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Aquaculture 271 (2007) 537–545
Aquaculture
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Cryoprotectant optimization for sperm of diploid Pacific oysters by use of commercial dairy sperm freezing facilities

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Received 18 November 2006; received in revised form 10 May 2007; accepted 4 July 2007

Abstract

Although sperm cryopreservation has been practiced in aquatic species for more than 50 years, viable markets for frozen sperm do not currently exist for commercial aquaculture. The present study suggests that the use of commercial cryopreservation facilities used for dairy bulls could be a cost-effective approach to initiate commercialization of frozen sperm in aquaculture, and the oyster industry could become one of the early adopters. To prove the technical feasibility of the use of a commercial freezing facility, this study adopted dairy freezing methods and emphasized cryoprotectant optimization for sperm from diploid Pacific oysters *Crassostrea gigas* with specific cooling methods employed for use with bull sperm. Specifically, the present study evaluated dimethyl sulfoxide (DMSO) at 5, 8, and 10%, ethylene glycol (E-glycol) at 2, 5, 8, and 10%, and methanol at 2, 4, 6, and 8%. Each cryoprotectant with its optimal concentration was chosen for subsequent selection of an optimal cryoprotectant. Previous results showed propylene glycol (P-glycol) at 5% yielded higher percent fertilization than did PG at 10 or 15%. Therefore, 5% of these cryoprotectants were compared and the highest percent fertilization was obtained with methanol (49±29%), followed by E-glycol (42±15%), DMSO (31±18%), and P-glycol (22±12%). Extensive evaluation for single and combined cryoprotectants and their concentrations were studied in our previous trials on a research scale (reported elsewhere), and 6% methanol and the combination of 4% methanol and 2% polyethylene glycol (PEG; FW 200) were shown to consistently yield the highest percent fertilization. Our last commercial-scale experiment compared 6% methanol with the combination of 4% methanol and 2% PEG (MET/PEG) with 20 oysters. There was no significant difference for percent fertilization between 6% methanol (39±29%) and 4% MET/2% PEG (42±26%). These findings demonstrate the technical feasibility of adopting dairy freezing protocols in commercial application for oyster sperm, and also provide a template for future commercialization of sperm cryopreservation for other aquatic species.

Keywords: Cryoprotectant; Sperm cryopreservation; Commercial; Oyster; *Crassostrea gigas*

1. Introduction

Sperm cryopreservation began in aquatic species more than 50 years ago (Blaxter, 1953) with study since then of approximately 200 fish species (Rana, 1995; Tiersch, 2000) and 30 invertebrates (Gwo, 2000). Although these research efforts have yielded protocols

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doi:10.1016/j.aquaculture.2007.07.005

that are being applied with varying levels of success, no markets for frozen sperm currently exist for application in aquaculture (Lang et al., 2003). Currently, human medicine and livestock agriculture are the only worldwide industries that have incorporated cryopreservation of semen into artificial insemination practices (Crister, 1998; Centola, 2002; Curry, 2000). The successful application of cryopreservation in the dairy industry is due to the many advantages associated with frozen bull semen: 1) economic benefits such as increased availability of semen and reduced transportation and holding costs; 2) ability of farmers to select bulls that exhibit the most desirable traits, and 3) facilitation of selective breeding and subsequent manipulation of the genetic makeup of dairy cows for increased milk production. Similar benefits could be applied to sperm cryopreservation in aquatic species, although markets for frozen aquatic sperm still lie somewhere beyond the early stages of commercialization (Caffey and Tiersch, 2000b). Based on the insights provided by the existing dairy model, economic and marketing analysis for the potential usage of frozen sperm from aquatic species revealed important factors that inhibit scaling-up to commercial production: 1) economic constraints (e.g., capital costs for required equipment and operating costs for supplies and labors) (Caffey and Tiersch, 2000a); 2) doubts concerning the benefits (e.g., aquaculture industries are not completely aware of the benefits associated with frozen sperm) (Boever, 2006) because evidence proving cryopreservation to be a more cost-effective option than traditional spawning is not available for aquatic species, and 3) lack of early adopters (e.g., producers of species with substantial economic impact, equipped with the necessary technical capabilities, and employing artificial spawning methods) (Caffey and Tiersch, 2000b).

If cryopreservation of fish or shellfish sperm is to be integrated into hatchery operations, one approach to overcome the economic barriers and to prove the effectiveness for aquaculture is the use of specialized cryopreservation centers such as dairy facilities (Tiersch et al., 2004). Years of research and refinement have resulted in a dairy industry that efficiently processes, stores, and tracks semen, making improved germplasm available for breeders. Use of existing dairy freezing facilities provides access to standardized procedures and expensive equipment such as labeling systems, automated straw fillers, bulk-freezing chambers, and storage and inventory capabilities. Therefore, hatcheries could be exempted from the initial investment (mainly the equipment capital and labor costs), and confine their investment to shipping, processing (freezing paid upon service), and storage expenses. Meanwhile, hatcheries would have the flexibility to determine production scale based on available budgets. This approach also provides the additional benefits of well-established methods for quality control, inventory, secure storage, and transport. Conversely, dairy facilities could benefit from the extra income resulting from providing services for aquatic species.

The realization of commercial production of frozen bull semen has been attributed to the worldwide development and application of artificial insemination (AI) in the dairy industry (Herman, 1981). This is because AI training made reproductive technicians receptive to collecting, transporting, storing, and using sperm, which in turn facilitated the later adoption of cryopreservation. Based on this, it was predicted that the logical beginning for commercial application of sperm cryopreservation would be with those aquatic species currently produced by artificial spawning methods (Caffey and Tiersch, 2000a,b). The Pacific oyster, *Crassostrea gigas*, fits into this category because artificial spawning is routinely practiced in oyster hatcheries, and sperm is dry-stripped, stored at 4 °C in the refrigerator, or shipped chilled for fertilization elsewhere (e.g., Dong et al., 2005a). In addition, the Pacific oyster has a world-wide market and certified (e.g., for quality, or disease-free status) frozen sperm would facilitate international distribution, especially to those more selective markets where import of seedstock or broodstock oysters is discouraged (e.g., Europe, Chile) or where direct production of triploid oysters by use of cryopreserved sperm from tetraploid oysters is desired (Dong et al., 2005a). Thus, oyster producers could be considered as potential early adopters for commercialization of frozen sperm, and this would be especially useful for high-intensity breeding programs in oyster industries.

It is worth mentioning that dairy freezing methods such as those used at the LSU T.E Patrick Dairy Improvement Center in Baton Rouge, Louisiana (detailed below) employ a chamber that can freeze 660 0.5-mL standard French straws within 8 min, and can be operated by technicians with minimal training. Although such a freezing curve may or may not provide optimal cooling rates for a specific aquatic species, its cooling rate (on average of 16 °C/min) is within a workable range of cooling rates for most aquatic species (a magnitude of 10, Leung and Jamieson, 1991; Zhang, 2004). This provides the logic for applying dairy freezing methods to sperm of aquatic organisms. In particular with oyster sperm, a wide range of cooling rates have been reported: from 1 °C per min (Hughes, 1973) to direct plunging in liquid nitrogen (Hwang and Chen, 1973).

Thus, the adoption of dairy freezing methods and the use of specialized cryopreservation facilities could be a

mutually beneficial and cost-effective option for marketing of frozen oyster sperm, with the remaining task to be the establishment that such an application is technically feasible (Caffey and Tiersch, 2000b). Our goal was to develop cryopreservation protocols for commercial production of sperm from diploid Pacific oysters. Because we were not able to alter the existing dairy bull cooling method and cooling rate, and our previous analysis of thawing method (Dong et al., 2005c) did not reveal significant differences, one of the remaining main parameters that could be modified was cryoprotectant. We believe this to be a general constraint in widespread application of commercial livestock cryopreservation facilities for aquatic species (reluctance or inability to change freezing conditions), and thus selection of cryoprotectant and handling protocols become increasingly important. Accordingly, in the present study we evaluated optimal concentrations for dimethyl sulfoxide (DMSO), ethylene glycol (E-glycol), methanol, and propylene glycol (P-glycol), and optimized the cryoprotectant type with oyster sperm for use with a commercial dairy sperm freezing protocol. The findings proved the technical feasibility of adopting dairy protocols for commercial production of oyster sperm, and this study provides a template for future commercialization of sperm cryopreservation for other aquatic species.

2. Materials and methods

2.1. Animal sources and sperm collection

A total of 39 male diploid Pacific oysters were obtained from May to August, 2004, from Taylor Resources Quilcene Shellfish Hatchery (TRQSH; *www.taylorshellfish.com*) in Quilcene, Washington (47° 49′ 133″ N, 122° 49′ 523″ W). Sperm were collected by dry stripping of the gonad and placed separately into single 15-mL centrifuge tubes (undiluted) for each male, and were shipped chilled at 5–10 °C in a styrofoam shipper by overnight delivery from TRQSH to the Louisiana State University Agricultural Center, Aquaculture Research Station (ARS). 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). Sperm suspensions were adjusted to 2×10^9 cells/mL using spectrophotometer readings (Dong et al., 2005b), and were mixed with double-strength cryoprotectant solutions to obtain a final concentration of 1×10^9 cells/mL.

2.2. Freezing and thawing procedures

Sperm samples were cryopreserved at the LSU T.E. Patrick Dairy Improvement Center through Genex Custom Collection, Inc. in Baton Rouge (*www.crinet.com/collect.htm*). Existing commercial freezing methods developed for dairy bulls (detailed in Chandler et al., 1984; Dong et al., 2005a) were used in this study. In brief, sperm samples were mixed with the appropriate cryoprotectant in serial addition fashion (9 additional increments during a 45-min equilibration, e.g, for 4.5 mL cryoprotectant solution, 0.5 mL was added to sperm samples every 5 min) 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 International 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). Samples were cooled at a rate of 16 °C/min, and once the chamber reached −140 °C, the samples were removed and placed under liquid nitrogen for sorting and preparation for long-term storage. For fertilization trials, straws were thawed in a 40 °C water bath for 7 s.

2.3. Effect of cryoprotectant concentration

There were three trials in this experiment. For the first trial, sperm from four males were used to evaluate DMSO concentration at 5, 8, and 10%, and samples were stored in liquid nitrogen for 38 d prior to fertilization. For the second trial, sperm from five males were used to test E-glycol at 2, 5, 8, and 10%, and samples were stored in liquid nitrogen for 313 d prior to fertilization. For the third trial, sperm from four males were used to test methanol at 2, 4, 6, and 8%, and samples were stored in liquid nitrogen for 318 d prior to fertilization.

2.4. Effect of the type of cryoprotectant

There were two trials in this experiment. The first trial compared the effect of 5% of DMSO, E-glycol, methanol, and propylene glycol (P-glycol) on percent fertilization. Four batches of sperm samples were used with the first three batches collected from individual males, and the last batch of sperm samples pooled from three males. Samples were stored in liquid nitrogen for 365 d, and fertilization trials were replicated twice for each batch of sperm samples. Based on the results of the first trial and our previous research-scale findings on the optimal cryoprotectant for diploid Pacific oysters (Dong et al., 2005c), the second trial employed 20 males to

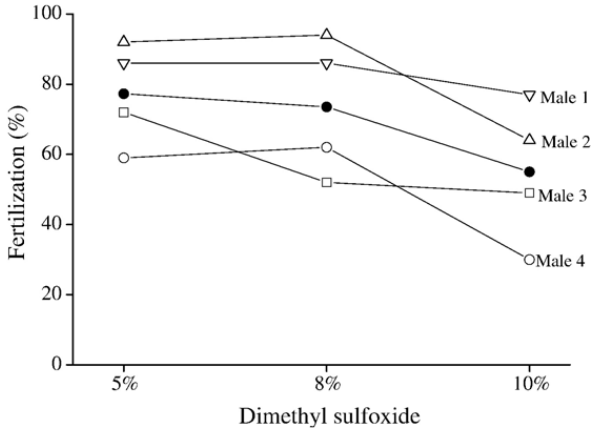

Fig. 1. Percent fertilization of sperm samples suspended in dimethyl sulfoxide at 5, 8, and 10%. Samples were frozen using commercial dairy methods with a 45-min pre-freeze equilibration. Different open symbols represent sperm samples from different males ($n=4$), and filled circles represent the average values for each treatment.

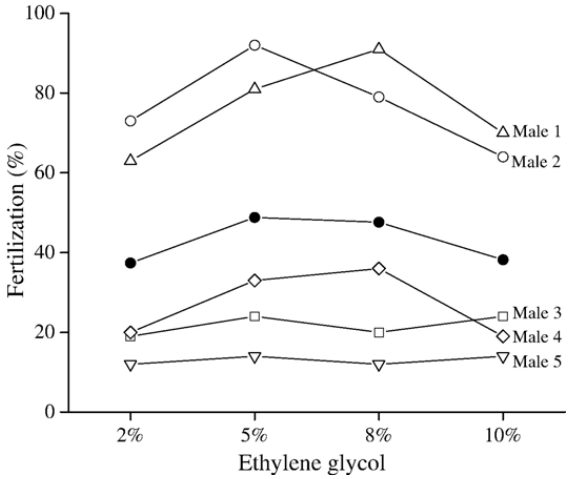

Fig. 2. Percent fertilization of sperm samples suspended in ethylene glycol at 2, 5, 8, and 10%. Samples were frozen using commercial dairy methods with a 45-min pre-freeze equilibration. Different open symbols represent sperm samples from different males ($n=5$), and filled circles represent the average values for each treatment.

compare 6% methanol with the combination of 4% methanol and 2% polyethylene glycol (PEG, FW 200), thus abbreviated as 4% MET/2% PEG. Samples were stored in liquid nitrogen for 301 d prior to fertilization.

2.5. Fertilization trials

Fertilization trials were performed as detailed previously (Dong et al., 2005c). In brief, frozen sperm samples were transported in shipping dewars (CP35, Taylor-Wharton, Theodore, Alabama) to the TRQSH, 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, pooled from three females) held in 250 mL of seawater, yielding approximately 10,000 spermatozoa per egg. Ten straws were heated simultaneously in a 37 °C water bath and immediately after thawing sperm were added to eggs. The gametes were incubated at 25 °C and percent fertilization was calculated by counting the number of developing embryos at 2 h after insemination. For a negative control, eggs were monitored without addition of sperm. For controls of egg quality, fresh (non-frozen) sperm from diploid males were added to fresh eggs to yield about 100 spermatozoa per egg.

2.6. Data analysis

Data were analyzed using the independent-sample *t*-test (comparisons between two means), and one-way analysis of variance (ANOVA). When significance ($\alpha=0.05$) was observed among treatments, Tukey's Honestly Significant Difference Procedure was used for pairwise comparisons. Results were presented as mean± SD, and probability values of $P<0.05$ were considered to be significant. Data for percent of fertilization were arcsine transformed prior to analysis.

3. Results

3.1. Effect of cryoprotectant concentration

In the first trial with DMSO (Fig. 1), the highest fertilization (77±15%) was found at 5%, which was not significantly different from that at 8% (74±20%; $P>0.05$), but both were significantly higher than at 10% (55±20%; $P<0.05$). In the second trial with E-

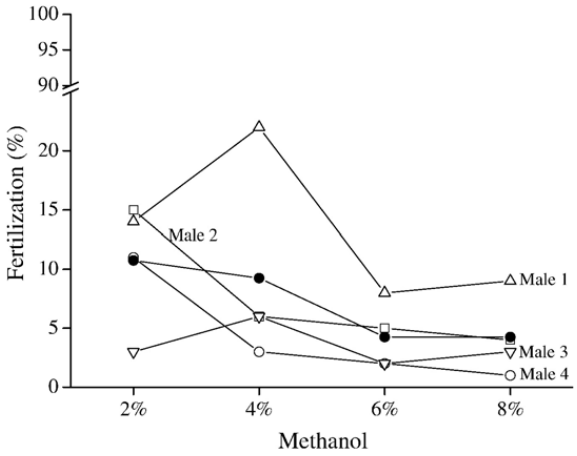

Fig. 3. Percent fertilization of sperm samples suspended in methanol at 2, 4, 6, and 8%. Samples were frozen using commercial dairy methods with a 45-min pre-freeze equilibration. Different open symbols represent sperm samples from different males ($n=4$), and filled circles represent the average values for each treatment.

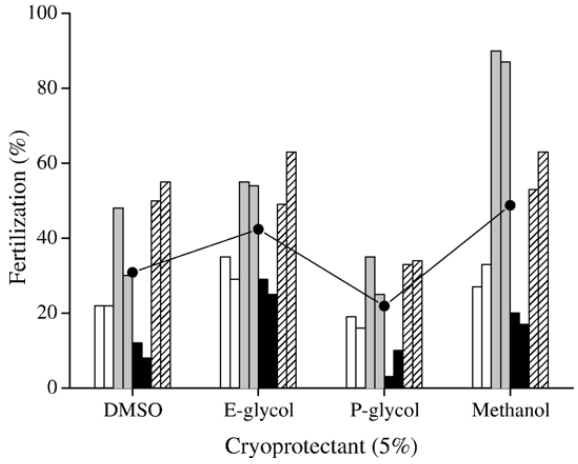

Fig. 4. Percent fertilization of sperm samples suspended in 5% of dimethyl sulfoxide, ethylene glycol, propylene glycol, and methanol. Samples were frozen using commercial dairy methods with a 45-min pre-freeze equilibration. Four replicated batches of sperm samples were used, with the first three batches collected from individual males (white, light grey, and dark grey bars), and the last batch of sperm samples pooled from three males (hatched bars). Filled circles represent the average values for each treatment.

glycol (Fig. 2), the highest fertilization was at 5% (49±35%), which was not significantly different from that at 8% (48±35%; $P>0.05$), but was significant higher than percent fertilization at 2% (37±28%; $P<0.05$) and at 10% (38±27%; $P<0.05$). In the third trial with methanol (Fig. 3), percent fertilization at 2% (11±5%) and 4% (9±9%) were significantly higher than at 6 and 8% (<5%). Therefore, 5% DMSO and 5% E-glycol were chosen for further comparison among different cryoprotectants. Due to the overall low percent fertilization for the methanol trial, selection of the optimal concentration for methanol was derived based on previous studies (Dong et al., 2005a,b,c), and 5% methanol was selected for further comparison.

3.2. Effect of the type of cryoprotectant

Our previous results showed P-glycol at 5% yielded higher percent fertilization than P-glycol at 10 or 15% (Dong et al., 2005a), thus 5% P-glycol was also included in this trial (Fig. 4). The highest percent fertilization was obtained with methanol (49±29%), followed by E-glycol (42±15%), DMSO (31±18%), and P-glycol (22±12%). No significant difference was found for percent fertilization between 5% methanol and 5% E-glycol ($P>0.05$), however, percent fertilization of 5% methanol was significantly higher than those of 5% DMSO and 5% P-glycol. There were minimal variations of percent fertilization (e.g., <13%) between replicates from the same male or same batch of sperm pooled from different males.

The above trial showed that 5% methanol yielded the highest percent fertilization, which was in close agreement with our previous findings on the optimal cryoprotectant for diploid Pacific oysters on a research scale (cooling at 5 °C/min) (Dong et al., 2005c), in which 6% methanol and the combination of 4% MET/2% PEG were shown to consistently yield the highest percent fertilization. Thus, the second trial compared these two protocols by using the commercial dairy freezing method with sperm of 20 males (Fig. 5). There was no significant difference ($P=0.483$) for percent fertilization between 6% methanol (39±29%) and 4% MET/2% PEG (42±26%). However, significant male variation was observed among these 20 males with extremes of percent fertilization of 0% for male 8 and >90% for male 13.

For the above experiments, negative control treatments of fresh eggs without sperm addition yielded 0% fertilization, indicating that eggs were not contaminated with extraneous sperm. Positive controls using fresh sperm without addition of cryoprotectant yielded 99% fertilization, which indicated that the eggs were of good quality.

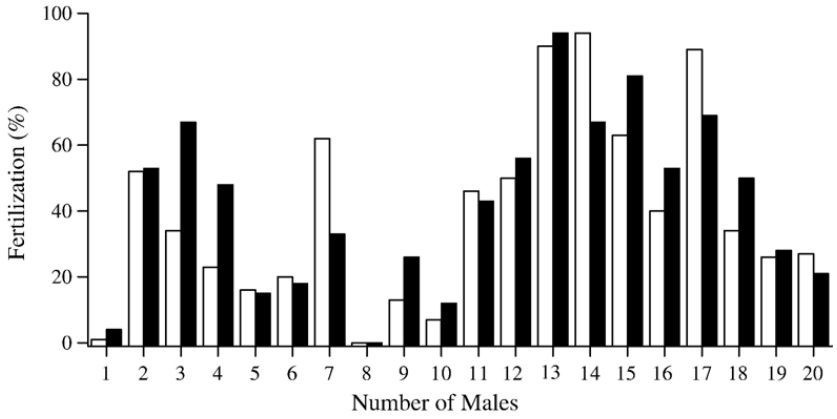

Fig. 5. Percent fertilization of sperm samples ($n=20$) suspended in 6% methanol (white bars) and combined cryoprotectants of 4% methanol and 2% polyethylene glycol (FW 200) (black bars). Samples were frozen using commercial dairy methods with 45-min pre-freeze equilibration.

4. Discussion

Rapid advancement of the field of cryopreservation did not occur until the breakthrough discovery of the protective effect of glycerol for fowl spermatozoa (Polge et al., 1949). Since then, selection of optimal cryoprotectants has been the primary focus of many empirical studies for sperm cryopreservation. For aquatic organisms, DMSO, methanol, E-glycol, and P-glycol are among the most frequently used cryoprotectants (Tiersch, 2000). The present study first evaluated the optimal concentration for each cryoprotectant, and then compared the different cryoprotectants with each at their optimal concentrations. Although DMSO (5 to 20%) has been the main cryoprotectant used in oyster sperm cryopreservation (Dong, 2005), the present study and previous findings (Dong et al., 2005c) showed that methanol or methanol and PEG in combination consistently yielded highest percent fertilization among all tested cryoprotectants. Furthermore, the effectiveness of 4% MET/2% PEG combination for diploid Pacific oysters has been confirmed by four different freezing methods: cooling with a commercial dairy protocol (Dong et al., 2005c and present study), cooling at 5 °C/min using a programmable freezer (Dong et al., 2005c), cooling with a shipping dewar method (Wayman, 2003) that yielded percent fertilization ranging from 17 to 87% (mean±SD: 57±27%; n=6; our unpublished data), and cooling at a rate of ~20 °C/min with an individual quick freezing (IQF) freezer (a device used in the frozen food processing industry) used for half-shell oysters, which yielded 84% fertilization for pooled sperm from 2 oysters (our unpublished data).

Studies of the effectiveness of cryoprotectants for particular species often present problems such as when the superior cryoprotectant in one study is found to be inferior in other studies (e.g., Piironen, 1993; Richardson et al., 2000). Controversies of this kind can result from a lack of procedural standardization (Dong, 2005). Sperm cryopreservation in aquatic species seldom utilizes the same protocols (e.g., preparation methods, or cooling and thawing methods) for the same species among different studies. For example, review of 26 published reports that directly related to oyster sperm cryopreservation revealed inconsistency of various components of cryopreservation technology among and within studies (Dong, 2005). The consistency of the effectiveness of 4% MET/2% PEG in combination for diploid Pacific oysters across four different cooling methods (see above) provides presumptive evidence of the importance of procedural standardization because all of these studies utilized the same methods for sperm collection, suspension, equilibration, sample loading, thawing, and fertilization, with the cooling method being the sole parameter that varied among them.

The optimal cryoprotectant for any particular species is often selected by eliminating non-effective cryoprotectants with limited preliminary trials. The present study raised cautions about this. First, sperm quality varied among different males, batches, or experimental trials. For example, in the trial with methanol in this study, all four males yielded less than 22% fertilization regardless of the methanol concentration, while in the subsequent two trials methanol was found to be superior to E-glycol, DMSO, and P-glycol (Fig. 4), and it was also found to be effective when tested with multiple males (Fig. 5). In addition, all four males used to evaluate DMSO concentration yielded >60% fertilization, while subsequent direct comparison revealed lower effectiveness of 5% DMSO than for 5% methanol (Fig. 4). Thus, it would be risky to eliminate a cryoprotectant simply because of one unsuccessful trial. Second, the effectiveness of a given cryoprotectant mainly relies on its concentration. Comparison among 10% DMSO, E-glycol, P-glycol, and methanol showed the highest percent fertilization (64%) was with 10% E-glycol, and the lowest (2%) with 10% methanol (unpublished data), suggesting that methanol was effective only at low concentrations, which is in agreement with earlier findings of the narrow effective range in terms of cryoprotection and toxic effects for methanol (Lahnsteiner et al., 1996; Dong et al., 2005c). A 10% concentration is generally considered as an intermediate value for most cryoprotectants, however, it could represent an upper limit for others. Therefore, for any given cryoprotectant, range-finding experiments should be evaluated before any choice of concentration is made. Third, the effectiveness of a given cryoprotectant also depends on cryopreservation protocols. For example, DMSO at 5% (Smith et al., 2001), 8% (Iwata et al., 1989), and 10% (Staeger, 1974; Yankson and Moyse, 1991; Li et al., 2002), 10% E-glycol (Ieropoli et al., 2004), 5% P-glycol (Dong et al., 2005a), and 0.45 M trehalose (Adams et al., 2004) all have been found to effectively preserve sperm from diploid Pacific oysters. In contrast, despite the effectiveness of methanol in the present and previous studies (Dong et al., 2005c), other studies with oyster sperm have excluded methanol from the list of suitable cryoprotectants (e.g., Smith et al., 2001). Given these problems of inconsistency, future studies should compare selected optimal protocols among different studies or laboratories to further optimize cryopreservation procedures for a particular species.

Fertility can be considered as 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). However, there can be significant male variation. An average market-sized diploid female oyster (3 inches in height) can produce 50-100 million eggs in a single spawning (Quayle, 1969), a 20% fertilization rate on average would result in at least 10 million eggs being fertilized. In the present trial with 20 males, six had fertilization rates between 20% and 50%, and 10 had fertilization rates higher than 50%. Thus, the methods and results yielded fair success to enable commercial production for oyster hatcheries. Pooling of commercially relevant numbers of males (e.g., 50–100), and using of large freezing containers (e.g., 10 mL straws or cryobags) should be explored in future cryopreservation research, as this would enable bulk processing and would simplify quality control and assessment of gamete quality, and facilitate fertilization with large egg batches. For breeding purposes such as developing new lines, sperm from individual males should be used.

The present study demonstrated the feasibility of commercial production of frozen oyster sperm with the use of cryoprotectant optimization at existing dairy facilities. Accordingly, a potential business structure (Fig. 6) for production and sale of frozen oyster sperm could be illustrated as follows: 1) Technicians at the commercial oyster hatchery would collect sperm, and ship it along with extenders, cryoprotectant solutions, and instructions for sample preparation to an existing dairy facility (freezing center), where other technicians would be responsible for reading the instructions, preparing the sperm suspensions, and freezing them properly; 2) Frozen samples could be transferred back to the hatchery for quality examination (e.g., percent fertilization) and pricing, and this information would be sent back to the freezing center for storage and inventory management (e.g., disposal of samples with poor quality while keeping those with high quality); 3) Users of sperm (e.g., private oyster hatcheries, research laboratories, or federal government) could contact the commercial hatchery for frozen sperm information (strains, quality, price, and availability), and place orders. When the commercial hatchery receives an order, they would notify the freezing center to send the frozen sperm directly to users; 4) In cases when users required preservation of valuable lines or strains, they could ship either the broodstock or undiluted sperm (based on Dong et al., 2005a) to the commercial hatchery or freezing center and have them arrange the cryopreservation. Property issues could be addressed through material transfer agreements and contracts.

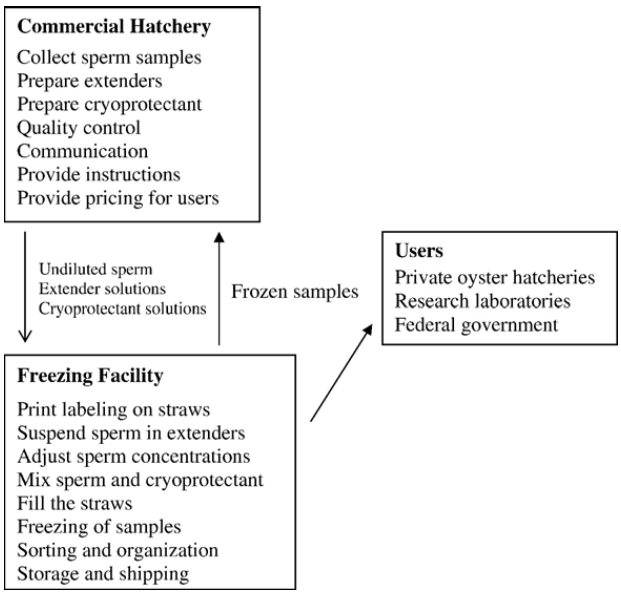

Fig. 6. Diagramatic representation of potential interactions among a commercial hatchery, a commercial freezing facility (existing dairy facility), and users of cryopreserved oyster sperm. Arrows indicate shipping of solutions and fresh samples (open arrow heads) or frozen sperm (filled arrow heads).

The successful adoption of a dairy freezing method allows the oyster hatchery to hire existing commercial facilities for cryopreservation services. Therefore, the commercial oyster hatchery could start a frozen sperm business with low investment costs and minimal economic risk as the major capital investment would be limited to processing and storage expenses. At present, specific processing and storage costs for aquatic sperm have not been determined, but it could be assumed that they would similar to those of dairy sperm because of the similarity in services being provided including economies of scale. In addition, cryopreservation service costs could be reduced if the oyster hatchery maximized the freezing capacity (e.g., 660 straws in the present study) for each batch, as the processing costs are often charged by the batch instead of the number of straws (J. Chenevert, Genex Inc., personal communication). Future studies should address the economics for use of dairy facilities to provide cryopreservation services for aquatic species.

In summary, the present study demonstrated that sperm from Pacific oysters can be shipped from an oyster hatchery to a dairy freezing facility and be frozen at a commercial scale through cryoprotectant optimization (i.e., without changing of the cooling methods and rate). It is possible that dairy freezing methods can be

adopted to freeze sperm from other aquatic species. In fact, the general applicability of dairy protocols for fish species has been indicated previously with sperm from blue catfish *Ictalurus furcatus* (Lang et al., 2003) and red snapper *Lutijanus campechanus* (Riley et al., 2004). These findings show that this approach could be cost-effective and technically feasible for application in aquaculture to provide necessary commercial cryopreservation services, and that oyster hatcheries could become an early adopter for commercialization of frozen sperm. Pacific oysters are an especially promising candidate for early adoption given the large-scale availability of sperm from tetraploid oysters (e.g., Eudeline et al., 2002). The diploid (female) by tetraploid (male) cross offers direct production of triploid offspring, and when performed with frozen sperm does not require shipping of live oysters that could be used to establish unauthorized tetraploid populations elsewhere (Dong et al., 2005a).

Acknowledgements

We thank J. Chenevert, S. Pelitz, C. Jeansonne, and B. Schexnayder of Genex Custom Collection Services, Inc. for assistance with cryopreservation, and S. Leibo for discussion and advice. This work was supported in part by funding from the USDA-SBIR program, 4Cs Breeding Technologies, Inc., the Louisiana Sea Grant College Program, and the Wenzhou Medical College Faculty Research Grant Program. This manuscript was approved for publication by the Director of the Louisiana Agricultural Experiment Station as number 07-11-0232.

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