Cryopreservation of Sperm and Larvae of the Eastern Oyster

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Introduction

In aquaculture, cryopreservation studies have primarily addressed spermatozoa of three groups of commercially important fishes (salmonids, tilapias and carps) (Stoss 1983). However, work with larvae of fish or other organisms such as mollusks is scarce (Gwo 1995). In mollusks, the Japanese oyster *Crassotrea gigas* has been the species most used to study cryopreservation of larvae (Table 1). Other species of oysters, including those of commercial importance, have not been studied. Cryopreservation of oyster gametes and larvae has been tested only in the laboratory, and has yielded only a single report of oyster growth beyond planktonic stages (Paniagua-Chavez et al. 1998a). Given the benefit that this technique offers to research and the commercial oyster industry, cryopreservation of oyster gametes and larvae should be developed for application in the hatchery.

In the United States the most important oyster is the eastern oyster *Crassostrea virginica*. In Louisiana the production reached a value of $49,000,000 in 1998 (Avery et al. 1999). Along the Atlantic and Gulf coasts, production of eastern oysters has declined due to a variety of reasons including a lack of consistent seed supply, excessive harvest, disease and natural predation (Supan and Wilson 1993). The production of cryopreserved gametes or larvae would improve hatchery production of seedstock oysters, thus allowing distribution of improved or genetically modified stocks.

This study represents the first successful production of seedstock from cryopreserved larvae of any aquatic food organism cultured for human consumption (Paniagua-Chavez et al. 1998a) and it documents for the first time the production of oyster seedstock from eggs fertilized with thawed sperm.

Materials and Methods

Gamete Collection and Larvae Production

Collection of high quality gametes was necessary for reliable production of eastern oyster larvae suitable for cryopreservation. *Crassostrea virginica* can tolerate a wide range of salinities (osmolalities) in the natural environment. In the laboratory, osmolality must be controlled for gametes and larvae to be used for cryopreservation (Figure 1). Oysters from their natural environments were held in recirculating systems in the laboratory for at least 5 d at 18 to 20 °C. This temperature inhibited the Gulf coast oysters from spawning, but allowed maintenance of ripe gametes when the oysters were fed the marine microalgae *Isochrysis galbana* (T-iso) and *Chaetoceros calcitrans*. This acclimation period also allowed oysters that were stressed or weakened in transport to recover or die (reducing bacterial contamination of gametes and larvae). A marked

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Table 1. Relevant cryopreservation studies of gametes and larvae of oyster species.

Species	Frozen material	Citation
Crassostrea gigas	Sperm	Bougrier and Rabenomanana 1986
C. tulipa, *C. iredalei* and *C. gigas*,	Sperm	Yankson and Moyse 1991
Saccostrea cucullata	Sperm	Yankson and Moyse 1991
C. gigas	Sperm	McFadzen 1995
C. gigas	Larvae	Renard and Cochanrd 1989
C. gigas	Larvae	Renard 1991
C. gigas	Larvae	McFadzen 1993
C. gigas	Larvae	Lin et al. 1993
C. gigas	Larvae	Chao et al. 1994
C. gigas	Larvae	Lin et al. 1994
C. gigas	Larvae	Gwo 1995
C. gigas	Larvae	Chao et al. 1997
C. gigas	Larvae and eggs	Naidenko 1997
C. virginica	Sperm	Huges 1973
C. virginica	Sperm	Zell et al. 1979
C. virginica	Larvae	Paniagua-Chavez et al. 1998a

increase in production of larvae was noted after acclimation (Figure 2), and in some cases larvae could not be produced until after 5 d of acclimation. Oysters were held for 2 months at 18 to 20 °C without spawning while allowing gamete maturation and retention (Buchanan et al. 1998).

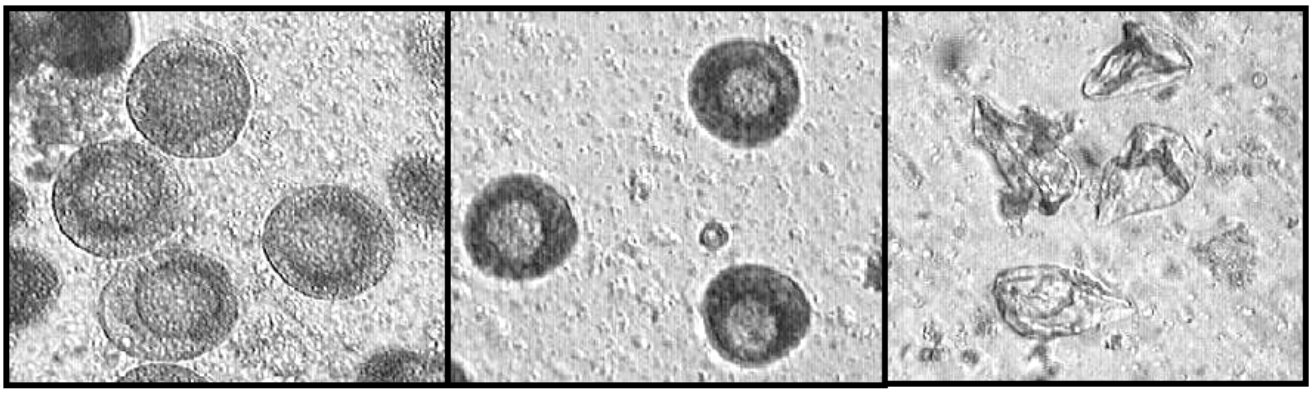

Figure 1. Eggs of *Crassostrea virginica* exposed for 5 min to artificial seawater (ASW) of various osmolalities. Prior to exposure, eggs were held at laboratory osmolalities (475 mOsmol/Kg) for 5 d. Left panel: eggs placed in hypotonic ASW at 115 mOsmol/Kg; middle panel: eggs placed in isotonic ASW at 468 mOsmol/Kg; right panel: eggs placed in hypertonic ASW at 705 mOsmol/Kg.

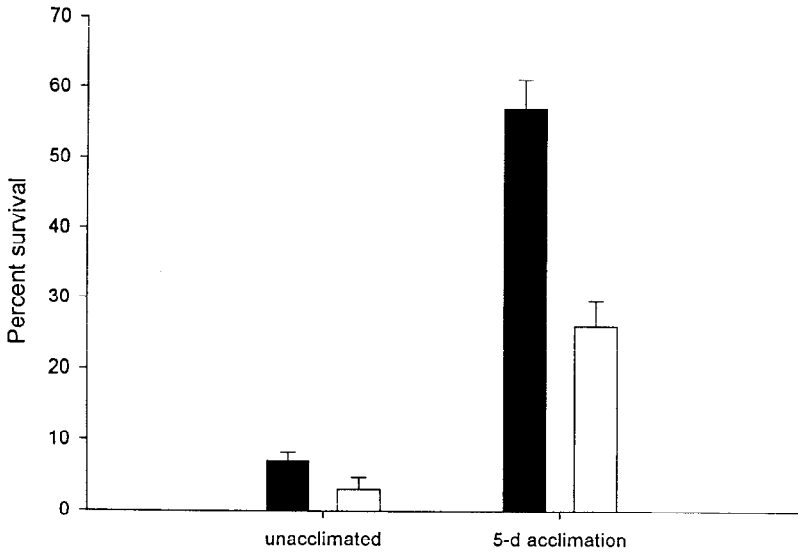

Figure 2. Larval survival of *Crassotrea virginica* at 12 hr (black bars) and 24 hr (open bars) after fertilization. Oysters were selected for spawning at random before and after laboratory acclimation (5 d). Percent survival was the average for eggs of four females. Error bars indicate one standard deviation.

Before gamete collection, acclimated oysters were scrubbed with a mild bleach solution (5%) or were washed with 70% ethanol. Oysters were opened and inspected visually for ripeness, indicated by a creamy color with prominent genital canals running throughout the gonad. Mature oysters were selected and rinsed with 70% ethanol, followed by rinsing with filtered artificial seawater (ASW). To determine sex of the oysters, a sample from each gonad was taken by puncture with a capillary tube. This sample was smeared onto a glass slide and observed at 100-X magnification with phase-contrast microscopy. The gonads were lacerated with a sterile scalpel and the gametes were removed with a glass Pasteur pipette and placed into clean plastic beakers. All egg samples were passed through a 70-μm Nitex screen and were collected on a 13-μm screen. Eggs were washed with ASW and collected in a clean beaker. For sperm, samples were washed with calcium-free Hanks' balanced salt solution (C-F HBSS) (Paniagua-Chavez et al. 1998b) through 70-μm and 13-μm screens, and were re-suspended in C-F HBSS. Only actively swimming sperm were considered to be motile, and only males with sperm motility greater than 90% were selected for experimentation. To produce larvae, eggs were mixed with ~ 500 sperm per egg in 1 L of ASW. Embryos were cultivated at a concentration of ~ 100 embryos/mL in ASW. Trochophore larvae were collected ~ 12 hr after fertilization (incubated at 21 °C) on a 13-μm screen. Osmolality of all solutions was ~ 475 mOsmol/Kg. A 0.45-μm filter was used to sterilize the CF-HBSS. The ASW was passed through a 1-μm filter, sterilized with ultraviolet irradiation and passed through an activated carbon filter before use.

Cryopreservation Protocol

A single protocol was used to cryopreserve sperm and larvae (described below). Trochophore larvae were frozen at a concentration of 10,000/mL and sperm were frozen at ~ 1 x 10^9 mL. After suspension of sperm in C-F HBSS, or trochophore larvae in ASW, they were placed in a cryoprotectant solution composed of ASW and 15% propylene glycol (Sigma Chemical Corp. St. Louis, Missouri). Five-mL straws were filled with the sperm or larvae suspensions and allowed to equilibrate for 20 min at 21 °C. The straws were frozen in a controlled-rate freezer (Kryo 10 series II, Planer, England). The cooling rate was -2.5 °C per min until reaching a final temperature of -30 °C, which was held for 5 min. Straws were plunged into LN_2 and stored for one week. A water bath was used to thaw the straws at 70 °C for 15 sec.

Hatchery Experiments

Samples were transported in nitrogen vapor shipping dewars (model CP-65, Taylor Wharton, Theodore, Alabama). In the hatchery, the straws were thawed in a water bath at 70 °C for 15 sec and drained into 1-L plastic beakers containing an equal volume of fresh filtered seawater. Thawed sperm was used to fertilize fresh eggs. A 30-μm mesh screen was used to remove thawed larvae from the water with cryoprotectant. Larvae collected on the screen were ready to culture in tanks.

Experimental Safeguards

Working at a hatchery on the coast created benefits and problems. We benefited from the availability of natural seawater, ambient conditions and natural (heterogeneous) food sources for grow-out of oysters after settlement. On the other hand, we needed to

ensure that no extraneous natural larvae contaminated the experiment before settlement, and that natural spatfall did not influence our results during growth.

The procedures we employed to ensure that no natural larvae contaminated the experiment were: 1) working during late September which is at the end of the spawning season in Louisiana; 2) all oyster and algae tanks were segregated from sources of natural water and other sources of contamination; 3) all equipment and mesh screens were used exclusively for this experiment and were washed with filtered water before and after use; 4) all water used for rearing of oyster larvae and for culture of algae was filtered repeatedly through mesh sizes down to 1 μm; 5) the embryos and larvae in the experiments were collected on appropriate-sized mesh screens every 2 d, to perform water exchanges and counts; 6) experimental larvae were counted at each handling; 7) the larvae were also monitored to ensure that the populations were of the correct developmental stage; 8) counts of eyed pediveligers were performed as they were collected to be moved to the tanks containing cultch; 9) the number of larvae collected for settlement was compared with the larval census at that developmental stage, and 10) two additional mesh bags containing clean cultch were suspended among the experimental bags to estimate the occurrence of natural spatfall.

Results

The experiments were performed in two consecutive years starting on September 18, 1996 and September 20, 1997 (Table 2). In the first year, the experiment was terminated after 10 d due to a hurricane and flooding on the experiment site (Grand Isle, Louisiana). After 24 hr of incubation, 10% of the control larvae had developed to D-stage. Subsequently, survival of the control larvae decreased rapidly and after 10 d larvae were not found. Two percent of thawed larvae developed to D-stage and after 10 d of incubation 1,000 pediveligers were counted. Eggs fertilized with thawed sperm yielded better survival. After 24 hr, 1.1×10^6 D-stage larvae were counted, and after 10 d of incubation, 2.4×10^4 pediveligers were found.

In the second year, larvae were incubated in 200-L tanks for 10 d, fed daily with 20 L of the algae *I. galbana* and were reared using normal hatchery procedures. After 24 hr of incubation, 28% of the control larvae, 24% of thawed larvae and 88% of larvae produced with thawed sperm had developed to D-stage. Larval numbers decreased throughout development, indicating absence of contamination with wild larvae. Larvae were collected by screening daily on 210-μm mesh beginning after 8 d of incubation and were transferred to a system containing cleaned shells of the common clam *Rangia cuneata* used as cultch material. A total of 500,000 control larvae, 28,000 thawed larvae and 64,000 larvae produced with thawed sperm were placed in the cultch system. After settlement, spat were secured in plastic bags and suspended in Caminada Bay (29º 15' 12" N, 90º 03' 26" W) to evaluate survival and growth. After 2 wk of suspension in the Bay, a subsample of 80 *R. cuneata* shells were obtained at random from each bag to count spat. One thousand spat were counted from the control group, 200 spat from the thawed larvae, 80 from larvae produced with thawed sperm and none from clean shells available for wild spat. After 4 months of suspension in the Bay, all of the cultch material was examined and a total of 1,000 seed oysters (2.5 to 5 cm) were counted from the control group, 850 were counted from the thawed larvae and 230 from larvae produced with thawed sperm.

At this time, a total of 57 wild spat were collected from the bags containing clean cultch (23 on one bag and 34 on the other).

Table 2. Number and percent of survival beyond settlement of Eastern oysters *Crassostrea virginica* produced from control larvae, thawed larvae and larvae produced with thawed sperm during 2 yr of research.

1996					
Date	**Control larvae**	**Thawed Larvae**	**Survival (% control)**	**Thawed Sperm**	**Survival (% control)**
9/18	3.0×10^6	3.0×10^6	100%	3.0×10^6	100%
9/20	3.1×10^5	5.0×10^3	2%	1.1×10^6	354%
9/22	3.8×10^4	3.6×10^3	9%	2.8×10^5	737%
9/24	6.0×10^3	2.0×10^3	33%	7.9×10^4	1,316%
9/26	4.6×10^3	2.0×10^3	43%	3.4×10^4	739%
9/30*	0	1.0×10^3		2.4×10^4	
1997					
Date	**Control Larvae**	**Thawed larvae**	**Survival (% control)**	**Thawed Sperm**	**Survival (% control)**
9/20	6.0×10^6	3.0×10^6	50%	3.0×10^6	50%
9/22	1.7×10^6	4.0×10^5	24%	1.5×10^6	88%
9/24	1.0×10^6	2.0×10^5	20%	6.3×10^5	63%
9/26	1.0×10^6	5.3×10^4	5%	5.0×10^5	50%
9/28	5.6×10^5	2.8×10^4	5%	6.4×10^4	11%
Set on cultch					
10/97**	1.0×10^3	2.0×10^2	20%	8.0×10^2	8%
1/98***	1.0×10^3	8.5×10^2	85%	2.3×10^2	23%

*Experiment terminated due to hurricane.
**Total number of spat counted on a subsample of 80 *R. cuneata* shell (cultch). No natural spatfall was observed on any cultch samples on this date.
***Natural spatfall was observed only on clean cultch (not in control or thawed treatments). Oysters from wild spatfall were visibly smaller than those from the experimental groups.

The wild spat were less than 0.5 to 1 cm in length, while the experimental spat (from control, thawed larvae and sperm) were more than 2.5 cm at this time, allowing identification of naturally produced spat. The combination of few natural spat at this time of year and a greater suitability for settlement on the clean cultch may explain the

lack of wild spat in the experimental bags. During the experiments all larvae and spat appeared to be morphologically normal and healthy (Figure 3).

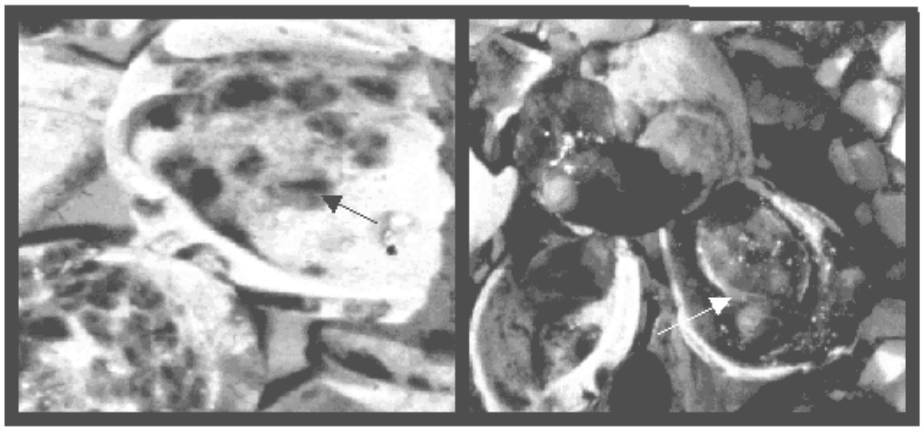

Figure 3. Eastern oysters produced from thawed larvae. Left: spat 2 wk after settlement on shell of *Rangia cuneata* clam. Right: Oysters 4 months after settlement.

Discussion

The goal of applied research is the transfer of technology from the laboratory to the industrial or commercial level. In aquaculture, technology transfer involves modification of laboratory techniques for use in hatcheries or other culture environments. Cryopreservation of gametes and embryos is a good example of technology that can be used in aquaculture. However, most of the studies on cryopreservation of oyster sperm and embryos have been limited to a few days in the laboratory and have not been evaluated for scaling up for production. It is important to evaluate laboratory studies because often they do not yield the same results as those performed in the hatchery or natural environment. The growth of thawed larvae and larvae produced from eggs fertilized with thawed sperm for a few hour or days does not ensure survival through settlement or production of adult oysters. Genetic or physiological damage could result in production of abnormal larvae, excessive mortality, or the inability to complete metamorphosis and attach to cultch.

In our study, we found that it was possible to obtain settlement and to produce normal seed oysters from thawed trochophore larvae and eggs fertilized with thawed sperm. Survival, although apparently low, was not different from the control group, and was not different from normal survival in the hatchery. Extremely large numbers of larvae (in the billions) are routinely used to produce seedstock at the commercial level. With additional research, cryopreservation could be scaled up to allow production of commercially relevant numbers of larvae.

This is the first successful production of seedstock from cryopreserved larvae of any species of aquatic organism commercially grown for human consumption. This is also the first production of seed oysters from eggs fertilized with thawed sperm. The production of oysters capable of being planted on oyster grounds for growth and harvest demonstrates that cryopreservation can be applied for use in the hatchery. This is especially important for mollusks because at present maintenance of broodstock requires

considerable space and expense within a hatchery, or entails the risk of placing valuable stocks into natural waters where they are susceptible to diseases, predators and contamination with wild spatfall.

The availability of frozen larvae and sperm offers obvious benefits. The frozen material would ensure protection of valuable stocks and would facilitate transport and availability of improved (e.g. disease-resistant) lines. The management of genetic resources of endangered species (Tiersch et al. 1998), and the techniques for larval cryopreservation may be applicable to highly endangered mollusks, such as freshwater mussels. Research efforts would benefit from standardization (over distance and time) due to the availability through cryopreservation of control lines of oysters and economical storage of specific research populations (e.g. tetraploid larvae). Work could proceed year-round given the availability of larvae outside of the spawning season. This point has special relevance to cytogenetic studies of oysters given that transformed cell lines do not exist, and larval material is often used to yield primary cultures with sufficient mitotic activity to reliably produce metaphase chromosomes (Zhang et al., Pp. 314-318, this volume). However, future studies should evaluate the complete life cycle of oysters produced from cryopreserved larvae or sperm, and address optimization of production.

Over the last 20 yr, the production of Japanese oyster seed in hatcheries has been economically viable in Washington state (Lipovsky 1980). However, for the eastern oyster, economic feasibility studies of hatcheries in the Chesapeake Bay have shown that hatcheries operating solely as seed oyster facilities, rather than as a component of a harvesting operation, have the ability to generate profit. However, this potential relies heavily on favorable growth conditions in the hatchery. Salinity fluctuations or equipment failure could drastically reduce the hatchery output (Lipschultz and Krantz 1980). A good option to optimize production is to use cryopreserved sperm and larvae when favorable growth conditions are found in the hatchery. A cost analysis has been performed for the integration of cryopreservation into existing fish hatcheries (Caffey and Tiersch 1999, and Caffey and Tiersch, pp 388-408, this volume). Given the possibility of using cryopreserved oyster larvae and sperm in the hatchery, a similar analysis should be performed for the application of cryopreservation to oyster production.

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