CRYOPRESERVATION OF EASTERN OYSTER SPERM

C.G. Paniagua-Chavez and T.R.Tiersch

1. INTRODUCTION

Crassostrea virginica (formerly the "American oyster") was designated in 1985 as the "eastern oyster" by the Committee on Scientific and Vernacular Names of Mollusks of the Council of Systematic Malacologists [1]. The eastern oyster is a lamellibranch with pronounced bilateral asymmetry [2] and usually spawns as a male in the first year, a condition called protandry. Fecundity has been estimated to be between 500,000 and 66 million eggs per female depending upon body size [3,4]. Within 8 to 12 h (depending on temperature) fertilized eggs develop into free-swimming larvae or trochophores (50 to 60 μm in width). After ~24 h, the trochophore larvae develop into veliger or D-stage larvae (70 to 125 μm). After metamorphosis, the settled larvae develop into adults within 1 to 2 years. Adult oysters mature and spawn in the summer months (April to September in the Gulf of Mexico) for the next cycle [5,6]. The eastern oyster is the most important bivalve species in the United States [7]. However, along the Atlantic and Gulf coasts, oyster production has declined over the past century due to reasons including a lack of consistent seed supply, excessive harvest, loss of suitable habitat, disease, and natural predation [8]. The use of cryopreserved gametes and larvae can improve hatchery production of seedstock to increase production for the oyster industry. Cryopreservation of oyster sperm and larvae has been tested at the laboratory level, but given the benefit that this technique offers, cryopreservation of oyster sperm and larvae should be developed for commercial application at the hatchery level. The procedures outlined below are suitable for application at the hatchery scale, and could be scaled up for commercial application.

2. PROTOCOL FOR FREEZING AND THAWING

2.1 Equipment

- Oyster knife
- Dissection kit
- Capillary tubes
- Microscope and slides
- Osmometer
- Nitex screens (15 μm, and 70 μm)
- Micropipettes (20 μl, 1000 μl, and 5000 μl)
- 50 ml beakers
- Hematocytometer

- 5 ml macrotubes and sealing balls
- 30 ml syringes without needles
- Controlled-rate freezer
- Water bath
- Dewars
- Basic laboratory glassware

2.2 Reagents

- Distilled water
- Natural seawater, artificial seawater, or calcium-free Hanks' balanced salt solution FHBSS: 24 g/l NaCl, 1.20 g/l KCl, 0.60 g/l $MgSO_4$ x $7H_2O$, 0.36 g/l Na_2HPO_4 x $7H_2O$, 0.18 g/l KH_2PO_4, 1.05 $NaHCO_3$, 3 g/l $C_6H_{12}O_6$ adjusted to 600 mOsm/kg
- Propylene glycol
- Liquid nitrogen

2.3 Semen Collection

- Gamete preparation: A gonad sample is collected with a capillary tube and smeared on a glass microscope slide for examination at 200 x magnification. Sex is identified based on the presence of eggs or sperm. Gamete samples are removed from each oyster by the dry stripping method [9]. The gonad is gently disrupted and gonadal material is collected with a Pasteur pipette. A 10 µl sample is removed from the gonad to measure osmolality with a vapor pressure osmometer (model 5500, Wescor Inc., Logan, Utah).
- Sperm samples are placed in 50 ml beakers until suspension in an extender. After suspension, the sperm samples are washed through 70 µm and 15 µm Nitex screens (Aquacenter, Leland, Mississippi), with artificial seawater (ASW), natural seawater, or calcium-free Hanks' balanced salt solution (C-F HBSS) adjusted to 600 mOsm/kg [10].

2.4 Sperm Motility Estimation

- Unlike sperm of most fishes, oyster sperm can remain continuously motile for hours or days. A 10 µl sample is removed from the sperm suspension to estimate motility. The percentage of sperm exhibiting vigorous forward movement is estimated at 200 x magnification using dark-field microscopy. Sperm vibrating in place are not considered to be motile. Only males with actively swimming sperm (> 90%) are selected for experimentation [10,11].

2.5 Description of the Protocol for Refrigerated Storage of Sperm

- After microscopic identification, undiluted sperm samples can be placed in sealed tubes and be stored in a refrigerator at 4°C for as long as 4 days. Sperm should maintain >50% motility in this condition. Extended sperm (1:1, v:v) may be refrigerated for as long as 2 days if diluted in C-F HBSS. Sperm can maintain 50% motility in this condition [10].

2.6 Freezing and Thawing Procedures

- Sperm may be suspended at 1 x 10^9 cells per ml in ASW, natural seawater, or C-F HBSS. The cryoprotectant can be prepared in these extenders to yield a final concentration of 10% or 15% propylene glycol (PG).
- After suspension, sperm are equilibrated for 20 min, and 5 ml aliquots are used to fill 5 ml macrotubes (Minitube of America, Inc., Madison, Wisconsin).
- The macrotubes are cooled in a controlled-rate freezer (Kryo 10 series II; Planer Products, Sunbury-on-Thames, UK). The initial temperature is 15°C, and the samples are cooled at a rate of 2.5°C per min until reaching a final temperature of –30°C, which is held for 5 min. Macrotubes are plunged into liquid nitrogen and stored in a dewar until use.
- A water bath is used to thaw the samples at 70°C for 15 s. After thawing, the samples are resuspended in 5 ml of fresh ASW, natural seawater or C-F HBSS. Sperm should be used immediately after thawing to fertilize eggs [11,12,13].

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