15
7/23/2002	CG02M83	3.34	7/16/2002	CG02M81	5.70
7/23/2002	CG02M84	2.59	7/16/2002	CG02M82	5.96
7/23/2002	CG02M85	2.99	7/23/2002	CG02M89	6.30
7/23/2002	CG02M86	2.95	7/23/2002	CG02M90	5.84
7/23/2002	CG02M87	3.23	7/23/2002	CG02M91	6.09
7/23/2002	CG02M88	3.01	7/23/2002	CG02M92	5.66
7/23/2002	CG02M83	2.05	7/23/2002	CG02M89	4.56
7/23/2002	CG02M86	2.18	7/23/2002	CG02M90	5.05
7/30/2002	CG02M93	2.98	7/23/2002	CG02M91	4.55
7/30/2002	CG02M94	2.65	7/23/2002	CG02M92	5.49
7/30/2002	CG02M95	2.19	7/30/2002	CG02M99	5.99
7/30/2002	CG02M96	2.62	7/30/2002	CG02M100	4.44
7/30/2002	CG02M97	2.91	7/30/2002	CG02M101	5.24
7/30/2002	CG02M98	2.40	7/30/2002	CG02M102	6.52
8/13/2002	CG02M103	2.05	8/13/2002	CG02M120	5.25
8/13/2002	CG02M105	1.14	8/13/2002	CG02M123	5.40
8/13/2002	CG02M108	2.95	8/13/2002	CG02M124	3.92
8/13/2002	CG02M118	2.48	8/13/2002	CG02M126	4.44
8/20/2002	CG02M139	2.59	8/20/2002	CG02M131	4.97
8/20/2002	CG02M142	2.68	--	--	--
8/20/2002	CG02M145	2.40	--	--	--
8/20/2002	CG02M146	2.96	--	--	--

Table B.8 The initial percent motility of sperm from 27 diploid oysters received from June 4 to July 7, 2004.

Received date	Shippment format	Oyster identification	Initial motility
6/4/2004	oyster	CG04M26	5
6/4/2004	oyster	CG04M27	80
6/4/2004	oyster	CG04M28	80
6/4/2004	oyster	CG04M29	90
6/4/2004	oyster	CG04M30	85
6/4/2004	oyster	CG04M31	50
6/10/2004	oyster	CG04M59	90
6/10/2004	oyster	CG04M60	80
6/18/2004	sperm	CG04M69	90
6/18/2004	sperm	CG04M70	95
6/23/2004	sperm	CG04M77	90
6/23/2004	sperm	CG04M78	90
6/23/2004	sperm	CG04M79	90
6/23/2004	sperm	CG04M80	90
6/23/2004	sperm	CG04M81	90
6/29/2004	sperm	CG04M83	90
6/29/2004	sperm	CG04M84	90
6/29/2004	sperm	CG04M87	90
6/29/2004	sperm	CG04M88	90
6/29/2004	sperm	CG04M92	90
7/2/2004	sperm	CG04M95	95
7/2/2004	sperm	CG04M96	90
7/2/2004	sperm	CG04M97	90
7/2/2004	sperm	CG04M98	20
7/7/2004	sperm	CG04M105	90
7/7/2004	sperm	CG04M106	95
7/7/2004	sperm	CG04M111	80

Table B.9 The post-thaw percent motility of sperm samples cooled at different rates.

Cryoprotectant (concentration)	Oyster identification	Cooling rate (°C/min)					
		50	45	30	16	5	0.5
MeOH 10	CG04M26	1	2	2	1	5	5
MeOH 10	CG04M26	1	2	5	5	10	1
MeOH 10	CG04M26	1	2	2	5	1	5
MeOH 10	CG04M27	5	1	1	1	1	1
MeOH 10	CG04M27	5	1	1	1	1	1
MeOH 10	CG04M27	5	1	1	1	1	1
MeOH 10	CG04M28	0	5	1	5	10	1
MeOH 10	CG04M28	1	1	5	5	5	5
MeOH 10	CG04M28	1	5	5	0	5	5
MeOH 10	CG04M29	1	1	1	1	1	1
MeOH 10	CG04M29	1	1	1	0	1	1
MeOH 10	CG04M29	1	1	1	0	1	1
P-glycol 10	CG04M26	1	1	2	1	1	2
P-glycol 10	CG04M26	1	1	2	2	1	2
P-glycol 10	CG04M26	1	1	5	5	5	2
P-glycol 10	CG04M27	10	10	10	10	20	1
P-glycol 10	CG04M27	10	10	10	1	10	1
P-glycol 10	CG04M27	10	10	10	1	10	5
P-glycol 10	CG04M28	10	10	10	10	20	5
P-glycol 10	CG04M28	10	10	10	1	10	10
P-glycol 10	CG04M28	10	10	10	10	15	10
P-glycol 10	CG04M29	10	10	20	1	20	15
P-glycol 10	CG04M29	10	15	15	30	30	20
P-glycol 10	CG04M29	10	10	20	30	35	20
E-glycol 10	CG04M26	1	1	1	1	1	1
E-glycol 10	CG04M26	1	1	1	1	1	1
E-glycol 10	CG04M26	1	0	1	1	2	1
E-glycol 10	CG04M27	1	5	5	5	1	5
E-glycol 10	CG04M27	2	1	10	1	5	10
E-glycol 10	CG04M27	2	1	5	10	5	5
E-glycol 10	CG04M28	10	20	10	20	30	20
E-glycol 10	CG04M28	5	1	20	20	30	30
E-glycol 10	CG04M28	10	10	10	0	30	15
E-glycol 10	CG04M29	1	10	15	4	20	20
E-glycol 10	CG04M29	10	1	15	10	10	15
E-glycol 10	CG04M29	5	10	15	1	5	10
DMSO 10	CG04M26	1	1	1	1	2	1
DMSO 10	CG04M26	1	1	1	1	2	1
DMSO 10	CG04M26	1	5	5	1	2	2
DMSO 10	CG04M27	10	10	10	10	10	1
DMSO 10	CG04M27	10	10	10	5	10	1
DMSO 10	CG04M27	10	10	15	10	15	1
DMSO 10	CG04M28	20	20	10	20	15	1
DMSO 10	CG04M28	10	20	20	5	15	1
DMSO 10	CG04M28	10	20	20	1	15	5
DMSO 10	CG04M29	10	20	40	20	30	5
DMSO 10	CG04M29	5	20	30	1	30	5
DMSO 10	CG04M29	10	20	40	15	30	5

Table B.10 The post-thaw percent motility of sperm samples suspended in different cryoprotectants and cooled at 5 or 30 °C/min.

5 °C/min		**Cryoprotectant**							
Oyster identification	**Concentration**	**MeOH**	**P-glycol**	**E-glycol**	**DMSO**	**Glycerol**	**DMA**	**PEG200**	**PEG600**
CG04M30	5%	15	5	10	10	5	5	5	1
CG04M30	5%	15	5	15	10	5	10	10	1
CG04M30	10%	1	20	10	30	1	5	10	1
CG04M30	10%	1	10	15	30	1	10	10	1
CG04M31	5%	15	10	10	10	1	1	5	1
CG04M31	5%	15	5	15	10	1	1	10	1
CG04M31	10%	1	10	5	10	1	5	10	1
CG04M31	10%	1	5	10	10	1	5	10	1

30 °C/min		**Cryoprotectant**							
Oyster identification	**Concentration**	**MeOH**	**P-glycol**	**E-glycol**	**DMSO**	**Glycerol**	**DMA**	**PEG200**	**PEG600**
CG04M59	5%	1	10	1	5	1	1	5	0
CG04M59	5%	1	5	1	5	0	1	1	0
CG04M59	10%	1	5	1	5	0	1	1	0
CG04M59	10%	0	2	0	1	0	1	1	0
CG04M60	5%	5	0	5	2	1	1	1	1
CG04M60	5%	0	1	5	5	0	1	1	1
CG04M60	10%	1	1	1	1	0	1	0	1
CG04M60	10%	1	1	1	5	0	1	1	0

Table B.11 The post-thaw percent motility of sperm samples suspended in single or combined cryoprotectants and cooled at 5 °C/min.

First cryoprotectant (%)	Second cryoprotectant (%)	Oyster identification							
		CG04M69	CG04M69	CG04M69	CG04M69	CG04M70	CG04M70	CG04M70	CG04M70
PEG200 (4)	MeOH (6)	30	0	10	20	0	1	20	1
PEG200 (4)	P-glycol (6)	20	20	20	1	40	20	40	20
PEG200 (4)	E-glycol (6)	5	20	5	20	5	2	1	10
PEG200 (4)	DMSO (6)	30	30	25	10	30	1	0	0
PEG600 (4)	MeOH (6)	10	5	10	1	5	10	5	1
PEG600 (4)	P-glycol (6)	10	5	1	1	20	10	10	20
PEG600 (4)	E-glycol (6)	5	5	1	1	2	5	5	1
PEG600 (4)	DMSO (6)	10	5	5	5	1	0	1	0
DMSO (4)	MeOH (4)	0	0	0	0	1	0	10	1
DMSO (4)	P-glycol (4)	5	15	10	1	1	0	0	5
DMSO (4)	E-glycol (4)	0	5	10	0	30	15	30	10
P-glycol (4)	E-glycol (4)	10	1	10	20	0	1	0	1
--	MeOH (8)	0	1	0	0	0	0	5	0
--	P-glycol (8)	0	20	10	5	0	0	0	0
--	E-glycol (8)	0	0	0	0	0	0	5	5
--	DMSO (8)	30	20	10	5	5	1	0	0

Table B.12 The post-thaw percent motility of sperm samples suspended in single or combined cryoprotectants (CPA) and cooled at 5 °C/min.

Concentration	PEG (first cryoprotectant)/MeOH (second CPA)															
First CPA	**2**	**4**	**6**	**2**	**4**	**6**	**2**	**4**	**6**	**0**	**0**	**0**	**2**	**4**	**6**	**8**
Second CPA	**4**	**4**	**4**	**6**	**6**	**6**	**2**	**4**	**8**	**4**	**6**	**8**	**0**	**0**	**0**	**0**
CG04M80	40	50	15	40	10	2	5	--	1	50	50	15	2	20	20	20
CG04M80	40	50	15	50	10	2	5	2	1	40	40	20	2	10	15	25
CG04M80	40	40	15	40	10	2	5	1	1	30	40	15	2	10	15	20
CG04M80	40	30	15	30	10	2	5	1	1	30	40	15	5	10	10	20
CG04M81	40	30	10	50	10	5	10	5	10	20	40	20	1	10	10	15
CG04M81	35	35	10	40	10	5	10	5	10	25	40	20	1	5	10	10
CG04M81	35	30	10	40	10	5	10	5	10	20	30	20	1	5	10	10
CG04M81	40	30	10	30	10	5	10	5	10	20	30	15	2	10	10	10
	PEG/P-glycol															
CG04M77	10	10	10	15	20	10	15	10	1	10	15	30	5	10	25	15
CG04M77	25	20	25	25	20	10	20	10	2	15	10	40	5	10	20	10
CG04M77	10	15	15	25	20	10	20	15	2	30	10	20	5	10	25	10
CG04M77	15	10	15	40	20	10	25	10	5	40	15	30	7	7	25	15
CG04M78	25	25	30	30	15	10	15	10	5	10	30	25	5	5	10	10
CG04M78	15	20	30	40	20	10	10	10	5	20	20	30	2	5	10	15
CG04M78	20	15	15	40	10	5	10	10	2	10	10	20	2	10	25	20
CG04M78	10	20	15	40	10	5	15	10	2	25	10	20	1	5	10	20
	PEG/DMSO															
CG04M78	20	20	20	30	15	10	20	10	5	10	20	20	2	5	15	10
CG04M78	15	20	20	30	10	10	20	10	5	10	20	20	1	10	10	15
CG04M78	20	20	20	30	10	10	20	10	5	5	20	20	1	5	10	15
CG04M78	20	20	20	30	10	10	20	10	5	10	20	20	1	5	15	15
CG04M79	5	5	5	10	5	2	5	1	2	2	5	5	1	2	5	2
CG04M79	5	5	5	10	2	2	5	1	2	1	5	5	1	2	5	2
CG04M79	5	5	5	10	5	2	5	1	2	1	5	5	1	1	2	2
CG04M79	5	5	5	10	2	2	5	1	2	2	5	5	1	1	2	2

Table B.13 The post-thaw percent motility of sperm samples suspended in single or combined cryoprotectants and cooled at 5 °C/min.

PEG200 (%)	Second cryoprotectant (%)	Oyster identification							
		CG04M87	CG04M87	CG04M87	CG04M87	CG04M88	CG04M88	CG04M88	CG04M88
0	MeOH (4)	25	25	25	20	10	5	5	5
0	MeOH (6)	40	45	35	45	10	20	20	15
0	P-glycol (4)	5	2	2	5	1	1	1	1
0	P-glycol (8)	15	20	10	20	5	5	5	5
0	DMSO (6)	1	5	5	5	5	5	2	2
0	DMSO (8)	15	10	5	5	10	15	7	10
2	MeOH (4)	40	40	35	30	15	15	20	20
2	MeOH (6)	40	35	35	35	15	5	15	7
2	P-glycol (4)	10	10	10	15	5	5	5	5
2	P-glycol (6)	25	20	20	25	10	15	5	5
2	DMSO (4)	10	10	10	5	5	5	5	5
2	DMSO (6)	20	20	15	15	10	10	10	10
4	MeOH (4)	45	40	40	35	10	15	15	10
4	P-glycol (4)	15	20	20	15	5	5	5	10
4	DMSO (4)	25	20	20	20	10	15	5	10
8	--	5	5	7	5	5	7	10	5

Table B.14 The post-thaw percent motility of sperm samples cooled with 0.25 and 0.5-ml straws.

			Cryoprotectant						
Straw size	**Thawing water bath (°C)**	**Oyster identification**	**2% PEG/ 4% MeOH**	**6% MeOH**	**2% PEG/ 6% P-glycol**	**8% P-glycol**	**2% PEG/ 6% DMSO**	**8% DMSO**	**8% PEG**
0.25-ml	40	CG04M83	25	25	10	5	25	20	10
0.25-ml	40	CG04M83	25	25	10	10	20	15	10
0.25-ml	40	CG04M83	20	30	10	7	15	30	10
0.25-ml	40	CG04M83	25	20	15	7	25	25	10
0.25-ml	40	CG04M84	25	30	30	20	40	30	10
0.25-ml	40	CG04M84	25	40	20	15	35	30	10
0.25-ml	40	CG04M84	30	35	25	25	40	30	10
0.25-ml	40	CG04M84	30	30	30	25	30	20	10
0.25-ml	60	CG04M83	20	30	10	10	20	25	5
0.25-ml	60	CG04M83	20	25	10	10	20	15	7
0.25-ml	60	CG04M83	25	25	10	15	20	20	5
0.25-ml	60	CG04M83	30	35	10	5	25	15	7
0.25-ml	60	CG04M84	35	35	30	35	30	30	10
0.25-ml	60	CG04M84	40	30	20	30	40	40	15
0.25-ml	60	CG04M84	50	50	30	30	40	20	10
0.25-ml	60	CG04M84	40	30	20	25	30	40	10
0.5-ml	40	CG04M83	25	15	5	2	30	20	10
0.5-ml	40	CG04M83	15	25	5	10	20	20	10
0.5-ml	40	CG04M83	20	15	5	5	20	20	10
0.5-ml	40	CG04M83	25	15	10	5	20	25	10
0.5-ml	40	CG04M84	25	30	20	20	20	10	10
0.5-ml	40	CG04M84	25	40	20	30	20	40	10
0.5-ml	40	CG04M84	25	35	15	25	35	20	10
0.5-ml	40	CG04M84	20	30	20	25	20	30	10
0.5-ml	60	CG04M83	25	15	15	20	25	20	15
0.5-ml	60	CG04M83	25	20	10	15	25	20	10
0.5-ml	60	CG04M83	30	20	20	5	30	20	10
0.5-ml	60	CG04M83	20	25	--	10	30	25	7
0.5-ml	60	CG04M84	35	30	20	20	30	20	15
0.5-ml	60	CG04M84	50	30	30	20	30	30	10
0.5-ml	60	CG04M84	45	25	25	25	30	10	15
0.5-ml	60	CG04M84	30	35	30	30	40	20	15

Table B.15 The post-thaw percent motility of sperm samples suspended in selected cryoprotectants and cooled at 0.5, 5, 16 and 30 °C/min.

Cooling rate (°C/min)	Oyster identification	Cryoprotectant				
		2% PEG/ 4% MeOH	2% PEG/ 6% P-glycol	2% PEG/ 6% DMSO	6% MeOH	8% PEG
0.5	CG04M97	5	5	7	2	1
0.5	CG04M97	5	10	7	2	2
0.5	CG04M97	5	10	5	2	1
0.5	CG04M97	2	5	5	2	2
0.5	CG04M98	1	1	1	1	1
0.5	CG04M98	1	1	2	1	1
0.5	CG04M98	1	1	2	1	1
0.5	CG04M98	1	1	1	1	1
5	CG04M97	30	40	30	20	30
5	CG04M97	30	30	30	20	20
5	CG04M97	20	25	30	15	20
5	CG04M97	30	30	35	20	15
5	CG04M98	1	1	1	1	1
5	CG04M98	1	1	2	1	1
5	CG04M98	1	1	2	1	1
5	CG04M98	1	1	2	1	1
16	CG04M97	20	20	30	10	15
16	CG04M97	15	25	20	20	15
16	CG04M97	15	20	20	12	20
16	CG04M97	25	20	20	12	10
16	CG04M98	1	1	1	1	1
16	CG04M98	1	1	1	1	1
16	CG04M98	1	1	1	1	1
16	CG04M98	1	1	1	1	1
30	CG04M97	20	5	20	20	5
30	CG04M97	5	5	10	10	10
30	CG04M97	30	5	30	10	5
30	CG04M97	10	5	20	5	10
30	CG04M98	1	1	2	1	1
30	CG04M98	1	1	2	1	1
30	CG04M98	1	1	2	1	1
30	CG04M98	1	1	2	1	1

Table B.16 The post-thaw percent motility of sperm samples equilibrated in selected cryoprotectants with different time periods and cooled at 5 and 30 °C/min.

Cooling rate (°C/min)	Cryoprotectant	Oyster identification	Equilibration time (min)			
			60	30	20	10
5	2% PEG/4% MeOH	CG04M106	10	5	5	5
5	2% PEG/4% MeOH	CG04M106	10	7	5	2
5	2% PEG/4% MeOH	CG04M106	7	5	5	5
5	2% PEG/4% MeOH	CG04M106	7	5	5	5
5	2% PEG/4% MeOH	CG04M111	25	10	10	10
5	2% PEG/4% MeOH	CG04M111	20	15	10	5
5	2% PEG/4% MeOH	CG04M111	20	15	7	5
5	2% PEG/4% MeOH	CG04M111	20	15	7	5
5	6% MeOH	CG04M106	10	7	5	5
5	6% MeOH	CG04M106	10	5	10	5
5	6% MeOH	CG04M106	7	5	5	2
5	6% MeOH	CG04M106	10	5	5	5
5	6% MeOH	CG04M111	10	7	7	5
5	6% MeOH	CG04M111	15	10	10	5
5	6% MeOH	CG04M111	15	10	10	5
5	6% MeOH	CG04M111	15	10	5	2
30	2% PEG/4% MeOH	CG04M106	7	10	5	5
30	2% PEG/4% MeOH	CG04M106	2	2	7	2
30	2% PEG/4% MeOH	CG04M106	2	10	5	2
30	2% PEG/4% MeOH	CG04M106	2	5	2	2
30	2% PEG/4% MeOH	CG04M111	20	20	15	10
30	2% PEG/4% MeOH	CG04M111	15	20	15	10
30	2% PEG/4% MeOH	CG04M111	20	15	15	10
30	2% PEG/4% MeOH	CG04M111	25	15	10	10
30	6% MeOH	CG04M106	2	10	2	2
30	6% MeOH	CG04M106	5	5	5	7
30	6% MeOH	CG04M106	10	5	2	5
30	6% MeOH	CG04M106	2	5	5	5
30	6% MeOH	CG04M111	30	20	5	12
30	6% MeOH	CG04M111	15	20	5	10
30	6% MeOH	CG04M111	20	15	7	7
30	6% MeOH	CG04M111	15	10	5	10

Table B.17 The post-thaw percent motility, percent fertilization and hatch of sperm samples equilibrated in selected cryoprotectants and cooled at 5 °C/min with controlled-rate freezer (CRF) and commercial freezing method (CMF).

		Cryoprotectant (CPA)							
Criterion	**Oyster identification**	**2% PEG/ 4% MeOH**	**6% MeOH**	**2% PEG/ 6% P-glycol**	**8% P-glycol**	**2% PEG/ 6% DMSO**	**8% DMSO**	**8% PEG**	**No CPA**
Cooled with CRF method									
Motility	CG04M87	50	60	30	20	30	7	15	--
Motility	CG04M87	50	60	30	20	30	10	20	--
Motility	CG04M87	30	50	30	25	30	10	7	--
Motility	CG04M87	50	70	40	30	20	10	10	--
Fertilization	CG04M87	95	98	82	92	82	90	20	--
Hatch	CG04M87	52	59	61	67	55	58	13	--
Motility	CG04M88	40	20	15	10	10	10	7	--
Motility	CG04M88	40	30	15	10	15	10	10	--
Motility	CG04M88	45	30	10	20	20	10	7	--
Motility	CG04M88	30	30	25	10	15	7	10	--
Fertilization	CG04M88	25	52	36	25	26	26	3	--
Motility	CG04M92	15	20	15	15	10	5	5	--
Motility	CG04M92	15	20	10	7	15	7	7	--
Motility	CG04M92	10	20	10	7	10	10	7	--
Motility	CG04M92	20	15	15	10	10	5	5	--
Fertilization	CG04M92	30	29	9	16	8	8	3	--
Cooled with CFM method									
Motility	CG04M95	1	1	1	1	2	--	--	--
Motility	CG04M95	1	1	1	1	5	--	--	--
Motility	CG04M95	1	1	1	1	2	--	--	--
Motility	CG04M95	1	1	1	1	2	--	--	--
Fertilization	CG04M95	4	1	2	2	4	--	--	--
Motility	CG04M96	2	2	2	1	5	--	--	--
Motility	CG04M96	2	2	2	1	5	--	--	--
Motility	CG04M96	5	5	--	1	5	--	--	--
Motility	CG04M96	2	5	--	1	2	--	--	--
Fertilization	CG04M96	53	52	23	16	19	--	--	--
Fresh sperm (non-frozen)									
Fertilization	Control	99	98	98	97	92	90	100	99

Table B.18 The post-thaw percent motility, percent fertilization and hatch of sperm samples equilibrated in selected cryoprotectants and cooled at 5 °C/min with controlled-rate freezer (CRF) and commercial freezing method (CMF).

Cooling method	Criterion	Oyster identification	Cryoprotectant (CPA)			
			2% PEG/ 4% MeOH	6% MeOH	5% E-glycol	No CPA
CFM	Motility	CG04M105	20	10	20	--
CFM	Motility	CG04M105	20	15	20	--
CFM	Motility	CG04M105	--	15	20	--
CFM	Motility	CG04M105	--	15	20	--
CFM	Fertilization	CG04M105	90	91	87	--
CFM	Hatch	CG04M105	--	--	43	--
CFM	Motility	CG04M106	1	1	5	--
CFM	Motility	CG04M106	2	1	5	--
CFM	Motility	CG04M106	1	1	2	--
CFM	Motility	CG04M106	5	--	2	--
CFM	Fertilization	CG04M106	61	12	31	--
CRF	Motility	CG04M105	25	20	25	--
CRF	Motility	CG04M105	30	35	20	--
CRF	Motility	CG04M105	20	20	25	--
CRF	Motility	CG04M105	20	20	25	--
CRF	Fertilization	CG04M105	95	96	93	--
CRF	Hatch	CG04M105	--	--	55	--
CRF	Motility	CG04M106	5	10	5	--
CRF	Motility	CG04M106	10	10	5	--
CRF	Motility	CG04M106	5	5	2	--
CRF	Motility	CG04M106	5	5	2	--
CRF	Fertilization	CG04M106	84	93	73	--
CRF	Hatch	CG04M106	--	--	54	--
--	Fertilization	Fresh sperm	95	94	96	92
--	Fertilization	Eggs only	--	--	--	0

Table B.19 The initial percent motility of sperm from 29 tetraploid oysters received from June 10 to July 7, 2004.

Received date	Shippment format	Oyster identification	Initial motility
6/10/2004	oyster	CG04M35	30
6/10/2004	oyster	CG04M36	50
6/10/2004	oyster	CG04M37	40
6/10/2004	oyster	CG04M38	5
6/10/2004	oyster	CG04M56	80
6/10/2004	oyster	CG04M57	40
6/10/2004	oyster	CG04M58	60
6/18/2004	sperm	CG04M64	50
6/18/2004	sperm	CG04M65	40
6/23/2004	sperm	CG04M71	80
6/23/2004	sperm	CG04M72	50
6/23/2004	sperm	CG04M73	85
6/23/2004	sperm	CG04M74	80
6/23/2004	sperm	CG04M75	40
6/29/2004	sperm	CG04M85	85
6/29/2004	sperm	CG04M86	50
6/29/2004	sperm	CG04M89	90
6/29/2004	sperm	CG04M90	70
6/29/2004	sperm	CG04M91	90
7/2/2004	sperm	CG04M93	90
7/2/2004	sperm	CG04M94	90
7/2/2004	sperm	CG04M99	90
7/2/2004	sperm	CG04M100	85
7/2/2004	sperm	CG04M101	70
7/2/2004	sperm	CG04M102	50
7/7/2004	sperm	CG04M107	90
7/7/2004	sperm	CG04M108	60
7/7/2004	sperm	CG04M109	95
7/7/2004	sperm	CG04M110	20

Table B.20 The post-thaw percent motility of sperm samples cooled at different rates.

Cryoprotectant (concentration)	Oyster identification	Cooling rate (°C/min)					
		50	45	30	16	5	0.5
MeOH 5	CG04M35	0	0	1	0	1	1
MeOH 5	CG04M35	0	0	1	0	0	1
MeOH 5	CG04M36	0	0	0	0	2	1
MeOH 5	CG04M36	0	0	1	1	2	1
MeOH 5	CG04M37	0	0	1	10	0	1
MeOH 5	CG04M37	0	1	1	5	0	1
MeOH 5	CG04M38	0	0	0	1	0	1
MeOH 5	CG04M38	1	0	1	1	1	1
P-glycol 10	CG04M35	1	0	1	5	5	5
P-glycol 10	CG04M35	2	1	1	1	10	2
P-glycol 10	CG04M36	1	0	0	2	5	1
P-glycol 10	CG04M36	1	1	1	1	1	1
P-glycol 10	CG04M37	1	1	1	0	5	5
P-glycol 10	CG04M37	1		1	10	1	5
P-glycol 10	CG04M38	0	2	0	1	1	1
P-glycol 10	CG04M38	1	1	0	5	1	1
E-glycol 10	CG04M35	0	0	0	0	0	2
E-glycol 10	CG04M35	0	0	0	0	0	2
E-glycol 10	CG04M36	0	0	0	0	1	1
E-glycol 10	CG04M36	0	0	0	0	0	1
E-glycol 10	CG04M37	0	0	0	0	0	5
E-glycol 10	CG04M37	0	0	1	1	2	5
E-glycol 10	CG04M38	0	0	0	0	0	1
E-glycol 10	CG04M38	0	0	0	1	0	5
DMSO 10	CG04M35	0	1	1	1	5	1
DMSO 10	CG04M35	0	1	5	1	1	1
DMSO 10	CG04M36	1	1	2	1	5	1
DMSO 10	CG04M36	1	1	3	1	1	1
DMSO 10	CG04M37	0	1	10	1	2	1
DMSO 10	CG04M37	1	1	5	1	2	1
DMSO 10	CG04M38	0	0	2	5	15	1
DMSO 10	CG04M38	0	0	2	5	1	1

Table B.21 The post-thaw percent motility of sperm samples suspended in different cryoprotectants and cooled at 5 or 30 °C/min.

5 °C/min Oyster identification	Concentration	Cryoprotectant							
		MeOH	P-glycol	E-glycol	DMSO	Glycerol	DMA	PEG200	PEG600
CG04M56	5%	15	1	1	10	1	2	15	1
CG04M56	5%	10	1	10	10	1	10	15	1
CG04M56	10%	1	5	1	15	1	10	1	0
CG04M56	10%	1	5	2	15	1	5	5	1
CG04M57	5%	1	5	1	5	1	2	1	0
CG04M57	5%	1	1	1	10	1	0	1	0
CG04M57	10%	1	1	0	5	0	0	5	1
CG04M57	10%	1	1	0	5	0	1	1	1

30 °C/min Oyster identification	Concentration	Cryoprotectant							
		MeOH	P-glycol	E-glycol	DMSO	Glycerol	DMA	PEG200	PEG600
CG04M58	5%	2	1	1	5	1	2	10	0
CG04M58	5%	5	5	5	1	1	2	2	1
CG04M58	10%	1	5	1	2	0	1	0	1
CG04M58	10%	1	1	1	1	0	1	0	1
CG04M56	5%	10	2	15	15	1	1	1	1
CG04M56	5%	5	1	1	10	0	1	1	1
CG04M56	10%	1	1	1	1	1	1	1	1
CG04M56	10%	0	1	1	5	0	7	2	1

Table B.22 The post-thaw percent motility of sperm samples suspended in single or combined cryoprotectants and cooled at 5 °C/min.

First cryoprotectant (%)	Second cryoprotectant (%)	Oyster identification							
		CG04M64	CG04M64	CG04M64	CG04M64	CG04M65	CG04M65	CG04M65	CG04M65
PEG200 (4)	MeOH (6)	1	0	1	1	35	30	5	10
PEG200 (4)	P-glycol (6)	5	1	5	5	10	30	30	20
PEG200 (4)	E-glycol (6)	1	1	5	1	15	20	2	10
PEG200 (4)	DMSO (6)	1	2	1	5	20	20	1	20
PEG600 (4)	MeOH (6)	5	1	1	1	10	1	5	10
PEG600 (4)	P-glycol (6)	1	1	1	1	2	10	1	1
PEG600 (4)	E-glycol (6)	0	1	2	1	10	10	1	5
PEG600 (4)	DMSO (6)	1	1	1	1	1	1	1	5
DMSO (4)	MeOH (4)	1	0	1	0	0	0	1	0
DMSO (4)	P-glycol (4)	5	2	0	1	1	5	2	0
DMSO (4)	E-glycol (4)	1	2	1	1	1	5	1	1
P-glycol (4)	E-glycol (4)	1	1	2	1	2	5	2	5
--	MeOH (8)	1	0	0	0	0	5	2	0
--	P-glycol (8)	0	1	2	0	0	0	2	5
--	E-glycol (8)	0	1	0	1	1	1	1	1
--	DMSO (8)	2	2	1	5	--	--	--	--

Table B.23 The post-thaw percent motility of sperm samples suspended in single or combined cryoprotectants and cooled at 5 °C/min.

Concentration	PEG (first cryoprotectant, CPA)/MeOH (second CPA)															
First CPA	**2**	**4**	**6**	**2**	**4**	**6**	**2**	**4**	**6**	**0**	**0**	**0**	**2**	**4**	**6**	**8**
Second CPA	**4**	**4**	**4**	**6**	**6**	**6**	**2**	**4**	**8**	**4**	**6**	**8**	**0**	**0**	**0**	**0**
CG04M74	15	20	25	15	20	10	--	10	15	--	10	10	--	--	--	5
CG04M74	15	20	25	15	20	10	15	10	15	--	15	10	--	--	--	5
CG04M74	15	20	25	10	20	10	10	10	15	--	10	10	--	--	--	5
CG04M74	15	25	25	10	20	10	10	10	20	--	10	10	--	--	--	5
CG04M75	15	5	35	30	20	15	10	10	5	5	15	15	1	5	10	15
CG04M75	15	5	30	20	20	15	15	7	5	5	10	10	1	5	15	20
CG04M75	10	5	35	20	20	15	10	7	5	5	10	7	1	5	10	15
CG04M75	10	5	30	25	20	15	10	7	5	5	10	10	1	5	10	15
	PEG/P-glycol															
CG04M71	10	30	40	40	30	20	45	20	10	--	5	20	--	--	--	15
CG04M71	20	20	30	40	30	15	40	20	10	--	5	30	--	--	--	10
CG04M71	15	35	40	40	20	10	40	25	5	--	5	40	--	--	--	20
CG04M71	10	30	30	40	30	10	40	20	5	--	5	20	--	--	--	20
CG04M72	10	35	40	50	30	20	50	40	10	1	5	40	1	10	15	40
CG04M72	10	30	30	50	30	20	40	30	10	1	5	40	1	10	15	25
CG04M72	10	30	40	50	30	20	40	25	10	1	5	30	1	10	20	25
CG04M72	10	40	35	50	30	20	40	40	5	1	5	30	1	10	20	25
	PEG/DMSO															
CG04M72	10	25	35	15	25	20	25	25	10	--	7	20	--	--	--	15
CG04M72	10	25	35	10	15	10	30	20	10	--	5	15	--	--	--	10
CG04M72	10	20	35	15	20	10	25	20	10	--	5	15	--	--	--	10
CG04M72	15	20	30	10	20	10	30	25	10	--	5	20	--	--	--	10
CG04M73	1	5	10	10	10	5	10	10	2	1	2	5	1	2	5	10
CG04M73	1	5	5	5	5	5	5	10	2	1	1	5	1	2	5	5
CG04M73	1	5	10	5	10	10	10	10	5	1	1	1	1	2	5	10
CG04M73	5	10	5	1	5	10	5	10	5	1	2	5	1	2	5	5

Table B.24 The post-thaw percent motility of sperm samples suspended in single or combined cryoprotectants and cooled at 5 °C/min.

PEG200 (%)	Second cryoprotectant (%)	Oyster identification							
		CG04M85	CG04M85	CG04M85	CG04M85	CG04M86	CG04M86	CG04M86	CG04M86
0	MeOH (6)	10	7	5	10	10	10	5	10
0	MeOH (8)	5	1	5	5	7	7	7	7
0	P-glycol (8)	2	1	5	1	5	5	5	5
0	DMSO (8)	5	5	5	10	5	5	1	5
2	MeOH (6)	10	15	15	15	10	10	10	15
2	P-glycol (6)	10	5	5	10	10	15	10	5
2	P-glycol (8)	5	5	10	10	15	15	10	10
2	DMSO (8)	15	10	10	10	10	5	5	5
4	MeOH (6)	15	10	10	5	20	20	20	20
4	P-glycol (4)	15	10	10	10	5	5	5	5
4	DMSO (4)	7	7	10	10	10	5	10	10
4	DMSO (6)	15	15	10	15	15	15	10	10
6	MeOH (4)	5	10	10	5	20	25	20	20
6	P-glycol (4)	10	5	10	10	25	20	20	20
6	DMSO (4)	10	10	15	20	15	20	15	15
8	--	5	10	5	10	5	10	7	5

Table B.25 The post-thaw percent motility of sperm samples cooled with 0.25 and 0.5-ml straws.

		Cryoprotectant				
Straw size	**Oyster identification**	**6% PEG/ 4% MeOH**	**6% MeOH**	**6% PEG/ 4% P-glycol**	**6% PEG/ 4% DMSO**	**8% PEG**
0.25-ml	CG04M99	20	15	40	35	20
0.25-ml	CG04M99	20	10	40	25	20
0.25-ml	CG04M99	15	10	40	35	20
0.25-ml	CG04M99	20	5	40	35	15
0.25-ml	G04M100	10	5	30	10	10
0.25-ml	G04M100	10	7	30	20	10
0.25-ml	CG04M100	5	2	15	15	10
0.25-ml	CG04M100	5	2	20	10	10
0.5-ml	CG04M99	20	15	40	35	15
0.5-ml	CG04M99	20	15	35	35	20
0.5-ml	CG04M99	15	20	35	30	20
0.5-ml	CG04M99	20	10	40	30	20
0.5-ml	CG04M100	15	5	30	10	5
0.5-ml	CG04M100	10	10	15	20	10
0.5-ml	CG04M100	10	7	20	15	10
0.5-ml	CG04M100	10	7	30	20	10

Table B.26 The post-thaw percent motility of sperm samples suspended in selected cryoprotectants and cooled at 0.5, 5, 16 and 30 °C/min.

		Cryoprotectant				
Cooling rate (C/min)	**Oyster identification**	**6% PEG/ 4% MeOH**	**6% PEG/ 4% P-glycol**	**6% PEG/ 4% DMSO**	**6% MeOH**	**8% PEG**
0.5	CG04M99	2	5	5	0	2
0.5	CG04M99	2	5	2	0	2
0.5	CG04M99	2	5	5	0	1
0.5	CG04M99	2	5	5	0	1
0.5	G04M100	5	2	5	0	1
0.5	CG04M100	2	2	5	0	2
0.5	CG04M100	2	4	5	0	1
0.5	CG04M100	2	5	2	0	1
5	CG04M99	20	40	35	15	20
5	CG04M99	20	40	25	10	20
5	CG04M99	15	40	35	10	20
5	CG04M99	20	40	35	5	15
5	CG04M100	10	30	10	5	10
5	CG04M100	10	30	20	7	10
5	CG04M100	5	15	15	2	10
5	CG04M100	5	20	10	2	10
16	CG04M99	15	20	25	7	10
16	CG04M99	20	20	20	7	10
16	CG04M99	15	20	20	10	10
16	CG04M99	10	15	20	5	10
16	CG04M100	10	10	15	2	5
16	CG04M100	10	20	10	2	10
16	CG04M100	10	10	10	5	10
16	CG04M100	--	10	10	2	5
30	CG04M99	10	25	20	10	10
30	CG04M99	20	30	20	10	10
30	CG04M99	15	20	20	5	10
30	CG04M99	10	20	20	5	10
30	CG04M100	10	20	10	5	7
30	CG04M100	1	15	10	2	5
30	CG04M100	10	15	20	2	7
30	CG04M100	10	15	15	5	7

Table B.27 The post-thaw percent motility of sperm samples equilibrated in selected cryoprotectants with different time periods and cooled at 5 and 30 °C/min.

Cooling rate (°C/min)	Cryoprotectant	Oyster identification	Equilibration time (min)			
			60	30	20	10
5	6% PEG/4% P-glycol	CG04M101	15	10	10	20
5	6% PEG/4% P-glycol	CG04M101	15	15	15	15
5	6% PEG/4% P-glycol	CG04M101	10	10	15	20
5	6% PEG/4% P-glycol	CG04M101	10	10	10	10
5	6% PEG/4% P-glycol	CG04M102	20	20	15	20
5	6% PEG/4% P-glycol	CG04M102	20	15	15	20
5	6% PEG/4% P-glycol	CG04M102	10	10	20	20
5	6% PEG/4% P-glycol	CG04M102	15	15	15	15
5	6% PEG/4% DMSO	CG04M101	15	10	20	15
5	6% PEG/4% DMSO	CG04M101	12	5	20	20
5	6% PEG/4% DMSO	CG04M101	20	15	10	10
5	6% PEG/4% DMSO	CG04M101	15	10	10	10
5	6% PEG/4% DMSO	CG04M102	20	20	15	15
5	6% PEG/4% DMSO	CG04M102	15	15	20	20
5	6% PEG/4% DMSO	CG04M102	15	15	20	10
5	6% PEG/4% DMSO	CG04M102	10	10	10	5
30	6% PEG/4% P-glycol	CG04M101	15	15	20	15
30	6% PEG/4% P-glycol	CG04M101	20	20	15	10
30	6% PEG/4% P-glycol	CG04M101	10	10	10	20
30	6% PEG/4% P-glycol	CG04M101	15	10	15	10
30	6% PEG/4% P-glycol	CG04M102	20	20	15	15
30	6% PEG/4% P-glycol	CG04M102	20	20	15	15
30	6% PEG/4% P-glycol	CG04M102	15	15	15	15
30	6% PEG/4% P-glycol	CG04M102	15	15	10	10
30	6% PEG/4% DMSO	CG04M101	20	25	20	20
30	6% PEG/4% DMSO	CG04M101	20	25	20	20
30	6% PEG/4% DMSO	CG04M101	20	25	15	10
30	6% PEG/4% DMSO	CG04M101	15	20	15	10
30	6% PEG/4% DMSO	CG04M102	20	20	15	15
30	6% PEG/4% DMSO	CG04M102	20	20	20	10
30	6% PEG/4% DMSO	CG04M102	10	10	10	10
30	6% PEG/4% DMSO	CG04M102	15	15	10	10

Table B.28 The post-thaw percent motility and percent fertilization of sperm samples equilibrated in selected cryoprotectants for 10, 30 and 60 min.

Equilibration (min)	Criterion	Oyster identification	Cryoprotectant (CPA)			
			6% PEG/ 4% MeOH	6% PEG/ 4% P-glycol	6% PEG/ 4% DMSO	No CPA
60	Motility	CG04M107	15	10	15	--
60	Motility	CG04M107	20	25	15	--
60	Fertilization	CG04M107	21	--	30	--
60	Motility	CG04M108	5	2	1	--
60	Motility	CG04M108	5	5	1	--
60	Fertilization	CG04M108	5	7	8	--
60	Fertilization	Fresh sperm	99	88	63	99
30	Motility	CG04M107	10	20	10	--
30	Motility	CG04M107	10	20	10	--
30	Fertilization	CG04M107	24	23	10	--
30	Motility	CG04M108	5	2	1	--
30	Motility	CG04M108	5	2	1	--
30	Fertilization	CG04M108	4	2	3	--
30	Fertilization	Fresh sperm	51	90	92	--
10	Motility	CG04M107	10	20	10	--
10	Motility	CG04M107	10	20	10	--
10	Fertilization	CG04M107	8	13	8	--
10	Motility	CG04M108	5	2	1	--
10	Motility	CG04M108	5	2	1	--
10	Fertilization	CG04M108	1	9	6	--
10	Fertilization	Fresh sperm	72	94	97	--

Table B.29 The post-thaw percent motility and percent fertilization of sperm samples equilibrated in selected cryoprotectants for 10, 30 and 60 min.

Equilibration (min)	Criterion	Oyster identification	Cryoprotectant (CPA)			No CPA
			6% MeOH	8% PEG	8% DMSO	
60	Motility	CG04M109	10	7	10	--
60	Motility	CG04M109	10	10	10	--
60	Fertilization	CG04M109	48	2	13	--
60	Motility	CG04M110	5	7	5	--
60	Motility	CG04M110	7	7	5	--
60	Fertilization	CG04M110	1	1	3	--
60	Fertilization	Fresh sperm	96	99	99	100
30	Motility	CG04M109	10	10	7	--
30	Motility	CG04M109	5	7	2	--
30	Fertilization	CG04M109	17	2	5	--
30	Motility	CG04M110	5	5	5	--
30	Motility	CG04M110	5	5	2	--
30	Fertilization	CG04M110	1	0	3	--
30	Fertilization	Fresh sperm	96	97	98	--
10	Motility	CG04M109	5	7	7	--
10	Motility	CG04M109	5	7	5	--
10	Fertilization	CG04M109	7	0	4	--
10	Motility	CG04M110	5	5	5	--
10	Motility	CG04M110	2	5	5	--
10	Fertilization	CG04M110	0	0	1	--
10	Fertilization	Fresh sperm	97	99	98	--

Table B.30 The percent post-thaw motility, percent fertilization and hatch of sperm samples equilibrated in selected cryoprotectants and cooled at 5 °C/min with controlled-rate freezer (CRF) and commercial freezing method (CMF).

		Cryoprotectant (CPA)							
Criterion	**Oyster identification**	**6% PEG/ 4% MeOH**	**6% MeOH**	**6% PEG/ 4% P-glycol**	**8% P-glycol**	**6% PEG/ 4% DMSO**	**8% DMSO**	**8% PEG**	**No CPA**
Cooled with CRF method									
Motility	CG04M89	20	15	20	10	15	10	20	--
Motility	CG04M89	20	15	15	7	15	10	15	--
Fertilization	CG04M89	3	6	1	2	1	3	0	--
Motility	CG04M90	10	5	15	5	20	5	10	--
Motility	CG04M90	10	5	15	5	15	2	5	--
Fertilization	CG04M90	21	2	3	9	2	5	3	--
Motility	CG04M91	10	5	15	10	15	7	5	--
Motility	CG04M91	15	10	20	10	15	5	5	--
Fertilization	CG04M91	7	15	18	--	8	15	7	--
Cooled with CFM method									
Motility	CG04M93	5	1	1	--	2	--	1	--
Motility	CG04M93	5	1	1	--	2	--	1	--
Motility	CG04M93		1	1	--	2	--	1	--
Fertilization	CG04M93	0	8	3	--	0	--	1	--
Motility	CG04M94	5	1	5	--	2	--	2	--
Motility	CG04M94	5	2	2	--	2	--	2	--
Motility	CG04M94	5	2	2	--	2	--	2	--
Motility	CG04M94	10	2	--	--	2	--	--	--
Fertilization	CG04M94	1	3	1	--	5	--	4	--
Fresh sperm (non-frozen)									
Fertilization	Fresh sperm	95	97	99	93	96	98	97	100
Hatch	Fresh sperm	65	55	72	72	73	75	66	56

Table B.31 Agglutination scale of sperm samples equilibrated with different cryoprotectants and cooled at 5 cooling rates, and thawed at room temperature (RT) and in a 40 °C water bath.

Cooling rate (°C/min)	Cryoprotectant at 5%	Thawing temperature (°C)	Ploidy	Scale	Cryoprotectant at 10%	Thawing temperature (°C)	Ploidy	Scale
1	DMSO	RT	2C	5	DMSO	RT	2C	4
1	DMSO	RT	2C	5	DMSO	RT	2C	4
1	DMSO	RT	4C	5	DMSO	RT	4C	2
1	DMSO	40	2C	5	DMSO	RT	4C	1
1	DMSO	40	4C	5	DMSO	RT	4C	2
1	DMSO	40	4C	5	DMSO	40	2C	1
1	E-glycol	RT	2C	5	E-glycol	RT	2C	2
1	E-glycol	RT	4C	5	E-glycol	RT	4C	1
1	E-glycol	40	2C	4	E-glycol	40	2C	2
1	E-glycol	40	2C	4	E-glycol	40	2C	2
1	E-glycol	40	4C	4	E-glycol	40	4C	1
1	E-glycol	40	4C	5	E-glycol	40	4C	1
1	MeOH	RT	2C	5	MeOH	RT	2C	0
1	MeOH	RT	4C	5	MeOH	RT	4C	0
1	MeOH	40	2C	2	MeOH	40	2C	0
1	MeOH	40	2C	2	MeOH	40	2C	0
1	MeOH	40	4C	3	MeOH	40	4C	0
1	MeOH	40	4C	2	MeOH	40	4C	0
1	P-glycol	RT	2C	5	P-glycol	RT	2C	4
1	P-glycol	RT	4C	5	P-glycol	RT	4C	2
1	P-glycol	40	2C	4	P-glycol	40	2C	2
1	P-glycol	40	2C	5	P-glycol	40	2C	2
1	P-glycol	40	4C	5	P-glycol	40	4C	1
1	P-glycol	40	4C	5	P-glycol	40	4C	1
5	DMSO	RT	2C	5	DMSO	RT	2C	1
5	DMSO	RT	4C	5	DMSO	RT	2C	1
5	DMSO	RT	4C	5	DMSO	RT	4C	0
5	DMSO	40	2C	2	DMSO	RT	4C	0
5	DMSO	40	2C	3	DMSO	40	2C	1
5	DMSO	40	4C	3	DMSO	40	4C	0
5	E-glycol	RT	2C	3	E-glycol	RT	2C	2
5	E-glycol	RT	2C	4	E-glycol	RT	2C	2
5	E-glycol	RT	4C	3	E-glycol	RT	4C	2
5	E-glycol	RT	4C	5	E-glycol	RT	4C	0
5	E-glycol	40	2C	1	E-glycol	40	2C	0
5	E-glycol	40	4C	1	E-glycol	40	4C	0
5	MeOH	RT	2C	5	MeOH	RT	2C	0
5	MeOH	RT	2C	5	MeOH	RT	2C	0
5	MeOH	RT	4C	5	MeOH	RT	4C	0
5	MeOH	RT	4C	5	MeOH	RT	4C	0
5	MeOH	40	2C	0	MeOH	40	2C	0
5	MeOH	40	4C	1	MeOH	40	4C	0
5	P-glycol	RT	2C	5	P-glycol	RT	2C	3

Cooling rate (°C/min)	Cryoprotectant at 5%	Thawing temperature (°C)	Ploidy	Scale	Cryoprotectant at 10%	Thawing temperature (°C)	Ploidy	Scale
5	P-glycol	RT	2C	5	P-glycol	RT	2C	2
5	P-glycol	RT	4C	5	P-glycol	RT	4C	1
5	P-glycol	RT	4C	5	P-glycol	RT	4C	1
5	P-glycol	40	2C	1	P-glycol	40	2C	0
5	P-glycol	40	4C	1	P-glycol	40	4C	0
16	DMSO	RT	2C	4	DMSO	RT	2C	0
16	DMSO	RT	4C	5	DMSO	RT	4C	0
16	DMSO	40	2C	2	DMSO	RT	4C	1
16	DMSO	40	2C	1	DMSO	40	2C	0
16	DMSO	40	4C	3	DMSO	40	2C	1
16	DMSO	40	4C	2	DMSO	40	4C	0
16	E-glycol	RT	4C	5	E-glycol	RT	2C	2
16	E-glycol	40	2C	2	E-glycol	RT	2C	2
16	E-glycol	40	2C	2	E-glycol	RT	4C	1
16	E-glycol	40	2C	2	E-glycol	40	2C	0
16	E-glycol	40	4C	2	E-glycol	40	4C	0
16	E-glycol	40	4C	2	E-glycol	40	4C	1
16	MeOH	RT	2C	5	MeOH	RT	2C	0
16	MeOH	RT	4C	4	MeOH	RT	4C	0
16	MeOH	40	2C	0	MeOH	40	2C	0
16	MeOH	40	2C	0	MeOH	40	2C	0
16	MeOH	40	4C	2	MeOH	40	4C	0
16	MeOH	40	4C	2	MeOH	40	4C	0
16	P-glycol	RT	2C	5	P-glycol	RT	2C	2
16	P-glycol	RT	4C	5	P-glycol	RT	4C	1
16	P-glycol	40	2C	1	P-glycol	40	2C	0
16	P-glycol	40	2C	2	P-glycol	40	2C	0
16	P-glycol	40	4C	2	P-glycol	40	4C	0
16	P-glycol	40	4C	2	P-glycol	40	4C	0
45	DMSO	RT	2C	3	DMSO	RT	2C	1
45	DMSO	RT	2C	5	DMSO	RT	2C	2
45	DMSO	RT	4C	5	DMSO	RT	4C	0
45	DMSO	RT	4C	5	DMSO	RT	4C	0
45	DMSO	40	2C	2	DMSO	40	2C	1
45	DMSO	40	4C	4	DMSO	40	4C	0
45	E-glycol	RT	2C	5	E-glycol	RT	2C	2
45	E-glycol	RT	2C	5	E-glycol	RT	2C	2
45	E-glycol	RT	4C	5	E-glycol	RT	4C	2
45	E-glycol	RT	4C	5	E-glycol	RT	4C	2
45	E-glycol	40	2C	2	E-glycol	40	2C	1
45	E-glycol	40	4C	2	E-glycol	40	4C	1
45	MeOH	RT	2C	4	MeOH	RT	2C	0
45	MeOH	RT	2C	5	MeOH	RT	2C	0
45	MeOH	RT	4C	4	MeOH	RT	4C	0
45	MeOH	RT	4C	4	MeOH	RT	4C	0
45	MeOH	40	2C	1	MeOH	40	2C	0
45	MeOH	40	4C	2	MeOH	40	4C	0
45	P-glycol	RT	2C	5	P-glycol	RT	2C	2

Cooling rate (°C/min)	Cryoprotectant at 5%	Thawing temperature (°C)	Ploidy	Scale	Cryoprotectant at 10%	Thawing temperature (°C)	Ploidy	Scale
45	P-glycol	RT	2C	5	P-glycol	RT	2C	2
45	P-glycol	RT	4C	5	P-glycol	RT	4C	1
45	P-glycol	RT	4C	5	P-glycol	RT	4C	1
45	P-glycol	40	2C	1	P-glycol	40	2C	0
45	P-glycol	40	4C	2	P-glycol	40	4C	0
50	DMSO	RT	2C	5	DMSO	RT	2C	3
50	DMSO	RT	4C	5	DMSO	RT	4C	2
50	DMSO	40	2C	2	DMSO	40	2C	0
50	DMSO	40	2C	2	DMSO	40	2C	0
50	DMSO	40	4C	1	DMSO	40	4C	0
50	DMSO	40	4C	1	DMSO	40	4C	0
50	E-glycol	RT	2C	5	E-glycol	RT	2C	5
50	E-glycol	RT	4C	5	E-glycol	RT	4C	3
50	E-glycol	40	2C	4	E-glycol	40	2C	1
50	E-glycol	40	2C	4	E-glycol	40	2C	1
50	E-glycol	40	4C	3	E-glycol	40	4C	1
50	E-glycol	40	4C	3	E-glycol	40	4C	0
50	MeOH	RT	2C	4	MeOH	RT	2C	0
50	MeOH	RT	4C	5	MeOH	RT	4C	0
50	MeOH	40	2C	4	MeOH	40	2C	0
50	MeOH	40	2C	2	MeOH	40	2C	0
50	MeOH	40	4C	1	MeOH	40	4C	0
50	MeOH	40	4C	2	MeOH	40	4C	0
50	P-glycol	RT	2C	5	P-glycol	RT	2C	5
50	P-glycol	RT	4C	5	P-glycol	RT	4C	5
50	P-glycol	40	2C	1	P-glycol	RT	4C	4
50	P-glycol	40	2C	2	P-glycol	40	2C	0
50	P-glycol	40	4C	1	P-glycol	40	2C	0
50	P-glycol	40	4C	1	P-glycol	40	4C	0

Table B.32 Agglutination scale of sperm samples suspended in DMSO at different concentrations and thawed at room temperature (RT) and in 20, 40, 60, and 80 °C water bath.

DMSO (%)	Thawing temperature (°C)	Ploidy	Scale	DMSO (%)	Thawing temperature (°C)	Ploidy	Scale
2	RT	2C	5	8	40	2C	1
2	RT	2C	5	8	40	4C	1
2	RT	4C	5	8	40	4C	2
2	RT	4C	5	8	60	2C	1
2	20	2C	5	8	60	2C	1
2	20	2C	5	8	60	4C	1
2	20	4C	5	8	60	4C	1
2	20	4C	5	8	80	2C	0
2	40	2C	5	8	80	4C	1
2	40	2C	5	10	RT	2C	0
2	40	4C	5	10	RT	2C	2
2	40	4C	5	10	RT	4C	2
2	60	2C	5	10	RT	4C	2
2	60	2C	4	10	20	2C	1
2	60	4C	5	10	20	2C	1
2	80	2C	2	10	20	4C	1
2	80	4C	5	10	20	4C	1
5	RT	2C	5	10	40	2C	0
5	RT	2C	5	10	40	2C	0
5	RT	4C	5	10	40	4C	1
5	RT	4C	5	10	40	4C	1
5	20	2C	3	10	60	2C	0
5	20	2C	2	10	60	2C	0
5	20	4C	5	10	60	4C	0
5	20	4C	5	10	60	4C	0
5	40	2C	4	10	80	2C	0
5	40	2C	3	10	80	4C	0
5	40	4C	5	12, 15, 20	RT	2C	0
5	40	4C	5	12, 15, 20	RT	2C	0
5	60	2C	2	12, 15, 20	RT	4C	0
5	60	2C	2	12, 15, 20	RT	4C	0
5	60	4C	5	12, 15, 20	20	2C	0
5	60	4C	4	12, 15, 20	20	2C	0
5	80	2C	2	12, 15, 20	20	4C	0
5	80	4C	5	12, 15, 20	20	4C	0
8	RT	2C	3	12, 15, 20	40	2C	0
8	RT	2C	3	12, 15, 20	40	2C	0
8	RT	4C	5	12, 15, 20	40	4C	0
8	RT	4C	5	12, 15, 20	40	4C	0
8	20	2C	3	12, 15, 20	60	2C	0
8	20	2C	2	12, 15, 20	60	2C	0
8	20	4C	3	12, 15, 20	60	4C	0
8	20	4C	3	12, 15, 20	60	4C	0
8	40	2C	1	12, 15, 20	80	2C	0
				12, 15, 20	80	4C	0

Table B.33 Agglutination scale of sperm samples frozen at various sperm concentrations and suspended in DMSO at different concentrations, thawed at room temperature (RT) and in a 40°C water bath.

Diploid				Tetraploid			
Spern concentration (cells/ml)	**DMSO (%)**	**Thawing temperature (°C)**	**Scale**	**Spern concentration (cells/ml)**	**DMSO (%)**	**Thawing temperature (°C)**	**Scale**
2.5×10^7	0	RT	0	2.5×10^7	0	RT	0
2.5×10^7	0	RT	0	2.5×10^7	0	RT	0
5.0×10^7	0	RT	0	5.0×10^7	0	RT	0
5.0×10^7	0	RT	0	5.0×10^7	0	RT	0
2.5×10^8	0	RT	5	2.5×10^8	0	RT	5
2.5×10^8	0	RT	5	2.5×10^8	0	RT	5
5.0×10^8	0	RT	5	5.0×10^8	0	RT	5
5.0×10^8	0	RT	5	5.0×10^8	0	RT	5
2.5×10^9	0	RT	5	2.0×10^9	0	RT	5
2.5×10^9	0	RT	5	2.0×10^9	0	RT	5
5.0×10^9	0	RT	5	2.5×10^7	2	RT	0
5.0×10^9	0	RT	5	2.5×10^7	2	RT	0
2.5×10^7	2	RT	0	5.0×10^7	2	RT	1
2.5×10^7	2	RT	0	5.0×10^7	2	RT	0
5.0×10^7	2	RT	0	2.5×10^8	2	RT	5
5.0×10^7	2	RT	0	2.5×10^8	2	RT	5
2.5×10^8	2	RT	5	5.0×10^8	2	RT	5
2.5×10^8	2	RT	5	5.0×10^8	2	RT	5
5.0×10^8	2	RT	5	2.0×10^9	2	RT	5
5.0×10^8	2	RT	5	2.0×10^9	2	RT	5
2.5×10^9	2	RT	5	2.5×10^7	5	RT	1
2.5×10^9	2	RT	5	2.5×10^7	5	RT	0
5.0×10^9	2	RT	5	5.0×10^7	5	RT	1
5.0×10^9	2	RT	5	5.0×10^7	5	RT	1
2.5×10^7	5	RT	0	2.5×10^8	5	RT	2
2.5×10^7	5	RT	0	2.5×10^8	5	RT	4
5.0×10^7	5	RT	0	5.0×10^8	5	RT	4
5.0×10^7	5	RT	0	5.0×10^8	5	RT	4
2.5×10^8	5	RT	2	2.0×10^9	5	RT	5
2.5×10^8	5	RT	3	2.0×10^9	5	RT	5
5.0×10^8	5	RT	4	2.5×10^7	8	RT	0
5.0×10^8	5	RT	5	2.5×10^7	8	RT	0
2.5×10^9	5	RT	5	5.0×10^7	8	RT	1
2.5×10^9	5	RT	5	5.0×10^7	8	RT	1
5.0×10^9	5	RT	5	2.5×10^8	8	RT	2
5.0×10^9	5	RT	5	2.5×10^8	8	RT	1
2.5×10^7	8	RT	0	5.0×10^8	8	RT	2
2.5×10^7	8	RT	0	5.0×10^8	8	RT	1
5.0×10^7	8	RT	0	2.0×10^9	8	RT	3
5.0×10^7	8	RT	0	2.0×10^9	8	RT	3
2.5×10^8	8	RT	1	2.5×10^7	10	RT	0
2.5×10^8	8	RT	1	2.5×10^7	10	RT	0

Diploid				Tetraploid			
Spern concentration (cells/ml)	**DMSO (%)**	**Thawing temperature (°C)**	**Scale**	**Spern concentration (cells/ml)**	**DMSO (%)**	**Thawing temperature (°C)**	**Scale**
5.0×10^{8}	8	RT	3	5.0×10^{7}	10	RT	0
5.0×10^{8}	8	RT	3	5.0×10^{7}	10	RT	0
2.5×10^{9}	8	RT	3	2.5×10^{8}	10	RT	0
2.5×10^{9}	8	RT	4	2.5×10^{8}	10	RT	0
5.0×10^{9}	8	RT	4	5.0×10^{8}	10	RT	1
5.0×10^{9}	8	RT	4	5.0×10^{8}	10	RT	1
2.5×10^{7}	10	RT	0	2.0×10^{9}	10	RT	1
2.5×10^{7}	10	RT	0	2.0×10^{9}	10	RT	1
5.0×10^{7}	10	RT	0	2.5×10^{7}	12	RT	0
5.0×10^{7}	10	RT	0	2.5×10^{7}	12	RT	0
2.5×10^{8}	10	RT	0	5.0×10^{7}	12	RT	0
2.5×10^{8}	10	RT	0	5.0×10^{7}	12	RT	0
5.0×10^{8}	10	RT	0	2.5×10^{8}	12	RT	0
5.0×10^{8}	10	RT	1	2.5×10^{8}	12	RT	0
2.5×10^{9}	10	RT	2	5.0×10^{8}	12	RT	0
2.5×10^{9}	10	RT	0	5.0×10^{8}	12	RT	0
5.0×10^{9}	10	RT	0	2.0×10^{9}	12	RT	0
5.0×10^{9}	10	RT	2	2.0×10^{9}	12	RT	0
2.5×10^{7}	12	RT	0	2.5×10^{7}	0	40	0
2.5×10^{7}	12	RT	0	2.5×10^{7}	0	40	0
5.0×10^{7}	12	RT	0	5.0×10^{7}	0	40	0
5.0×10^{7}	12	RT	0	5.0×10^{7}	0	40	0
2.5×10^{8}	12	RT	0	2.5×10^{8}	0	40	5
2.5×10^{8}	12	RT	0	2.5×10^{8}	0	40	5
5.0×10^{8}	12	RT	0	5.0×10^{8}	0	40	5
5.0×10^{8}	12	RT	0	5.0×10^{8}	0	40	5
2.5×10^{9}	12	RT	0	2.0×10^{9}	0	40	5
2.5×10^{9}	12	RT	0	2.0×10^{9}	0	40	5
5.0×10^{9}	12	RT	0	2.5×10^{7}	2	40	0
5.0×10^{9}	12	RT	0	2.5×10^{7}	2	40	0
2.5×10^{7}	0	40	0	5.0×10^{7}	2	40	0
2.5×10^{7}	0	40	0	5.0×10^{7}	2	40	1
5.0×10^{7}	0	40	0	2.5×10^{8}	2	40	5
5.0×10^{7}	0	40	0	2.5×10^{8}	2	40	4
2.5×10^{8}	0	40	5	5.0×10^{8}	2	40	5
2.5×10^{8}	0	40	5	5.0×10^{8}	2	40	5
5.0×10^{8}	0	40	5	2.0×10^{9}	2	40	5
5.0×10^{8}	0	40	5	2.0×10^{9}	2	40	5
2.5×10^{9}	0	40	5	2.5×10^{7}	5	40	0
2.5×10^{9}	0	40	5	2.5×10^{7}	5	40	0
5.0×10^{9}	0	40	5	5.0×10^{7}	5	40	0
5.0×10^{9}	0	40	5	5.0×10^{7}	5	40	0
2.5×10^{7}	2	40	0	2.5×10^{8}	5	40	1
2.5×10^{7}	2	40	0	2.5×10^{8}	5	40	2
5.0×10^{7}	2	40	0	5.0×10^{8}	5	40	4
5.0×10^{7}	2	40	0	5.0×10^{8}	5	40	3
2.5×10^{8}	2	40	5	2.0×10^{9}	5	40	4

Diploid				Tetraploid			
Spern concentration (cells/ml)	**DMSO (%)**	**Thawing temperature (°C)**	**Scale**	**Spern concentration (cells/ml)**	**DMSO (%)**	**Thawing temperature (°C)**	**Scale**
2.5×10^8	2	40	5	2.0×10^9	5	40	4
5.0×10^8	2	40	5	2.5×10^7	8	40	0
5.0×10^8	2	40	5	2.5×10^7	8	40	0
2.5×10^9	2	40	5	5.0×10^7	8	40	1
2.5×10^9	2	40	5	5.0×10^7	8	40	1
5.0×10^9	2	40	5	2.5×10^8	8	40	1
5.0×10^9	2	40	5	2.5×10^8	8	40	1
2.5×10^7	5	40	0	5.0×10^8	8	40	1
2.5×10^7	5	40	0	5.0×10^8	8	40	1
5.0×10^7	5	40	0	2.0×10^9	8	40	1
5.0×10^7	5	40	0	2.0×10^9	8	40	1
2.5×10^8	5	40	3	2.5×10^7	10	40	0
2.5×10^8	5	40	2	2.5×10^7	10	40	0
5.0×10^8	5	40	5	5.0×10^7	10	40	0
5.0×10^8	5	40	4	5.0×10^7	10	40	0
2.5×10^9	5	40	5	2.5×10^8	10	40	0
2.5×10^9	5	40	5	2.5×10^8	10	40	0
5.0×10^9	5	40	5	5.0×10^8	10	40	0
5.0×10^9	5	40	5	5.0×10^8	10	40	0
2.5×10^7	8	40	0	2.0×10^9	10	40	0
2.5×10^7	8	40	0	2.0×10^9	10	40	0
5.0×10^7	8	40	0	2.5×10^7	12	40	0
5.0×10^7	8	40	0	2.5×10^7	12	40	0
2.5×10^8	8	40	1	5.0×10^7	12	40	0
2.5×10^8	8	40	1	5.0×10^7	12	40	0
5.0×10^8	8	40	1	2.5×10^8	12	40	0
5.0×10^8	8	40	1	2.5×10^8	12	40	0
2.5×10^9	8	40	2	5.0×10^8	12	40	0
2.5×10^9	8	40	2	5.0×10^8	12	40	0
5.0×10^9	8	40	2	2.0×10^9	12	40	0
5.0×10^9	8	40	2	2.0×10^9	12	40	0
2.5×10^7	10	40	0				
2.5×10^7	10	40	0				
5.0×10^7	10	40	0				
5.0×10^7	10	40	0				
2.5×10^8	10	40	0				
2.5×10^8	10	40	0				
5.0×10^8	10	40	0				
5.0×10^8	10	40	1				
2.5×10^9	10	40	0				
2.5×10^9	10	40	1				
5.0×10^9	10	40	0				
5.0×10^9	10	40	1				
2.5×10^7	12	40	0				
2.5×10^7	12	40	0				
5.0×10^7	12	40	0				
5.0×10^7	12	40	0				

Diploid				Tetraploid			
Spern concentration (cells/ml)	DMSO (%)	Thawing temperature (°C)	Scale	Spern concentration (cells/ml)	DMSO (%)	Thawing temperature (°C)	Scale
2.5×10^8	12	40	0				
2.5×10^8	12	40	0				
5.0×10^8	12	40	0				
5.0×10^8	12	40	0				
2.5×10^9	12	40	0				
2.5×10^9	12	40	0				
5.0×10^9	12	40	0				
5.0×10^9	12	40	0				

Table B.34 Concentration of non-agglutinated sperm available after thawing for each agglutination scale for sperm from tetraploid Pacific oysters.

Oyster identification	Scale	Sperm concentration (cells/ml)	
		Before crushing	After crushing
CG03M22	0	6.97×10^8	--
CG03M22	0	1.20×10^9	--
CG03M22	0	1.29×10^9	--
CG03M22	0	1.28×10^9	--
CG03M22	1	9.27×10^8	--
CG03M22	1	7.20×10^8	--
CG03M22	4	6.10×10^8	--
CG03M22	4	6.43×10^8	--
CG03M22	5	4.85×10^7	--
CG03M22	5	1.15×10^8	--
CG03M22	5	7.97×10^7	3.46×10^8
CG03P16	0	7.50×10^8	--
CG03P16	0	6.06×10^8	--
CG03P16	0	5.22×10^8	--
CG03P16	0	6.47×10^8	--
CG03P16	2	3.72×10^8	--
CG03P16	2	5.76×10^8	--
CG03P16	3	4.82×10^8	--
CG03P16	3	2.02×10^8	--
CG03P16	4	1.68×10^8	2.84×10^8
CG03P16	5	4.97×10^7	--
CG03P16	5	7.08×10^7	--
CG03P16	5	3.42×10^7	2.21×10^8

Table B.35 Percent fertilization of samples from diploid and tetraploid oysters with different agglutination scale after thawing.

Diploid			Tetraploid		
Oyster identification	Scale	Fertilization (%)	Oyster identification	Scale	Fertilization (%)
CG04M17	0	1	CG04M22	0	0
CG04M17	0	1	CG04M89	0	0
CG04M16	0	2	CG04M93	0	0
CG04M92	0	8	CG04M93	0	0
CG04M16	0	20	CG04M101	0	0
CG04M87	0	20	CG04M102	0	0
CG04M50	0	64	CG04M114	0	0
CG04M51	0	77	CG04M114	0	0
CG04M51	0	86	CG04M122	0	0
CG04M92	0	3	CG04M89	0	1
CG03M52	0	10	CG04M89	0	1
CG04M92	1	8	CG04M94	0	1
CG04M16	1	64	CG04M94	0	1
CG04M87	1	82	CG04M101	0	1
CG04M87	1	90	CG04M21	0	2
CG04M17	1	1	CG04M21	0	2
CG04M88	1	3	CG04M90	0	2
CG04M48	1	49	CG04M101	0	2
CG04M88	1	26	CG04M89	0	3
CG04M92	2	9	CG04M89	0	3
CG04M92	2	16	CG04M90	0	3
CG04M92	2	29	CG04M93	0	3
CG04M92	2	30	CG04M102	0	3
CG04M49	2	30	CG04M43	0	3
CG04M17	2	1	CG04M94	0	5
CG04M50	2	94	CG04M91	0	7
CG04M87	2	95	CG04M107	0	8
CG04M87	2	98	CG04M107	0	8
CG04M88	2	26	CG04M40	0	13
CG04M126	2	33	CG04M41	0	15
CG04M16	3	27	CG04M107	0	24
CG04M51	3	86	CG04M22	0	26
CG04M105	3	90	CG04M42	0	36
CG04M129	3	12	CG04M42	0	45
CG04M88	3	25	CG04M122	0	0
CG04M87	3	82	CG04M102	1	1
CG04M87	3	92	CG04M108	1	6
CG04M50	3	92	CG04M91	1	8
CG04M95	3	4	CG04M107	1	10
CG04M128	3	13	CG04M90	1	21
CG04M49	3	62	CG04M107	1	21
CG04M106	3	84	CG04M114	1	0
CG04M106	3	93	CG04M108	1	1
CG04M95	4	4	CG04M110	1	1

Diploid			Tetraploid		
Oyster identification	Scale	Fertilization (%)	Oyster identification	Scale	Fertilization (%)
CG04M121	4	20	CG04M21	1	3
CG04M96	4	23	CG04M90	1	3
CG04M88	4	52	CG04M108	1	3
CG04M48	4	52	CG04M21	1	4
CG04M126	4	62	CG04M108	1	4
CG04M95	4	2	CG04M91	1	7
CG04M88	4	36	CG04M108	1	9
CG04M49	4	59	CG04M107	1	13
CG04M95	4	2	CG04M91	1	15
CG04M121	4	18	CG04M122	1	3
CG04M96	4	19	CG04M94	1	4
CG04M106	4	61	CG04M22	2	21
CG04M119	4	23	CG04M107	2	23
CG04M88	4	25	CG04M109	2	0
CG04M128	4	26	CG04M110	2	0
CG03M52	4	27	CG04M110	2	0
CG04M95	5	1	CG04M110	2	1
CG04M106	5	31	CG04M101	2	2
CG04M119	5	48	CG04M101	2	2
CG04M96	5	53	CG04M109	2	2
CG04M118	5	67	CG04M110	2	3
CG04M48	5	72	CG04M108	2	8
CG04M105	5	95	CG04M41	2	12
CG04M106	5	12	CG04M109	2	13
CG04M129	5	7	CG04M91	2	18
CG04M120	5	15	CG04M40	2	22
CG04M118	5	34	CG04M107	2	30
CG04M105	5	96	CG04M110	3	1
CG04M127	5	0	CG04M110	3	1
CG04M127	5	0	CG04M110	3	3
CG04M96	5	16	CG04M109	3	4
CG04M120	5	16	CG04M90	3	5
CG03M52	5	19	CG04M108	3	5
CG04M96	5	52	CG04M109	3	5
CG04M106	5	73	CG04M110	3	0
CG04M105	5	87	CG04M89	3	2
CG04M105	5	91	CG04M108	3	2
CG04M105	5	93	CG04M108	4	7
			CG04M93	4	1
			CG04M43	4	4
			CG04M22	4	12
			CG04M42	4	44
			CG04M90	5	2
			CG04M109	5	2
			CG04M94	5	3
			CG04M89	5	6
			CG04M109	5	7

Diploid			Tetraploid		
Oyster identification	**Scale**	**Fertilization (%)**	**Oyster identification**	**Scale**	**Fertilization (%)**
			CG04M90	5	9
			CG04M91	5	15
			CG04M109	5	17
			CG04M43	5	7
			CG04M93	5	8
			CG04M40	5	20
			CG04M41	5	29
			CG04M109	5	48

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Appendix C
Letter of Permission

January 24, 2005

Dr. Ronald W. Hardy
Aquaculture Research, Editor
UI Hagerman Fish Culture Experiment Station
3059 F National Fish Hatchery Road
Hagerman, Idaho 83332, USA

Dear Dr. Hardy,

I am preparing my dissertation and would like to request permission to reproduce material from my published manuscript entitled "Standardization of photometric measurement of sperm cell concentration from diploid and tetraploid Pacific oysters, *Crassostrea gigas* (Thunberg)" published and copyrighted by the *Aquaculture Research* Vol. 36.

With thanks,

Qiaoxiang Dong

Qiaoxiang Dong

Dear Qiaoxiang Dong,
As original author, you may use this material in your PhD dissertation. Good luck & best regards,
Ronald W. Hardy

Vita

Qiaoxiang Dong was born in March 1975, in Jinhua, Zhejiang, People's Republic of China. She was the youngest of the four in her family. She attended Jinhua First High School in Jiangtang, Zhejiang. Upon graduating, she attended then Hangzhou University (now Zhejiang University), Hangzhou, Zhejiang, where she earned a Bachelor of Science degree in biology. She continued her master's program in water pollution ecology in Jinan University, Guangzhou, Guangdong, where she studied for 2.5 years before leaving to study in the United States. In January 2000, she enrolled in the graduate program at Florida Institute of Technology, Melbourne, Florida. A year and a half later, she earned the degree of Master of Science in marine biology and came to Louisiana State University. In September 2002, she delivered a baby boy, Calvin (Wenchang), and she is currently a candidate for the degree of Doctor of Philosophy from the School of Renewable Natural Resources with the major in wildlife and fisheries science, which will be awarded on May 20, 2005.