Cryo-Letters 19, 283-292(1998).
Published by Cryo-Letters, 7, Wootton Way, Cambridge CB3 9LX, U.K.

SETTLEMENT AND GROWTH OF EASTERN OYSTERS PRODUCED FROM CRYOPRESERVED LARVAE

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ABSTRACT

Previous reports on viability of cryopreserved oyster larvae have been limited to evaluation of planktonic stages for less than 6 d after thawing. However, for hatchery and aquacultural purposes the performance of larvae through settlement is of primary importance. The present experiments on production of oysters from cryopreserved larvae were conducted in two consecutive years. Observations through veliger larvae only were collected from the first year because of interruption by a hurricane. In the second year, growth was observed for 4 months. A total of 3 x 10^6 trochophore larvae (12 h post-fertilization) were diluted in a cryoprotectant solution composed of artificial sea water and 15% propylene glycol. Larvae were placed in 5-mL straws, frozen at –2.5 °C per min, and stored in liquid nitrogen. The frozen material was transported to an oyster hatchery at Grand Isle, Louisiana. Larvae were thawed at 70 °C for 15 sec and were incubated in a 200-L tank. For a control treatment, 6 x 10^6 eggs were fertilized and incubated in the same conditions as the thawed larvae. After 24 h of incubation, 28% of the control group developed into D-stage larvae, while 24% of the thawed larvae developed to this stage. Larvae were fed daily with 20 L of microalgae (Isocrysis galbana) for 10 d. After incubation, larvae were allowed to metamorphose and attach to substrate and were held for 8 d. Spat were placed in mesh plastic bags and suspended in Caminada Bay (29°15'12''N, 90°03'26''W) to evaluate survival and growth. After 4 months, 1,000 oysters from the control group, 850 from the thawed larvae, and 57 from naturally spawned oysters were found. The results indicate that oysters produced from thawed larvae developed normally in the hatchery, opening opportunities for use of cryopreserved larvae in oyster research and in commercial industry

KEY WORDS: Cryopreservation, Crassostrea virginica, trochophore larvae, propylene glycol.

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INTRODUCTION

Cryopreservation of embryos has increased in importance in recent years, offering advantages, in fields such as medicine, genetics, toxicology and aquaculture. Techniques for cryopreservation of embryos of several vertebrate species have been developed (4), but work on aquatic organisms has addressed only a few species such as the Japanese oyster, Crassostrea gigas (3, 5, 6, 8, 11), blue mussel (19), hard clam, Meretrix lusoria (3), manila clam Tapes philippinarum (6), sea urchin, (2), shrimps, Penaeid sp (12, 14), and rotifer, Brachionus plicatilis (20). None of these studies produced animals beyond larval stages. The only aquatic organism that has been successfully cryopreserved yielding development to adult stages has been the ragworm Nereis virens (9). This event marked the first step in cryopreservation of more complex organisms and potential for impact in aquaculture of polychaetes.

In aquaculture, cryopreservation studies have primarily addressed spermatozoa of three groups of commercially important fishes (salmonids, tilapias and carps) (13). However, work with embryos of fish or other organisms such as molluscs is scarce (5). In molluscs, the Japanese oyster has been the species most used to study larvae cryopreservation. Other species of oysters, including those of commercial importance, have not been studied. The most recent report we are aware of concerning cryopreservation studies of the Eastern oyster (C. virginica), for example, was on sperm in 1979 (22).

In the United States, the most important oyster species, representing >80% of total production, is the Eastern oyster (21). Along the Atlantic and Gulf coasts, oyster production has declined due to a lack of consistent seed supply, excessive harvest, disease, and natural predation (16). The production of cryopreserved oyster larvae would be useful to improve hatchery production of seedstock oysters, to allow distribution of improved or genetically modified stocks, and to provide feed for larval marine fish.

Oysters develop from planktonic (free-swimming) larval stages through metamorphosis and attachment to a substrate (settlement of spat on cultch) for subsequent growth as sessile organisms (Table 1). Cryopreservation of oyster gametes and larvae has been tested only in the laboratory, and has not yielded a report of oyster growth beyond planktonic stages (Table 2). 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.

The objective of this work was to evaluate the performance of thawed Eastern oyster larvae in the hatchery by setting and growing them in a natural environment. The purpose of this report is to document for the first time the settlement and growth of Eastern oysters from cryopreserved larvae, representing the first successful production of seedstock from cryopreserved larvae of any aquatic food organism cultured for human consumption.

MATERIALS AND METHODS

Chemicals

All chemicals were of reagent grade (Sigma Chemical Corp., St. Louis, Missouri, USA). Artificial sea water (ASW) was prepared using Instant Ocean® salts (Aquarium Systems,

Mentor, Ohio, USA). Osmolality of suspensions was measured with a vapor pressure osmometer (model 5500, Wescor Inc., Logan, Utah, USA).

Table 1. Larval (planktonic) development of oysters. Indicated below are the temperatures recorded during our experiments, approximate time after fertilization, and size for each developmental stage. The size of mesh used to collect experimental larvae for water exchanges and observation is indicated for each stage.

Developmental stage	Temperature (°C)	Time	Larval size (μ)	Mesh sizes (μ)
Four-cell	25	1 h	50	10, 15
Trochophore	21	12 h	60-70	15, 45
D-stage	28	1-4 d	90-120	40, 70
Veliger	28	5-7 d	130-200	60, 100
Pediveliger	28	8-16 d	>225	100, 210
Young spat	28	17 d	Up to 4 mm	Not screened, on cultch

Oyster collection

Adult oysters were obtained from the Louisiana Sea Grant Program hatchery on Grand Isle, Louisiana, and transported to the Louisiana State University Agricultural Center, Aquaculture Research Station (ARS) in Baton Rouge. Oysters were opened and inspected visually for the presence of gonad development and prominent genital canals (15). Gonad samples were removed with a capillary tube and spread on glass slides for microscopic examination at 200X. Sex was identified based on the presence of eggs or sperm.

Gamete preparation

Gamete samples were collected from each oyster by the dry stripping method (1). The gonad was gently disrupted and gonadal material collected with a Pasteur pipet. Egg or sperm samples were placed in 50-mL plastic beakers (VWR Scientific Inc., St. Louis, Missouri, USA) until suspension. Eggs were suspended in ASW at 642 mOsml/kg, and sperm were suspended in calcium-free Hanks' balanced salt solution (CF-HBSS) at 600 mOsml/kg (18, 10). After suspension, eggs were washed through a 70-μ Nitex screen (Aquacenter, Leland, Mississippi,

USA), collected on a 15-μ screen, and were resuspended in ASW. Sperm were washed through 70-μ and 15-μ screens and motility was estimated as described below.

Table 2. Studies of cryopreservation of larval oysters (genus Crassostrea).

Species	Developmental stage when cryopreserved	Development after thawing	Citation
C. gigas	2-4 cell stages	D-stage	11
C. gigas	24 h post-fertilization	48 h post-thaw	6
C. gigas	Morula, gastrula, trochophore, and veliger	Veliger	5
C. gigas	2-7 h post-fertilization, D-stage and veliger	1-2 h, 24h, 3-6 d post-thaw	8
C. gigas	Morula and trochophore	Immediate post-thaw	3
C. virginica	Trochophore	Seed oyster (to 5 cm)	Present study

Motility estimation

A 10-μL sample was removed from each sperm suspension to estimate motility. The sample was placed on a glass microscope slide and the percentage of sperm exhibiting vigorous forward movement was estimated at 200X using darkfield microscopy (Optiphot 2, Nikon Inc. Garden City, New York, USA). Sperm vibrating in place were not considered to be motile. Only samples with actively swimming sperm (> 90%) were selected for experiments.

Fertilization

Gametes from five females and three males were pooled in plastic beakers: 6.2 x 10^6 eggs (35 eggs/mL) were suspended in 2 L of ASW and fertilized with sperm in a ratio of 1 egg:5 to 10 sperm (Figure 1a). Concentrations of eggs were determined by counting of 1-mL aliquots in a Sedgewick-Rafter chamber (Hausser Scientific Partnership, Horsham, Pennsylvania, USA). Fertilized eggs were incubated until ~50% were in first cell division at 25°C (30 min). After incubation, fertilized eggs were placed in 15-L buckets at 21°C. Twelve h after fertilization, the number of trochophore larvae/mL was estimated with a Sedgewick-Rafter chamber. Gamete and larvae counts were performed in duplicate.

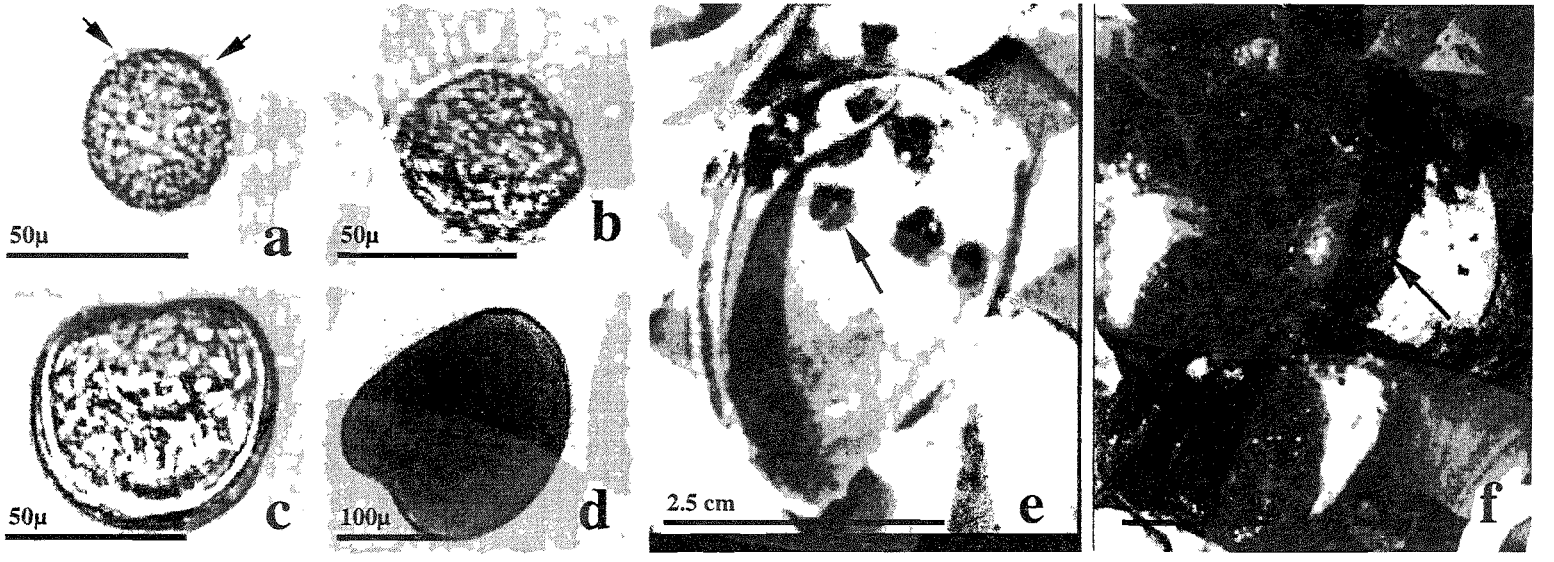

Figure 1: Development of the Eastern oyster Crassostrea virginica. **a:** Fertilized egg, arrows indicate sperm surrounding the egg; **b:** trochophore larvae; **c**: D-stage larvae; **d**: pediveliger (formalin-fixed); **e**: spat, arrow indicates spat 2 weeks after settlement on shell of Rangia clam; **f:** Seed oysters, arrow indicates oysters 4 months after setting.

Temperatures varied during the experiment based on the location of specific activities. Gametes were collected for cryopreservation studies and fertilizations were performed in wet laboratories at the ARS (25°C). Embryos were incubated on the laboratory bench (21°C) until cryopreservation at 12 h after fertilization (trochophore stage). All other work was carried out at the oyster hatchery on Grand Isle (28°C).

Pre-freezing

Detailed studies of extenders, cryoprotectants, cooling rates, thawing rates, containers, and larval concentrations were conducted prior to these experiments and will be reported elsewhere. Optimized conditions were chosen for the hatchery trials in the present report. The cryoprotectant solution (CPS) was prepared with fresh-made filtered ASW containing 15% (v/v) propylene glycol. Trochophore larvae were concentrated with a 30-μ Nitex screen yielding a total volume of 150 mL. After concentration, the embryos were mixed with the CPS. The final concentration of larvae was 12,000 larvae/mL. Osmotic pressure of the suspension was measured before (642 mOsmol/kg) and after (1620 mOsmol/kg) addition of CPS. Aliquots (5 mL) were placed in 5-mL macrotubes (Minitube of America, Inc., Madison, Wisconsin, USA) and equilibrated for 20 min at 21°C. After equilibration, the macrotubes were frozen in a controlled-rate freezer.

Freezing

Larvae were frozen in a controller-rate freezer (Kryo 10 series II, Planer products, Sunbury-on-Thames, United Kingdom). The initial temperature was 15 °C, the samples were cooled at a rate of –2.5 °C per min until reaching a final temperature of –30 °C which was held for 5 min. Straws were plunged into liquid nitrogen and held for one week before transport to the hatchery.

Thawing

Frozen material was transported in shipping dewars (Taylor Wharton, model CP-65, Theodore, Alabama, USA) to the oyster hatchery at Grand Isle. Straws were thawed in a water bath at 70°C for 15 sec. After thawing, the larvae were placed in 1-L plastic beakers containing 300 mL of filtered sea water (1-μ mesh) and were collected on a 30-μ screen before suspension in fresh sea water.

Hatchery experiments

The experiments were performed in two consecutive years starting on September 18, 1996, and September 20, 1997. In the first year, the experiment was terminated after 10 d due to a hurricane and flooding on Grand Isle. In the second year, thawed larvae were incubated in a 200-L tank for 10 d. For control treatments, 6×10^6 eggs were fertilized and incubated in the same conditions as the thawed larvae. During incubation, larvae were fed daily with 20 L of the marine algae Isochrysis galbana and were treated as a normal hatchery brood. Size and number were estimated every 2 d for larvae collected using appropriately sized mesh screens (Table 1). After 8 d of incubation, metamorphosis began. A foot developed indicating the change from veliger to pediveliger stages (Figure 1d). At this time, larvae were collected by screening daily on 210-μ mesh for 8 d and were transferred to a system containing cleaned shells of the common clam Rangia cuneata used as cultch material. After setting, spat were secured in plastic mesh bags and suspended in Caminada Bay to evaluate survival and growth.

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 growth of oysters after settling. On the other hand, we needed to ensure that no extraneous natural larvae contaminated the experiment before settling, and that natural spawn did not influence our results during growth.

Before metamorphosis and settlement of larval oysters, we used several approaches to exclude contamination by extraneous larvae. We chose to work during late September which is at the end of the spawning season in Louisiana, thereby reducing the numbers of natural larvae in the waters near the hatchery. All oyster and algae tanks were segregated from sources of natural water and other sources of contamination. All equipment and mesh screens were used exclusively for this experiment and were washed with filtered water before and after use. All water used for rearing of oyster larvae and for culture of algae was filtered repeatedly through mesh sizes down to 1 μ to prevent contamination. The embryo and larvae in the experiments were collected on appropriate-sized mesh screens (Table 1) every 2 d to perform water exchanges and counts. This eliminated contamination of experimental larvae by earlier (smaller) stages of natural larvae. Experimental larvae were counted at each handling, allowing us to note any increases in numbers due to contamination (Table 3). The larvae were also monitored to ensure that the populations were of the correct developmental stage. As a final check of the larvae, we performed counts of eyed pediveligers as they were collected to be moved to the tanks containing cultch. The number collected for setting was compared with the larval census at that developmental stage.

After setting, spat were placed in mesh bags and suspended in the waters of Caminada Bay for grow-out. Because some amount of natural spat settlement was unavoidable, we suspended two additional mesh bags containing clean cultch among the experimental bags to estimate the occurrence of natural spat fall. The oysters were inspected at 2 weeks and at 2-month intervals thereafter. Relative size was used to differentiate between experimental and wild spat.

RESULTS

Year 1

After 24 h of incubation 10% of the control larvae had developed to D-stage (Figure 1b), whereas 2% of the thawed larvae had developed to this stage. Subsequently, survival of the control larvae decreased rapidly. After 10 d control larvae were not found, while 1,000 pediveligers produced from thawed larvae were counted (Table 3). The larvae were fixed in 10% formalin at this stage when the experiment was terminated.

Year 2

After 24 h of incubation, 28% of the control larvae had developed to D-stage, while 24% of thawed larvae had developed to this stage. Larval numbers decreased throughout development, indicating absence of contamination with wild larvae. A total of 500,000 control larvae (>225 μ) and 28,000 thawed larvae were placed in the cultch system. After 2 weeks of suspension in Caminada Bay, a subsample of 80 Rangia 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, and none from clean shells available for wild spat (Figure 1e). After 4 months of suspension in the Bay, a total of 1,000 seed oysters (2.5 to 5 cm) were counted from the control group and 850 were counted from the thawed larvae (Table 3, Figure 1f). 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). This was the equivalent of about 30 wild spat per treatment (bag). The wild spat were less than 0.5 to 1 cm in length after 4 months of suspension in the Bay, while the experimental spat (from control and thawed larvae) were more than 2.5 cm at this time, allowing identification of naturally produced spat. No natural spat were observed on the cultch of the experimental oysters. This may have been due to a variety of reasons including competition, parasites, overgrowth by experimental (older) spat or biofouling.

Table 3. Number of oysters produced from control and thawed larvae of C. virginica during 2 years of research.

1996				1997			
Date	Control larvae	Thawed larvae	Survival (% control)	Date	Control larvae	Thawed larvae	Survival (% control)
9/18	3.0×10^6	3.0×10^6	100 %	9/20	6.0×10^6	3.0×10^6	50 %
9/20	3.1×10^5	5.0×10^3	2 %	9/22	1.7×10^5	4.0×10^5	24 %
9/22	3.8×10^4	3.7×10^3	9 %	9/24	1.0×10^6	2.0×10^5	20 %
9/24	6.0×10^3	2.0×10^3	33 %	9/26	1.0×10^6	5.3×10^4	5 %
9/26	4.7×10^3	2.0×10^3	43 %	9/28	5.7×10^5	2.8×10^4	5 %
9/30*	0	1.0×10^3		Set on cultch			
				10/97**	1.0×10^3	2.0×10^2	20 %
				1/98***	1.0×10^3	8.5×10^2	85 %

*Experiment terminated due to hurricane.

**Number of spat counted on a subsample of 80 Rangia (cultch) shell. No natural spat fall was observed on any cultch samples on this date.

***Total number of seed oysters counted for each treatment. Natural spat fall was observed only on clean cultch (not in control or thawed treatments). Oysters from wild spat fall were visibly smaller than the experimental oysters.

All of the cultch material came from the same source, although the clean cultch had spent no time in water prior to suspension in the Bay. 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.

DISCUSSION

In our study, we found that it was possible to set and grow normal seed oysters produced from thawed trochophore larvae of C. virginica. It has been reported that propylene glycol is a suitable cryoprotectant for embryos of C. gigas (5), although other reports suggest that DMSO is less toxic for embryos at eight different larval stages for this species (8). In our study, we found that 15% propylene glycol was a successful cryoprotectant for trochophore larvae of C. virginica. The use of 5-ml macrotubes (instead of standard 0.5-mL French straws) increased the numbers of larvae available for use, and suggests that containers of larger volume should be evaluated for purposes of scaling up production for hatchery application.

Often experiments in the laboratory do not yield the same results as those performed in the hatchery or natural environment. For aquacultural purposes, the growth of larvae for a few hours or days after thawing does not ensure survival through settlement or production of adult oysters. Genetic or physiological damage could result in production of abnormal larvae, excessive larval mortality, or the inability to complete metamorphosis and attach to cultch.

In this study, we made no effort to maximize survival of larvae, using only standard techniques in a working hatchery. Survival of thawed larvae through setting, although apparently low, was not different from the control group, and was not different than normal survival in the hatchery. Extremely large numbers of larvae (in the billions) are routinely used to produce seedstock at the commercial level. This high mortality of oyster larvae has been recognized as a constraint in genetic studies (segregation distortion) in which Mendelian ratios can be obscured by random survival of few individuals in specific crosses. With additional research, cryopreservation could be scaled up to allow production of commercially relevant numbers of larvae.

This is the first report of successful production of seedstock from cryopreserved larvae of any species of aquatic organism commercially grown for human consumption. This is not insignificant. More than 40 yr of research has yet to yield successful cryopreservation of fish embryos (7). More than 20 yr of cryopreservation research had not yielded a report of a single oyster completing metamorphosis and setting. The production in this report of seed oysters capable of being planted on oyster grounds for growth and harvest documents that cryopreservation can be applied for use in the hatchery. This is especially important for oysters because presently 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 spat fall. The availability of frozen larvae would ensure protection of valuable stocks and would facilitate transport and availability of improved (e.g. disease-resistant) lines. Cryopreservation offers obvious benefits for managing genetic resources of imperiled species (17), and the techniques for cryopreservation of larvae may be applicable to highly endangered species 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 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. For example, we take advantage of cryopreservation for use in genetic mapping studies in our laboratory (unpublished data). Future studies should evaluate the complete life cycle of oysters produced from cryopreserved larvae, and address optimization of production.

Acknowledgements
Supported in part by grants from the Louisiana Sea Grant College Program and the United States Department of Agriculture. We thank R. Becker, R. Cooper, R. Romaire, and J. La Peyre for manuscript review and discussion. We thank J. Arias, G. Yu, and M. Segovia for technical assistance. Approved for publication by the Director of the Louisiana Agricultural Experiment Station as manuscript number 98-22-0141.

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