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NONLETHAL SPERM COLLECTION AND CRYOPRESERVATION IN THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*

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ABSTRACT Cryopreservation can preserve genetic materials in perpetuity and can be applied to oyster culture for breeding programs. Protocols exist for sperm cryopreservation in the eastern oyster *Crassostrea virginica*, but nonlethal sample collection is needed for valuable individuals, such as tetraploids, or specific lines. The goal of this study was to develop nonlethal methods for sperm collection in the eastern oyster. The objectives were (1) to evaluate natural spawning as a collection method, (2) to evaluate anesthesia methods to induce shell opening for biopsy, (3) to evaluate mechanical notching for biopsy, and (4) to verify notching combined with biopsy for collection and cryopreservation. Five males (of 60 oysters) spawned naturally after 7 h, with an average sperm concentration of $1.9 \pm 1.0 \times 10^3$ cells/mL (in 2 L seawater). No oysters ($n = 30$) responded by opening during 36 h of treatment with 5% Dead Sea salt (containing 33.3% $MgCl_2$), and 22 oysters (of 30) opened during the 36-h treatment with 5% Epson salt ($MgSO_4$). Sperm collected by biopsy had fresh motility of 3%–80% and postthaw motility of 1%–5%; sperm production was 4.5×10^5 to 2.3×10^8 cells per male. Mechanical notching did not cause mortality to oysters ($n = 20$). After notching and biopsy with 18-G and 20-G needles, survival was 80% (16 of 20 for each). Sperm production was 5.42×10^7 cells by 18-G needle ($n = 8$) with fresh motility of $16 \pm 12\%$ and postthaw motility of $3 \pm 2\%$, and 1.35×10^8 cells by 20-G needle ($n = 9$) with fresh motility of $21 \pm 20\%$ and postthaw motility of $5 \pm 4\%$. No differences were observed between samples biopsied with the 2 needle sizes ($P \geq 0.074$). To verify notching and biopsy for nonlethal sperm collection, a total of 39 oysters were sampled to obtain 20 males, which averaged 99.48 ± 23.17 g total weight, 74.1 ± 6.0 mm shell height, and 60.9 ± 7.2 mm shell length. The sperm production was $3.6 \pm 2.1 \times 10^8$ cells per male. Biopsied sperm showed $23 \pm 12\%$ fresh motility, $13 \pm 6\%$ postequilibration motility (after equilibration with 10% of DMSO for 30–60 min before freezing), and $6 \pm 4\%$ postthaw motility. Flow cytometry analysis indicated an average of $84 \pm 4\%$ of cells with intact plasma membranes for fresh sperm, and $59 \pm 9\%$ for postthaw sperm. Fertilization by thawed sperm averaged $20 \pm 22\%$ (from 1%–87%). No significant differences were observed between the biopsied samples and the dissected samples (lethal collection) for fresh motility ($P = 0.550$), postequilibration motility ($P = 1.000$), postthaw motility ($P = 0.101$), fresh membrane integrity ($P = 1.000$), or postthaw membrane integrity ($P = 1.000$), but a difference was observed in fertilization ($P = 0.039$; biopsied samples, 20 ± 22%; dissected samples, 68 ± 40%). Overall, this study developed notching combined with biopsy for sperm collection and cryopreservation in Eastern oysters that can be applied to valuable individuals and breeding programs.

KEY WORDS: eastern oyster, nonlethal collection, sperm cryopreservation, *Crassostrea virginica*

INTRODUCTION

The eastern oyster *Crassostrea virginica* industry in the United States has been affected greatly by challenges such as oyster disease, coastal erosion, and environmental pollution (i.e., the Deepwater Horizon oil spill in 2010). It is becoming increasingly important that research efforts focus on improving and sustaining this industry. Currently, molecular techniques and cytogenetic biotechnologies in shellfish are fairly well developed (Liu & Cordes 2004, Reece et al. 2004, Guo et al. 2008). For the eastern oyster, a basic linkage map has been established (Yu & Guo 2003) and quantitative trait loci disease resistance has been identified (Yu & Guo 2006). With these molecular techniques, genetic analyses performed in different populations showed that strains subjected to long-term selection for disease resistance shared frequency shifts at only a few loci (Yu & Guo 2004), indicating that these markers were linked to disease-resistant genes and could be used for selective breeding programs. In addition, artificial selection for 4 generations based on survival, growth, and disease susceptibility has produced a strain with dual resistance to the parasites *Haplosporidium nelsoni* (i.e., MSX) and *Perkinsus marinus* (i.e., Dermo) (Ragone-Calvo et al. 2003), with higher growth and survival rates and low susceptibility to infection. In Louisiana, a regional Dermo–resistant line is being evaluated through field and laboratory challenges, and is currently in the fourth generation of production. These breeding effects and technologies are promising developments for the oyster industry (Guo 2004).

Cryopreservation is a technique that allows preservation of genetic materials in perpetuity (Fuller et al. 2004). It can be applied to triploid–tetraploid oyster programs to preserve sperm or larvae of valuable tetraploid oysters, and can also be applied to selective breeding programs for preserving specific lines or species. In addition, sperm cryopreservation can extend the commercialization of triploid–tetraploid technology in the oyster industry by sale of frozen sperm from tetraploids, providing cost savings for maintaining tetraploids, and protecting intellectual property. Sperm cryopreservation in Eastern oysters was first studied during the early 1970s (Hughes 1973), and 2 more studies followed (Zell et al. 1979, Paniagua-Chavez & Tiersch 2001). The cryoprotectants used were dimethylsulfoxide (5% or 10%) or propylene glycol (10% or 15%). The extenders were artificial seawater or calcium-free Hanks' balanced salt solution (HBSS) (Hanks 1975). The cooling rates used were 1–5.5°C/min, and the containers were 2-mL ampules or plastic straws (0.25 mL or 5 mL). The results varied from 2%–91%

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fertilization after thawing and were all based on laboratory-scale projects. We developed a reliable protocol for sperm cryopreservation of Eastern oysters with the potential for high-throughput application (such as for large-scale breeding programs) by systematic evaluation of cryoprotectants, cooling rates, and thawing temperatures suitable for adoption with automated processing equipment. Fertilization by thawed sperm from 16 individual males was 58 ± 24% for 0.5-mL French straws and 54 ± 21% for 0.5-mL CBS straws (Yang et al. 2012).

Previous studies of oyster cryopreservation were based on the ready availability of ripe broodstock for lethal collection of gametes. For valuable tetraploid oysters or specific valuable lines, nonlethal sample collection methods need to be developed and linked with the development of highly efficient cryopreservation techniques that would not require killing of the animals and would potentially save sperm through increased efficiency during fertilization. Specifically, nonlethal sperm collection in Eastern oysters can be applied for the following purposes. First, it can be applied for the establishment of tetraploid populations. Tetraploid production usually begins with a few individuals from single-cross families (Eudeline et al. 2000). Therefore, with these techniques, the few valuable tetraploid individuals produced each year can be preserved and used for breeding until a sufficient-size population (e.g., tens to hundreds) is assembled. This population can be used in a breeding program to develop reliable broodstock lines and larger broodstock populations. Second, it is a highly efficient use of valuable animals. Nonlethal sample collection can allow valuable individuals such as tetraploids to be used repeatedly for breeding, and potentially more sperm could be obtained without killing the oysters if repeated collection occurs. Third, it helps establish self-fertilized lines. Such lines have high levels of inbreeding and can enhance dramatically the progress for genome mapping. The eastern oyster is a protandrous species, beginning life as a male and changing its sex to female as the oyster ages (Galtsoff 1964). This characteristic allows the fertilization by cryopreserved sperm of eggs from the same oyster after sex change. Also, this approach can be applied to tetraploids for developing necessary founder populations, including the worst-case scenario, requiring the use of self-fertilization to propagate lines.

The goal of this study was to develop nonlethal methods for sperm collection and cryopreservation in the eastern oyster. The objectives were (1) to evaluate natural spawning as a collection method, (2) to evaluate anesthesia methods to induce shell opening for biopsy, 3) to evaluate mechanical notching for biopsy, and (4) to verify notching combined with biopsy for collection and cryopreservation.

MATERIALS AND METHODS

Oysters

Male oysters used in this study were originally collected from Bay Courant, LA, in January 2010 and held in an adjustable longline system (see bstoyster.com) at the Sea Grant Oyster Research and Demonstration Farm at Grand Isle, LA. Females used for artificial fertilization trials were from Cape Shore, NJ. The experiments for sperm collection were performed from April 23 to May 25, 2010, at the Aquaculture Research Station of the Louisiana State University Agricultural Center. The oysters at the Grand Isle farm were transported to Baton Rouge, LA, and were used directly for the sperm collection experiments after cleaning with fresh water. Artificial fertilization trials of frozen sperm were performed in June 11, 2010, at the Haskin Shellfish Research Laboratory of Rutgers University in New Jersey by overnight shipping of frozen samples in a shipping dewar (CX-100 Theodore; Taylor-Wharton, AL).

Motility Analysis

Sperm motility was assessed by computer-assisted sperm analysis (CASA system, CEROS model; Hamilton Thorne, Inc., Beverly, MA) with 20-μm, 2-cell slides (Hamilton Thorne, Inc.). The parameter settings used for CASA were as follows: minimum contrast, 30; minimum cell size, 3 pixels; number of frames for recording, 30; average path (VAP) cutoff, 20 μ/sec; straight line (VSL) cutoff, 10 μ/sec; static intensity gate, 0.15–2.82; static size gate, 0.72–3.29; elongation gate, 56–99; and slow cell, defined as static. For each sample, at least 3 CASA measurements of different fields were performed, and the average motility was calculated for that sample.

Evaluation of Membrane Integrity by Flow Cytometry

Sperm membrane integrity was measured with the fluorescent dyes SYBR-14 and propidium iodide (PI) (live/dead sperm viability kit; Molecular Probes). Sperm samples (250 μL) at a concentration of 1×10^6 cells/mL were stained with 100 nM SYBR-14 and 12 μM PI for 10 min, and analyzed using an Accuri C6 flow cytometer (BD bioscience) with a 10-μL sample size at the medium flow rate setting (35 μL/min). Membrane integrity was determined as the percentage of sperm stained with SYBR-14 (intact sperm) of the total number of sperm cells (stained with SYBR-14 and PI) by use of CFlow software (version 1.0.202.1; BD Bioscience).

Cryopreservation of Sperm

Sperm samples were suspended in Ca^{2+}-free HBSS at an osmolality of 650 mOsmol/kg (Ca-free HBSS650), and filtered through a 20-μm screen to remove large pieces of tissue. Calcium-free HBSS650 was prepared by adjusting the water volume for the standard 1-L recipe of HBSS to 450 mL without the addition of $CaCl_2$ (0.137 M NaCl, 5.4 mM KCl, 1.0 mM $MgSO_4$, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, and 5.55 mM glucose, with a pH of 7.8) (Hanks 1975). The osmolality was confirmed by measurement with a vapor pressure osmometer (model 5520; Wescor, Inc., Logan, UT). Sperm concentration was determined by measuring absorbance with a microspectrophotometer (NanoDrop, Wilmington, DE) (Yang et al. 2012). Sperm cryopreservation was performed by following the protocol developed previously in our laboratory (Yang et al. 2012). After adjustment of the sperm concentration to 2×10^8 cells/mL, sperm samples were mixed with an equal volume of 20% dimethylsulfoxide in Ca-free HBSS650, packaged into 0.5-mL French straws, and frozen at a cooling rate of 20–25°C/min (sample probe temperature) from 5°C to –80°C within 20 min after mixing with cryoprotectant (equilibration time). After reaching –80°C, straws were plunged into liquid nitrogen in goblets for storage in dewars at the Louisiana State University Agricultural Center.

Experiment I: Natural Spawning of Oysters for Sperm Collection

Oysters from the Grand Isle farm were transferred on ice (without direct contact with ice or melt water) in a cooler to Baton Rouge, LA (3.5 h, driving), were cleaned, and were held at 4°C overnight. The next morning, the oysters were placed individually into 2 L seawater (20 ppt salinity) at 25°C with aeration and a mix of 4 marine microalgae (*Isochrysis, Pavlova, Tetraselmis, Thalassiosira weissflogii*; Shellfish Diet 1800, Reed Mariculture, Inc., Campbell, CA). Gamete release was monitored each hour. If no gametes were released after 4 h, the water was changed and monitoring was continued for another 3 h, and experiment was ended if no spawning occurred. A total of 60 oysters were tested on 3 different dates. For each oyster, shell height, shell length, shell width, and total weight (body plus shell weight) were measured.

Experiment II: Anesthetizing Oysters for Biopsy of Sperm Samples

Two types of salts were used in this experiment to anesthetize oysters to induce shell opening: Ultra Epson ($MgSO_4$) and Bokek Dead Sea salt (containing 33.3% $MgCl_2$, 24.3% KCl, 5.5% NaCl, 0.2% $CaCl_2$, 0.5% Br^-, 0.15% sulfates, and 36.4% water of crystallization) purchased from SaltWorks, Inc. (Woodinville, WA). The salts were dissolved in 15 ppt seawater at a concentration of 5% (w/v) in a 25-L tank (1 oyster/L) with the addition of aeration and algae (5 mL concentrated food). The treatment was started at 22:30 HR, and oysters were observed after 8 h, and monitored every 2–3 h for 36 h total. The opened oysters were biopsied after 12 h and 36 h with an 18-G needle and a 1-mL syringe. Sperm collected from males were cryopreserved. After the treatments, oysters were returned to a 20-ppt seawater recirculating system after measurement of shell height, shell length, shell width, and total weight. A total of 30 oysters were treated in 1 tank with each salt.

Experiment III: Notching Oysters for Biopsy of Gamete Samples

Oysters were notched on the middle of the dorsal side by use of an angle grinder (Chicago Electric power tools, model 91223) with a standard metal 4.5-inch grinding wheel after measurement of total weight, shell height, shell length, and shell width. The notched oysters were treated in 3 ways: (1) returned to the holding system for culture, (2) sampled for gonadal biopsy with a 20-G needle, or (3) gonadal biopsy with an 18-G needle. Biopsies were acquired by syringe 3 times. The biopsied oysters were labeled individually and returned to the recovery holding system. Twenty oysters were used for each treatment.

Biopsied gonad samples were collected into 1.5-mL centrifuge tubes, and sex was identified by viewing the gametes with a bright-field microscope at a magnification of 40× (for eggs) or 100× (for sperm). Sperm samples were processed and cryopreserved as described earlier. To estimate the postthaw motility, frozen samples in French straws were thawed at 40°C in a water bath for 7–8 sec, and were released into a 1.5-mL centrifugation tube on ice. Postthaw motility was estimated with the CASA system (Yang et al. 2012) within 30 min.

Experiment IV: Evaluation of Motility and Fertility of Sperm Collected by Notching Biopsy

To evaluate the sperm obtained by nonlethal collection and cryopreservation, 20 male oysters were notched, and biopsied with an 18-G needle after measurement of total weight, shell height, and shell length. For comparison, sperm samples were collected by dissection (lethal method) from 3 males selected from 10 oysters after opening, and were processed in parallel with the nonlethal biopsied samples for motility estimation, cryopreservation, and fertility testing. Cryopreservation was performed by using the protocol described earlier.

Fertility testing of thawed sperm was performed at Rutgers University with frozen samples shipped overnight from Louisiana in a commercially available shipping dewar. Eggs were collected by dissection of six 1-y-old females with an average total weight of 8.18 ± 1.07 g, shell height of 42.0 ± 1.5 mm, and shell length of 30.5 ± 2.1 mm. Gonads were minced individually with a scalpel into 1-μm filtered seawater at a salinity of 20 ppt and allowed to hydrate for 1 h. The egg suspensions were filtered through a 200-μm screen to remove gonadal debris, and were collected and washed on a 20-μm screen using filtered seawater. Eggs from individual females were combined after viewing with a microscope (40× magnification) to ensure normal shape (i.e., rounded, oval, or pear shaped) and that they were free of sperm contamination. Egg concentration was determined by counting the number of eggs in a 1-mL sample with a Sedgewick-Rafter counting cell (S50; Pyser-sgi Ltd., Kent, UK). The eggs were aliquoted into 40-mL samples in 1-L beakers, which were used for fertilization within 1 h.

Frozen samples were thawed at 40°C in a water bath for 7–8 sec, and the sperm suspensions were released into 1.5-mL centrifuge tubes on ice for motility estimation and fertilization. For fertilization, thawed sperm (a pool of samples from 4 straws) were mixed with 40 mL egg suspension (yielding a concentration of ~10,000 eggs/mL) in a 1-L beaker at room temperature (23°C, yielding a sperm-to-egg ratio of ~500 sperm per egg). Additional seawater was added to produce a volume of 200 mL after 30 min, and to a 1-L final volume after 1 h. Embryos at the 4-cell stage or beyond were counted as fertilized eggs, and the percentage based on total eggs (of more than 100 eggs) was recorded as percentage fertilization. Two counts were performed for each fertilization group. To evaluate egg quality, 2 aliquots from the same suspension used for testing of cryopreserved sperm were fertilized with 2 mL fresh sperm (equal to volume of thawed sperm from 4 straws) at a similar sperm concentration (estimated by dilution ratio of testis weight in HBSS650) obtained from 2 male oysters from the same population as the females.

Data Analysis

The data collected in this project were analyzed with statistical software (SYSTAT 13 Software, Chicago, IL). Percentage data were arcsine square root transformed before data analysis. The significance level was set at $P < 0.05$.

RESULTS

Experiment I: Natural Spawning Trials

Only 1 of the 3 trials yielded natural spawning. In this trial, 5 oysters (of 20) spawned after 7 h (from 08:00–15:00 HR for oysters in seawater; Table 1). The sperm concentration of the 5 spawned oysters was $1.9 \pm 1.0 \times 10^3$ cells/mL (range, 0.7–3.2 × 10^3 cells/mL). Because of the low concentration, sperm cryopreservation was not performed for these samples.

TABLE 1.

Three trials of natural spawning of Eastern oysters *Crassostrea virginica* were evaluated by placing each oyster individually in 2 L of 20 ppt seawater (25°C) after being held dry overnight at 4°C.

Date	Oysters (*n*)	Total weight (g)	Shell height (mm)	Shell width (mm)	Oysters spawned (*n*)	Sperm concentration (cells/mL)
4/23/2010	20	104.2 ± 23.0	74.6 ± 5.8	63.4 ± 5.0	0	NA
5/3/2010	20	83.7 ± 17.6	69.9 ± 5.5	52.1 ± 6.7	5	$(1.9 \pm 1.0) \times 10^3$
5/4/2010	20	114.5 ± 29.3	70.0 ± 6.6	59.0 ± 4.3	0	NA

NA, not available.

Experiment II: Anesthesia of Oysters by Dead Sea Salt and Epson Salt

The oysters treated by the 2 types of salts came from the same population and had the same total weights ($P = 0.064$), shell height ($P = 0.542$), and shell length ($P = 0.052$). For the treatment with Dead Sea salt, none of the 30 oysters opened during the 36-h treatment. At 12 h after treatment with Epson salt, 12 of the 30 oysters opened and were biopsied: 5 males and 7 females (Table 2). At 36 h, 10 more oysters opened and were biopsied—4 males and 6 females—and the remaining 8 oysters (of 30) did not open their shells. All oysters exposed to anesthesia treatment without biopsy survived, and all oysters after anesthesia and biopsy at 12 h lived for 2 wk, but 2 oysters (of 10) died after anesthesia and biopsy at 36 h (at day 1 and day 7). There were no significant differences in total weight ($P = 0.299$), shell height ($P = 0.271$), or shell length ($P = 0.383$) between the oysters that opened and those that did not.

For the sperm samples biopsied after anesthesia, sperm motility ranged from 3%–75% (Table 3). The total sperm production was also different among individuals, ranging from 4.5×10^5 to 2.3×10^8 cells. Postthaw motility ranged from 1%–5%.

Experiment III: Effect of Notching and Gonad Biopsy on Oyster Survival

The oysters that were notched only, and those notched with biopsy by 18-G or 20-G needles came from the same batch, therefore there were no differences in total weight, shell height, or shell length ($P \geq 0.127$; Table 4). For the 20 oysters biopsied with 20-G needles, 9 were males. For the 20 oysters biopsied with 18-Ga needles, 8 were males. No differences were observed between females and males in total weight, shell height, or shell length ($P \geq 0.107$). All oysters survived notching at day 7 and day 30. After notching and biopsy, however, only 80% (16 of 20) survived at day 7.

For biopsied samples, sperm production with 20-G needles averaged 1.35×10^8 cells, and with 18-G needles was 5.42×10^7 cells (Table 5). The motility of fresh sperm biopsied with 20-G needles was 21 ± 20% (range, 2%–55%), and postthaw motility was 5 ± 4% (range, 1%–10%). For the samples biopsied with 18-G needles, fresh motility was 16 ± 12% (range, 2%–35%), and postthaw motility was 3 ± 2% (range, 1%–8%). No differences were observed in sperm production, fresh motility, or postthaw motility between samples biopsied with different-size needles ($P \geq 0.074$).

Experiment IV: Evaluation of Motility and Fertility of Sperm Collected by Notching and Biopsy

A total of 39 oysters were processed to ensure sampling of 20 males for this experiment (the other 19 oysters were females). The 20 males used for biopsy of sperm samples averaged 99.5 ± 23.2 g in total weight, 74.1 ± 6.0 mm in shell height, and 60.9 ± 7.2 mm in shell length. The sperm production from these males was $3.6 \pm 2.1 \times 10^8$ cells (Table 6). For the 3 males dissected, the total sperm production was $1.10 \pm 0.67 \times 10^{12}$ cells with the whole gonad dissected and minced, which was significantly higher than that by biopsy collection ($P = 0.000$), whereas there were no differences in total weight (121.1 ± 13.9 g) and body size (shell height, 73.9 ± 5.8 mm; shell length, 53.3 ± 4.5 mm; $P \geq 0.323$).

Fresh motility of biopsied sperm varied with an average of 23 ± 12% (range, 8%–44%); after equilibration with 10% DMSO for 30–60 min (before freezing), the postequilibration motility was 13 ± 6% (range, 1%–25%). After cryopreservation, the postthaw motility was 6 ± 4% (range, 1%–15%). Membrane integrity averaged 84 ± 4% (from 73%–93%) for fresh sperm, and 59 ± 9% (range, 41%–68%) for postthaw sperm. No significant male-to-male variation in membrane integrity was observed compared with that in the motility analysis ($P = 0.000$, variance analysis). Fertilization by thawed sperm averaged 20 ± 22% (range, 1%–87%), whereas aliquots of the same eggs had 80 ± 1% fertilization with fresh sperm (a control for evaluation

TABLE 2.

Anesthesia trials of Eastern oysters by using 2 types of salts (Ultra Epson and Bokek Dead Sea Salt; SaltWorks, Inc., Woodinville, WA) at a concentration of 5% in 15 ppt seawater (22°C).

Salt	Oysters (*n*)	Total weight (g)	Shell height (mm)	Shell width (mm)	Oysters (of 20) with opened shells after anesthesia exposure time (*n*)							
					8 h	9 h	10 h	12 h	14 h	17 h	19 h	36 h
Ultra Epson	30	79.6 ± 15.3	66.2 ± 8.8	49.4 ± 6.6	4	2	3	3	2	1	1	6
DEAD Sea Salt	30	89.0 ± 27.0	67.5 ± 7.0	52.6 ± 5.8	0	0	0	0	0	0	0	0

TABLE 3.

Sperm production from male Eastern oysters sampled with 18-G needle after anesthesia with 5% Ultra Epson ($MgCl_2$; SaltWorks, Inc., Woodinville, WA) on May 20, 2010.

Oyster no.	Biopsy time (h)	Sperm motility (%)	Concentration ($\times 10^7$ cells/mL)	Sperm volume collected (μL)	Sperm production (cells)	Postthaw motility (%)
3	12	55	7.00	950	6.65×10^7	1
6		15	0.10	450	4.50×10^5	NA
8		3	2.80	1,050	2.94×10^7	2
10		75	6.20	1,000	6.20×10^7	1
12		33	7.30	345	2.52×10^7	1
15		9	52.00	450	2.34×10^8	1
16	36	35	8.60	270	2.32×10^7	1
19		37	8.20	85	6.97×10^6	1
22		45	8.30	1,000	8.30×10^7	5

Biopsies were acquired at 12 h and 36 h after anesthesia. NA, not available; this sample was not cryopreserved because of the low sperm concentration.

of egg quality). Parallel analysis of dissected sperm samples did not show significant differences from biopsy samples in fresh motility ($P = 0.550$), postequilibration motility ($P = 1.000$), postthaw motility ($P = 0.101$), fresh membrane integrity ($P = 1.000$), or postthaw membrane integrity ($P = 1.000$). A significant difference was observed in fertility testing between dissected samples ($68 \pm 40\%$) and biopsied samples ($20 \pm 22\%$; $P = 0.039$). Overall, correlation analysis did not show relationships among sperm production, fresh motility, postequilibration motility, postthaw motility, fresh membrane integrity, postthaw membrane integrity, or fertility ($P \geq 0.278$).

DISCUSSION

The Limitations of Natural Spawning for Nonlethal Sperm Collection and Cryopreservation

Like most marine bivalve species, oysters have external fertilization (Kennedy et al. 1996). Gametes are released into natural seawater and fertilization occurs within several hours. Spawning usually takes place communally for 2–4 wk in the summer, and can be triggered by environmental factors such as water temperature, tides, or the presence of sperm in the water. In this study, the procedure for inducing spawning was to keep oysters out of water at 4°C overnight, followed by an increase in water temperature and addition of algae. This was adopted from routine oyster hatchery methods (Wang & Wang 2008), except that the oysters were placed individually.

During cryopreservation, sperm samples need to be maintained at a suitable volume and concentration for efficiency of sample handling and storage. Although oysters are especially fecund, and a single male can produce billions of sperm (Galtsoff 1964), the practical concentration for sperm suspensions is usually low ($<10^6$ cells/mL), because the oysters need to be in a certain volume (usually liters) to spawn. In this study, the sperm concentration obtained from natural spawning in 2 L of seawater was 1.9×10^3 cells/mL. Thus, natural spawning has limitations as a choice for nonlethal sperm collection because of the uncertainty of spawning and low sperm concentration obtained. However, it need not be completely excluded, especially when sexual maturity is guaranteed, and because oysters have high survival after natural spawning.

Opening of Oysters by Anesthesia or Mechanical Notching

Oysters are naturally distributed in the intertidal zone, which is characterized by harsh environmental extremes such as fluctuating temperatures and strong wave action. Therefore, oysters have physical and physiological adaptations to this environment, and have developed a strong adductor muscle to close their shells tightly to tolerate environmental conditions and predators (Kennedy et al. 1996). Therefore, anesthesia and notching

TABLE 4.

Sex composition, total weight, shell size, and survival of Eastern oysters after notching followed by gonadal biopsy with 20-G and 18-G needles.

Treatment	Oysters (*n*)	Sex (*n*)	Total weight (g)	Shell height (mm)	Shell length (mm)	Survival of oysters	
						Day 3	Day 7
Notch only	20	NA	98.5 ± 14.3	76.1 ± 7.3	59.7 ± 5.5	20/20	20/20
Notch, 20-G needle	20	Female (11)	105.1 ± 19.6	76.8 ± 7.7	61.1 ± 6.7		
		Male (9)	95.2 ± 13.8	71.9 ± 4.4	58.0 ± 2.9	20/20	16/20
Notch, 18-G needle	20	Female (12)	92.2 ± 23.0	70.4 ± 6.2	58.3 ± 6.6		
		Male (8)	90.5 ± 28.1	74.0 ± 11.7	58.0 ± 2.9	20/20	16/20

NA, not available because no biopsy was performed on these oysters.

TABLE 5.

Total weight, shell height, shell length, sperm production, fresh sperm motility, and fresh and postthaw sperm motility of Eastern oysters after mechanical notching followed by gonadal biopsy with 20-G and 18-G needles for sperm collection and cryopreservation.

Biopsy method	Male no.	Total weight (g)	Shell height (mm)	Shell length (mm)	Sperm production (cells)	Fresh sperm motility (%)	Postthaw sperm motility (%)
Notch, biopsy with 20-G needle	1	69.0	67.2	57.2	3.07×10^7	15	8
	2	93.2	69.0	60.3	5.03×10^7	20	5
	3	110.1	71.5	63.8	9.82×10^7	55	10
	5	101.0	73.7	56.8	7.25×10^7	10	5
	15	110.0	80.5	59.9	1.07×10^8	55	10
	16	85.8	65.6	57.0	2.00×10^8	10	5
	17	85.3	72.2	56.9	1.34×10^8	5	1
	18	108.1	73.3	54.2	2.48×10^8	15	2
	20	94.5	74.1	55.4	2.75×10^8	2	1
	Mean	95.2	71.9	57.9	1.35×10^8	21	5
	SD	13.8	4.4	2.9	8.72×10^7	20	4
Notch, biopsy with 18-G needle	22	89.2	84.6	51.5	1.82×10^7	2	2
	25	91.0	71.8	51.5	1.03×10^7	2	1
	27	138.1	77.6	76.1	2.07×10^7	10	2
	28	36.5	49.1	45.7	5.96×10^7	5	2
	30	100.0	76.7	54.8	2.45×10^8	20	8
	31	102.7	83.5	57.8	3.28×10^7	25	3
	34	81.0	81.6	55.8	1.30×10^7	35	1
	37	85.2	67.4	54.5	3.41×10^7	25	1
	Mean	90.5	74.0	56.0	5.42×10^7	16	3
	SD	26.3	10.9	8.4	7.37×10^7	12	2
P value		1.000	1.000	1.000	0.074	1.000	0.482

The *P* values represent differences between needle sizes.

were tested in this study to induce opening of oyster shells for gonad biopsy.

Dead Sea salt (33.3% $MgCl_2$) was chosen as an anesthetic because it has been applied to scallops *Pecten fumatus* (Heasman et al. 1995), Pacific oyster *Crassostrea gigas* (Namba et al. 1995, Suquet et al. 2009), Sydney rock oyster (Butt et al. 2008), and flat oyster *Ostrea edulis* (Suquet et al. 2010) with varied effectiveness from species to species. In this study, however, Dead Sea salt did not induce opening in any oysters within 36 h of treatment. This possibly was because the oyster size (79–89 g, Table 2) in this study was larger than that in previous studies (29.9 g for flat oysters; 9–22 g and 32.1 g for Pacific oysters), and large body size may require higher concentrations of salt. The other anesthetic chosen in this study was Epson salt ($MgSO_4$), which is a traditional bath salt used widely for biomedical anesthesiology (Sirvinskas & Laurinaitis 2002), and has been used for anesthesia of Pacific oysters (Wang et al. 2011) and American (Eastern) oysters (Galtsoff 1964). Compared with $MgCl_2$ (Dead Sea salt), Epsom salt was more effective (22 of 30 oysters opened within 36 h). Overall, anesthesia can induce oysters to open without physical damage, but it requires hours of treatment, and thus sperm samples cannot be collected from multiple males at the same time for the freezing process. In addition, it is unknown whether the salts used for anesthesia have effects on sperm quality. This method offers some potential but will require further study.

Notching can ensure the opening of all oysters, but mechanical notching can damage mantle tissues and also prevents tight closure until the shell reforms. Therefore, notching should be performed by a skilled technician to make sure that the cutting is only deep enough to allow entry of the needle for biopsy. The results in this study indicated that proper notching itself did not affect survival of the oysters. Besides notching, other mechanical approaches have been reported in bivalves for sample biopsy, such as drilling of a hole in the shell or prying apart of the valves in endangered mussels (Svardh 2003). For oysters, these 2 options are not suitable because the oyster shell is much thicker than that of mussels, and the thickness can vary individually.

Sperm Production and Oyster Survival After Gonad Biopsy

Biopsy of gametes for sex determination and assessment of gametogenesis has been accomplished with syringe or forceps in several bivalve species, such as the blacklip pearl oyster *Pinctada margaritifera* (Acosta-Salmon & Southgate 2004), freshwater mussels (*Elliptio dilatata* and *Actinonaias ligamentina*) (Saha & Layzer 2008), Pacific oysters (Suquet et al. 2009) and flat oysters (Suquet et al. 2010). In this study, the purpose of the biopsy was to collect sperm samples for cryopreservation and future artificial fertilization; therefore, relatively large samples needed to be collected.

An effective nonlethal sperm collection method should ensure survival of the oysters and maximization of sperm production. In this study, biopsy performance was defined with 3 punctures of the gonad for each oyster. Two relatively small-size needles (18 G and 20 G) were used, although larger needle sizes (12 G and 14 G) have been suggested for gonad biopsy for gametogenesis analysis (Acosta-Salmon & Southgate 2004). The needle sizes in this study did not yield differences in oyster survival or sperm production. This indicated that the tissue damage caused by biopsy affected survival, but the needle sizes

TABLE 6.

Total weight, shell height, shell length, and sperm collected from individual male Eastern oysters by gonadal biopsy with 18-G needle after notching.

Male no.	Total weight (g)	Shell height (mm)	Shell length (mm)	Sperm production ($\times 10^8$ cells)	Sperm motility (%)			Membrane integrity (%)		Postthaw fertility (%)
					Fresh	Postequilibration	Postthaw	Fresh	Postthaw	
1	113.6	79.2	60.0	1.96	30	18	5	86	60	5
3	95.8	79.7	68.0	1.03	14	6	2	93	68	15
6	141.1	82.1	74.5	5.56	8	1	1	87	59	1
7	69.9	70.6	58.8	1.98	15	15	1	85	59	1
11	92.9	74.1	66.1	4.99	22	9	3	85	66	45
14	113.1	83.3	58.8	3.58	38	14	5	83	68	55
16	90.2	70.1	61.4	2.52	44	17	8	87	59	1
17	110.3	81.6	65.3	3.84	33	5	5	80	61	15
18	66.7	64.7	59.1	4.34	42	10	8	86	66	12
21	88.3	65.2	53.8	5.64	14	9	8	84	67	33
22	91.8	67.2	66.1	2.61	10	8	5	84	68	1
23	115.8	72.5	56.0	1.16	16	16	5	83	56	10
25	85.7	74.4	56.3	4.06	15	11	2	81	51	2
26	94.3	72.0	63.4	0.85	33	25	5	86	66	87
27	145.9	80.5	57.0	2.25	39	18	15	87	62	35
28	129.2	64.5	57.9	6.73	10	8	5	83	41	2
36	70.7	79.7	51.8	9.02	12	19	15	83	55	12
39	78.0	74.3	56.3	3.06	20	19	10	81	46	21
40	119.1	75.7	78.0	4.51	25	22	10	80	65	8
41	77.2	70.5	48.8	1.50	14	10	8	73	41	40
Mean	99.5	74.1	60.9	3.56	23	13	6	84	59	1
SD	23.2	6.0	7.2	2.11	12	6	4	4	9	22
D1	69.0	48.8	133.5	3,440	35	18	10	88	62	21
D2	80.4	57.9	123.7	15,000	40	25	15	85	67	94
D3	72.4	53.3	106.1	15,000	40	22	15	86	65	87
Mean	73.9	53.3	121.1	11,100	38	22	13	86	65	68
SD	5.8	4.5	13.9	6,680	3	4	3	2	2	40

Motility estimation of fresh sperm, postequilibration sperm (after mixing with cryoprotectant for 30–60 min), and postthaw sperm; membrane integrity analysis of fresh sperm and postthaw sperm; and fertility testing of postthaw sperm. Dissected sperm samples (D1–D3, lethal method) from 3 individual oysters were used for parallel comparison.

used (18 G and 20 G) did not make a difference. Sperm collected by biopsy could be affected by the number and location of punctures made for biopsy, but the major factors should be gonad maturity and oyster size. During biopsy, gonad maturity cannot be observed and detected, and opening a small number of oysters from the same batch can provide a sample for evaluation of sexual maturity, such as the 3 dissected controls in this study that were selected from 10 opened oysters.

Male-to-Male Variation of Biopsied Samples for Cryopreservation

In this study, the sperm samples biopsied from 20 oysters showed significant male-to-male differences in motility, membrane integrity, and fertility. This phenomenon was also observed in dissected oyster sperm samples (Yang et al. 2012) and in fish species (Yang et al. 2009), although the reason for this variation is currently uncertain. In this study, the oysters used were obtained directly from a grow-out site at Grand Isle, LA, without indoor conditioning; therefore, the maturity of each individual could vary, and gonad development could not be observed for maturity evaluation. Thus, biopsy as a nonlethal sperm collection method could ensure the certainty of sample collection, but may not guarantee high sperm quality and quantity for every individual sampled. When dealing with extremely valuable samples, conditioning of oysters to accelerate or synchronize sexual maturity might be needed to ensure sperm sample quality and quantity when collections are acquired with biopsy.

Application of Nonlethal Sperm Collection and Cryopreservation to Oyster Breeding Programs

In typical practice, a breeding program encompasses the planned reproduction of groups of animals or plants that usually involves at least several individuals and extends over several generations, and traditionally includes selective breeding, backcrossing, hybridization, or inbred lines. These breeding approaches have been used in crops and livestock for hundreds or even thousands of years and have yielded superior strains and lines for agriculture. For the oyster industry, breeding programs have begun to be established (Guo 2004). Selective breeding based on molecular techniques and cytogenetic biotechnologies are currently being developed by oyster researchers (Ragone-Calvo et al. 2003, Nell & Perkins 2006), and valuable lines or strains have been established (DeBrosse 2011). Genome maps have been constructed for the eastern

oyster to identify disease-related genes and quantitative trait loci (Yu & Guo 2003, Reece et al. 2004, Yu & Guo 2004, Yu & Guo 2006). Triploid–tetraploid technology also offers promise for this industry (Guo et al. 2009). To accelerate the application of these breeding programs and biotechnologies in the oyster industry, sperm cryopreservation with nonlethal sample collection can be applied through different approaches, such as the establishment of self-fertilization inbred lines, preservation of valuable strains (e.g., tetraploids and disease-resistant lines), and preservation of broodstock at each generation for future backcrossing.

CONCLUSIONS

Overall, this study addressed the development of nonlethal methods for sperm collection and cryopreservation in the eastern oyster. Evaluation of currently available techniques revealed that each has strengths and weaknesses. Natural spawning did not provide reliable results and sperm concentration was not sufficient for cryopreservation. Considering time consumption, dependability, sperm production, sperm concentration, and oyster survival after sampling, the combination of notching and gonadal biopsy by syringe would appear to be the best choice for nonlethal sperm collection. This does not mean other approaches such as natural spawning should be excluded completely, especially when dealing with extremely valuable individuals; the use of indoor conditioning of maturity could improve sample quantity and quality. More investigation is needed, and studies could include evaluation of the effectiveness of anesthesia with different salt concentrations and its relationship to oyster size, effects on natural spawning on improving sex maturity by indoor conditioning, and the relationship of oyster size, sperm production, and survival after sampling by various methods.

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