The Need for Standardization in Cryopreservation: a Case Study with Oysters

Qiaoxiang Dong, Changjiang Huang and Terrence R. Tiersch

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 chapter refers to salt solutions only (e.g., calcium-free Hanks' balanced salt solution).

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 review calls attention to the importance of the cumulative and interacting 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 review 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.

A Case Study: Sperm Cryopreservation in Oysters

The beginning 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 yr later (Blaxter 1953), and since then more than 200 fish species have been studied (Rana 1995, Tiersch 2000) although this estimate requires updating. In contrast to the extensive studies in cryopreservation of fish semen, similar work for invertebrates has been limited to echinoderms (sea urchins, sand dollars, and starfishes),

Dong, Q., C. Huang and T. R. Tiersch. 2011. The Need for Standardization in Cryopreservation: a Case Study with Oysters. In: *Cryopreservation in Aquatic Species,* 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 581-593.

mollusks (oysters and abalone), polychaetes, and crustaceans (shrimps and crabs) (e.g., reviewed by Gwo 2000).

Despite the limited work in aquatic invertebrates overall, oysters are well studied reflecting their economic importance around the world. Indeed, the oyster cryopreservation literature provides a strong database to illustrate the types and breadth of problems inherent in the lack of consistency in methods and reporting for cryopreservation in all aquatic species. For the 35-yr period between 1971 and 2006, there were 26 reports directly related to oyster sperm cryopreservation since the first study some 40 yr ago (Lannan 1971). These reports comprised 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 1, next page). Except for the review articles, 19 of the 24 research reports (~80%) were produced for sperm from the Pacific oyster, *Crassostrea gigas*. These research efforts have yielded techniques with varying levels of success. 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.

As in most other aquatic species, one of the major obstacles to widespread application 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, Tiersch 2000). Lack of procedural standardization in the cryopreservation of oyster sperm is identified in detail in this chapter (see Appendix), but the same problems identified here would be routinely observable in other aquatic species.

Gamete Collection

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 (these are summarized in the Appendix at the end of the chapter). 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). Samples from individual males or pooled samples from several males were 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 assessed, motility was the sole criterion used.

Table 1. Literature published (between 1971 and 2006) on 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 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.

Extender Choice

The most commonly used extender (when specified) was sterilized or filtered seawater, followed by artificial seawater. 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), or osmolalities (mOsmol/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. 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.

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 were considered optimal for freezing.

Cryoprotectant and Equilibration Time

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 that was considered to be optimum varied among studies, ranging from 5 to 20%, with most reports referring to either 8 or 10%. In addition, ethylene glycol at 10%, propylene glycol at 5, 10, or 15%, and trehalose at 0.45 M were also considered in different studies 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 by some to avoid osmotic injury (e.g., Adams et al. 2004). Temperatures at which 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 equilibration was considered to be more effective in retaining post-thaw sperm quality, but long equilibration (e.g., 60 min) with low cryoprotectant concentrations (< 10%) may not decrease percent fertilization (Dong et al. 2005). The longest time reported between gamete collection and freezing was 4 h.

Freezing Methods

For freezing trials, glass ampules, 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. 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), commercial dairy bull sperm freezing methods (Dong et al. 2005), and direct plunging into liquid nitrogen. Various rates of success were reported with

each method (Table 1), but comparisons among them were made difficult or impossible 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, cooling rates reported ranged from 4.7 to 114 °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 of optimal cooling rates (from 6 to 80 °C/min) was reported in various studies.

Storage and Thawing

For storage, frozen samples were stored at -196 °C or lower in most studies except one, in which samples were stored at -170 °C (Staeger 1974), and storage time varied from 5 min to 4 yr before thawing. Sperm of Pacific oysters cryopreserved for 4 yr yielded 78% normal D-stage larvae and no negative effect was found in mean shell length of the larvae at 6 d after fertilization (Usuki et al. 1997). For thawing, samples were placed in a water bath in most studies, but the temperature of the bath varied from 4 to 75 °C. Thawing at higher temperatures (e.g., 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 at 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.

Post-thaw Sperm Quality Assessment

Various criteria were used to estimate post-thaw sperm quality, 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 in increments of 0.5 or 1.0. Similarly, percent survival or viability referred to results derived from different assays. In addition, results for percentage fertilization, larvae produced, and survival were reported as absolute values or as values relative to controls (Table 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, scale of the trials (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 (cessation of the 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.

Summary and Future Outlook

As shown in this review of sperm cryopreservation in oysters, a considerable and potentially troublesome lack of standardization was observed in methods and reporting for each step involved in the cryopreservation process. Comparisons among different studies were difficult to perform and could well be invalid in most cases due to the procedural and reporting variations across studies observed at each step. Studies utilizing the sperm agglutination phenomenon (Dong et al. 2007) clearly demonstrated the requirement for researchers to standardize sperm concentration and methods for oysters (and by extension, all aquatic species) 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. Controversy and inconsistency would be reduced if more congruent approaches were utilized and results among various studies could be directly compared. Suggestions for improvement include the creation and widespread acceptance of standard reference works to assist in harmonizing terminology, and the development and utilization of congruent educational programs. Standardization of research practices and reporting could be facilitated through establishment of guidelines for publication of results. Once in place the guidelines could be made available to journal editors and reviewers to assist in evaluation of research reports.

Acknowledgments

We thank H. Yang, R. Cuevas and E Hu for discussion. This work was supported in part by funding from the National Natural Science Foundation of China (No. 30800845), the Natural Science Fund for Distinguished Young Scholars of Zhejiang Province (No. R3100105), the USDA-SBIR program, 4Cs Breeding Technologies, Inc., and the Louisiana Sea Grant College Program. This manuscript was approved for publication by the Director of the Louisiana Agricultural Experiment Station as number 11-xx-xxxx.

References

Adams, S. L., J. F. Smith, R. D. Roberts, A. R. Janke, H. F. Kaspar, H. R. Tervit, P. A. Pugh, S. C. Webb and N. G. King. 2004. Cryopreservation of sperm of the Pacific oyster (*Crassostrea gigas*): development of a practical method for commercial spat production. Aquaculture 242:271-282.

Blaxter, J. H. S. 1953. Sperm storage and cross-fertilization of spring and autumn spawning herring. Nature 172:1189-1190.

Bougrier, S. and L. D. Rabenomanana. 1986. Cryopreservation of spermatozoa of the Japanese oyster, *Crassostrea gigas*. Aquaculture 58:277-280.

Chao, N. H. and I. C. Liao. 2001. Cryopreservation of finfish and shellfish gametes and embryos. Aquaculture 197:161-189.

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.

Dong, Q., C. Huang, T. R. Tiersch. 2007. Control of sperm concentration is necessary for standardization of sperm cryopreservation in aquatic species: evidence from sperm agglutination in oysters, Cryobiology 54:87-98.

Gwo, J. C. 2000. Cryopreservation of aquatic invertebrate semen: a review. Aquaculture Research 31:259-271.

Gwo, J. C., C. Y. Wu, W. P. Chang and H. Y. Cheng. 2003. Evaluation of damage in Pacific oyster (*Crassostrea gigas*) spermatozoa before and after cryopreservation using comet assay. CryoLetters 24:171-180.

He, Y., Q. Dong, T. R. Tiersch and R.V. Devireddy. 2004. 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 70:1428-1437.

Hughes, J. B. 1973. An examination of eggs challenged with cryopreserved spermatozoa of the American oyster, *Crassostrea virginica*. Cryobiology 10:342-344.

Hwang, S. W. and H. P. Chen. 1973. Fertility of male oyster gametes after freeze-thawing. Chinese-American Joint Commission on Rural Reconstruction Fisheries Series 15:1-5.

Ieropoli, S., P. Masullo, M. Do Espirito Santo and G. Sansone. 2004. Effects of extender composition, cooling rate and freezing on the fertilization viability of spermatozoa of the Pacific oyster (*Crassostrea gigas*). Cryobiology 49:250-257.

Iwata, N., H. Kurokura and R. Hirano. 1989. Cryopreservation of Pacific oyster, *Crassostrea gigas*, sperm. Suisanzoshoku 37:163-166 (our translation from Japanese with English abstract).

Kurokura, H., K. Namba and T. Ishikawa. 1990. Lesions of spermatozoa by cryopreservation in oyster *Crassostrea gigas*. Nippon Suisan Gakkaishi 56:1803-1806.

Lannan, J. E. 1971. Experimental self-fertilization of the Pacific oyster, *Crassostrea gigas*, utilizing cryopreserved sperm. Genetics 68:599-601.

Leibo, S. P. 2000. Sources of variation in cryopreservation. *In:* Cryopreservation in Aquatic Species. T. R. Tiersch and P. M. Mazik, editors. World Aquaculture Society, Baton Rouge, Louisiana, USA. Pp. 75-83.

Li, Y., P. Wang, G. He and Q. Zhao. 2002a. Cryopreservation of Pacific oyster (*Crassostrea gigas* spermatoza. Journal of Ocean University of Qingdao 32:207-211 (in Chinese with English abstract).

Li, Y., G. He and P. Wang. 2002b. The morphological and ultrastructural variation of Pacific oyster (*Crassostrea gigas* (Thunberg)) sperm after cryopreservation. Journal of Ocean University of Qingdao 32:526-532 (in Chinese with English abstract).

McFadzen, I. R. B. 1995. Cryopreservation of the sperm of the Pacific oyster *Crassostrea gigas*. *In:* Methods in Molecular Biology, Volume 38: Cryopreservation and Freeze-Drying Protocols. J. G. Day and M. R. McLellan, editors. Pp. 145-149.

Paniagua-Chavez C. 1999. Cryopreservation of gametes and larvae of the eastern oyster *Crassostrea virginica.* Doctoral dissertation. Louisiana State University, Baton Rouge, Louisiana, 145 pp.

Paniagua-Chavez, C.G., J. T. Buchanan, J. E. Supan and T. R. Tiersch. 2000. Cryopreservation of sperm and larvae of the Eastern oyster. *In:* Cryopreservation in Aquatic Species. T. R. Tiersch and P. M Mazik, editors. World Aquaculture Society, Baton Rouge, Louisiana, USA. Pp. 230-239.

Paniagua-Chavez, C. and T. R. Tiersch. 2001. Laboratory studies of cryopreservation of sperm and trochophore larvae of the eastern oyster. Cryobiology 43:211-223.

Polge, C., A. U. Smith and A. S. Parkes. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 164:666.

Rana, K. J. 1995. Cryopreservation of fish spermatozoa. *In:* Methods in Molecular Biology, Volume 38: Cryopreservation and Freeze-Drying Protocols. J. G. Day and M. R. McLellan, editors. Pp. 151-165.

Smith, J. F., P. A. Pugh, H. R. Tervit, R. D. Roberts, A. R. Janke, H. F. Kaspar and S. L. Adams. 2001. Cryopreservation of shellfish sperm, eggs and embryos. Proceedings of New Zealand Society of Animal Production 61:31-34.

Staeger, W. H. 1974. Cryobiological investigation of the gametes of the Pacific oyster *Crassostrea gigas.* Unpublished Master's Thesis. Oregon State University, 45 pp.

Tiersch, T. R. 2000. Introduction. *In:* Cryopreservation in Aquatic Species. T. R. Tiersch and P. M. Mazik, editors. World Aquaculture Society, Baton Rouge, Louisiana, USA. Pp. xix-xxvi.

Usuki, H., M. Hamaguchi and H. Ishioka. 1997. Long-term cryopreservation of Pacific oyster *Crassostrea gigas,* sperm. Bulletin of Nansei National Fisheries Research Institute 30:115-123 (our translation from Japanese with English abstract).

Usuki, H., M. Hamaguchi and H. Ishioka. 1999. Cryopreservation of Pacific oyster sperm and larvae. Bulletin of National Research Institute of Aquaculture Supplement 1:3-6.

Van der Horst, G., H. M. Dott, J. S. Samuels and A. Genade. 1985. Short- and long-term storage of viable oyster sperm. South African Journal of Science 81:404-405.

Yankson, K. and J. Moyse. 1991. Cryopreservation of the spermatozoa of *Crassostrea tulipa* and three other oysters. Aquaculture 97:259-267.

Zell, S. R., M. H. Bamford and H. Hidu. 1979. Cryopreservation of spermatozoa of the American oyster *Crassostrea virginica* Gmelin. Cryobiology 16:448-460.

Appendix. Variation in methods and reporting for 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 1.

Numbers in bold identify optimized protocols of that study.

Appendix. 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 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 1.

Numbers in bold identify optimized protocols of that study.

Appendix. 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	(8, 10, 12, 15, **17**, 20 cm above) x (2, **6**, 10, 14, 20, 30 min)[20]
sample and liquid	3 cm above for10 min (13.5 °C/min)[23]
nitrogen (reported	5 cm above: (3 min)[7], 10 min (114.3 °C/min)[12], with exposed straws (**79.8** °C/min
cooling rate)	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]
method (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 (-80 °C)	60 min (-6.1 °C/min)[12]
Commercial dairy 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 1.

Numbers in bold identify optimized protocols of that study.

Appendix. 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],
Control treatments	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 1.

Numbers in bold identify optimized protocols of that study.