CRYOPRESERVATION OF PACIFIC OYSTER SPERM

Q. Dong, C. Huang, B. Eudeline and T. R. Tiersch

1. INTRODUCTION

The Pacific oyster, *Crassostrea gigas* (Thunberg, 1793), also referred to as the Japanese oyster or giant oyster, is one of the most important species of bivalves cultured worldwide. The basic life history of the Pacific oyster is similar to that of the eastern oyster, *C. virginica* [1] with both functioning as protandrous hermaphrodites. The young are functionally male during their first spawning, while adults function as separate male or female animals in any given reproduction season [2]. Sexual maturity is reached during the first year, and spawning generally occurs in the summer months (June to August in the west coast of United States). Oysters are highly prolific, and males generally produce 5 x 10^{10} sperm per gram of gonad wet weight [3].

The first report of sperm cryopreservation in oysters was published [4] with *C. virginica.* Since then some 27 reports have been published to address oyster sperm cryopreservation, of which ~80% have addressed sperm from the Pacific oyster. Despite that, procedural standardization is lacking in the cryopreservation of oyster sperm [5]. Inconsistency of various components of cryopreservation technology have been observed 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 [5]. The procedures outlined below are mainly derived from our 4 years of research on this species.

2. PROTOCOL FOR FREEZING AND THAWING

2.1 Equipment

- Sperm collection: gloves, oyster knife, tweezers, scissors, scalpel, spatula, 15 ml centrifuge tubes, permanent marker, Kimwipes, and 40 μm cell strainer (BD Biosciences Discovery Labware, Bedford, Massachusetts).
- Solution preparation: top-loading balance, spatula, stir plate (Barnstead/ Thermolyne, Dubuque, Iowa), stir bar, 0.45 μm CA (cellulose acetate) filter (Corning Incorporated, Corning, New York), and vapor pressure osmometer (model 5500, Wescor Inc., Logan Utah).
- Motility estimation: micropipette and tips (10 and 100 μl), two-well glass microscope slide, and microscope with darkfield and 20 × objectives.

- Straw filling: 0.25 or 0.5 ml straws, 3 ml syringe without needle, goblets, cane holders, PVC powder, and paper towels.
- Freezing: a controlled-rate freezer with accompanying low-pressure liquid nitrogen cylinder, liquid nitrogen storage dewar, and cryogloves.
- Sample shipping: shipping Dewar (CP35, Taylor-Wharton, Theodore, Alabama), plastic cable tie wraps, and tape.
- Thawing: Safety glasses, water bath with temperature controls (Model 1141, VWR Scientific, Niles, Illinois), long tweezers, scissors, paper towels, and 1.5 ml microcentrifuge tubes.
- Fertilization: Constant supply of filtered seawater at 34 ppt salinity and 25°C, 25 µm and 60 µm meshes, 1 l beakers, and 100 l tanks.

2.2 Reagents

- C-F HBSS at 1000 mOsm/kg (calcium free Hanks' balanced salt solution: 26.32 g NaCl; 1.32 g KCl; 0.65 g $MgSO_4 \times 7H_2O$; 0.18 g $Na_2HPO_4 \times 7H_2O$; 0.18 g KH_2PO_4; 1.15 g $NaHCO_3$; 3.30 g $C_6H_{12}O_6$ (glucose) in sufficient distilled water to yield 1000 ml, pH 7.0-7.4) [5].
- Cryoprotectants: methanol, dimethyl sulfoxide, propylene glycol, ethylene glycol, and polyethylene glycol (formula weight of 200).

2.3 Sperm Collection

- Carefully remove the top shell by use of an oyster knife (wear gloves, at least on the hand holding the oyster).
- 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, and use tweezers to remove the heart and associated tissues.
- Use filtered sea water or C-F HBSS to rinse the gonad 3 times, and use Kimwipes to dry the gonad and clean the inside shell.
- Use the scalpel to cut openings on the gonad, and use the spatula to collect the gonad tissue into the 15 ml centrifuge tube. Avoid contamination with digestive gland (yellowish-green material).
- Label the tubes, and store the undiluted sperm samples at 4°C before shipping or use.
- After suspension with C-F HBSS, filter samples through a 40 µm cell strainer prior to adjusting cell concentration.

2.4 Sperm Motility Estimation

- Take 10 ml C-F HBSS from a stock solution stored at -20°C and allow it warm to room temperature (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) . Evaluate the C-F HBSS with microscopy for presence of bacterial contamination.

- Place 30 µl of C-F HBSS inside the wells on the two-well glass microscope slide.
- Determine initial motility by adding undiluted nonmotile sperm [6] or 1 µl of sperm suspension (after suspending in C-F HBSS at 1000 mOsm/kg) and gently mix with the tip of the pipette.
- Evaluate post-thaw motility by adding 2 µl of sperm suspension and gently mixing with the tip of the pipette. Equilibrate sperm suspensions inside the wells on the glass slide at 23°C for 2 min before motility estimation.
- Estimate the percent of sperm that are actively moving in a forward direction at 200 × magnification using darkfield microscopy. Estimate the percent motility in increments of 5%. Samples with motility below 5% that have motile sperm are recorded as 1%.
- Identify the treatments (to avoid bias associated with observer, it is especially important to estimate sperm motility without knowledge of the treatment of the straw).

2.5 Freezing and Thawing Procedure

- Prepare a double-strength cryoprotectant solution within 2 h of use with C-F HBSS at 1000 mOsm/kg, and store at 4°C.
- Adjust sperm concentration to 2 x 10^9 cells/ml with a spectrophotometer using standard curves, and add an equal volume of the cryoprotectant solution in a stepwise addition within 1 h (e.g., 10 or more increments).
- Selected cryoprotectants (final concentration) include 6% methanol, 5% dimethyl sulfoxide, 5% propylene glycol, 5% ethylene glycol, and combined cryoprotectants of 4% methanol and 2% polyethylene glycol at a formula weight of 200.
- Load sperm suspensions into 0.25 ml or 0.5 ml French straws (IMV International, Minneapolis) (for detailed instructions see Appendix A in [5]). Place eight 0.25 ml straws or five 0.5 ml straws into a 10 mm plastic goblet, and attach two goblets to a 10 mm aluminum cane. Insert the canes into the rack inside the freezing chamber.
- Equilibrate samples at 5°C for 5 min before cooling at the desired rate.
- Cool samples in two steps, initially to -30°C at 5°C per min, followed by cooling at 45°C per min from –30°C to –80°C. Hold straws at –80°C for 5 min.
- Remove samples swiftly from the freezing chamber and immediately plunge them into liquid nitrogen in a storage dewar.
- Thaw samples after a minimum of 12 h of storage in liquid nitrogen.
- Remove individual straws from the 10 mm goblets with the long tweezers.
- Thaw in a 40°C water bath for 7 s for 0.5 ml straws, or 6 s for 0.25 ml straws.

- Wipe the straws with paper towel and cut the PVC powder sealed end. Empty the straw into 1.5 ml microcentrifuge tubes by cutting the cotton plug end to release the contents into the tubes.
- In case of sperm agglutination, disrupt the agglutination before motility estimation or fertilization trials.

2.6 Fertilization

- Use the shipping dewar to ship frozen sperm samples to the hatchery.
- 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.
- Pool unfertilized eggs (fresh) from three females and determine the number of eggs per ml (by Coulter Counter if possible). Hold the eggs in seawater at 25°C for at least 30 min to observe germinal vesicle breakdown at 100 × magnification. Separate eggs into 1 liter beakers with each beaker containing 500,000 eggs (fresh) in 250 ml of seawater.
- Thaw ten straws of each treatment as described above.
- 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.
- Incubate the gametes at 25°C and calculate percent fertilization by counting developing embryos at 2 h after insemination (n = 100).
- Hold treatments for further evaluation of percent hatch by transferring to 100 liter tanks filled with fresh seawater. Drain the tanks 24 h after fertilization through a 45 μm mesh and concentrate larvae into 1 liter of seawater. After mixing, calculate percent hatch in five 1 ml subsamples by counting normal straight-hinge larvae with a dissecting scope. If the samples cannot be evaluated immediately, fix the subsamples with Lugol's solution (mix 100 ml acetic acid with 1 liter of Lugol solution, Sigma Chemical Corporation., St. Louis, Missouri) and analyze later.
- For a negative control, monitor eggs after treatment as described above without addition of sperm.
- For the evaluation of egg quality, collect fresh (nonfrozen) sperm from diploid males using the techniques described above, 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 curve [3].
- 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.
- 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.

3. GENERAL CONSIDERATIONS

The cryoprotectants suggested above were found to yield percent fertilization above 90% in different trials. Sperm samples frozen at the suggested concentration can lead to sperm agglutination, which was found to have no effect on fertilization success [5]. However, crushing of the sperm agglutination after thawing is required for motility estimation and fertilization trials.

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Complete affiliation:

Terrence R. Tiersch, Aquaculture Research Station, Louisiana Agricultural Experiment Station, Louisiana State University Agricultural Center, Baton Rouge, Louisiana 70820 USA. ttiersch@agcenter.lsu.edu.

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Edited by

Elsa Cabrita
Vanesa Robles
and Paz Herráez

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