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EFFECT OF EXTENDER SOLUTIONS AND DILUTION ON MOTILITY AND FERTILIZING ABILITY OF EASTERN OYSTER SPERM

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ABSTRACT Optimization of conditions for short-term storage of gametes is important for the production of seedstock and genetic management of broodstock in aquaculture. We conducted a series of experiments to evaluate refrigerated storage of sperm of the Eastern oyster (*Crassostrea virginica*). Our objectives were to: 1) compare motility of oyster sperm suspended in artificial seawater (ASW) at five osmotic pressures (22, 203, 403, 601, or 833 mOsm/kg) over 24 h; 2) compare motility of sperm suspended in solutions of ASW, Hanks' balanced salt solution (HBSS), or DCSB4 solution (all at 833 mOsm/kg) over 4 days of refrigerated storage; 3) compare motility of sperm suspended in ASW or HBSS (833 mOsm/kg) in six ratios of sperm:extender (1:0, 1:1, 1:3, 1:7, 1:15, or 1:31) over 4 days of refrigerated storage, and 4) compare motility and fertilizing capacity of oyster suspended in ASW (200 and 830 mOsm/kg), ASW with 6% glycine (ASW+G), HBSS, and calcium-free HBSS (C-F HBSS) (all at 830 mOsm/kg). Significant differences ($p < 0.001$) were found in the motility of sperm suspended in ASW of different osmotic pressures. No significant differences ($p = 0.267$) were found in motility of sperm suspended in ASW, HBSS, or DCSB4. Significant differences ($p < 0.001$) were found in the motility of sperm suspended in different ratios of sperm:extender. The highest motility was found in undiluted sperm and the lowest in the 1:31 dilution for sperm suspended in ASW or HBSS. Significant differences ($p = 0.0001$) were found in motility of sperm suspended in various extenders. The highest motility was found in sperm diluted in CF-HBSS (96%), and the lowest in sperm diluted in ASW at 200 mOsm/kg (12%). The highest percent fertilization (48%) (as measured by larval development at 12 h) was obtained when eggs were fertilized with sperm diluted in C-F HBSS. These results indicate that for storage (4 days), it is best to leave sperm samples undiluted. However, when sperm samples are diluted for use within 24 h, it is best to maintain high sperm concentrations and to use C-F HBSS as an extender.

KEY WORDS: *Crassostrea virginica*, sperm, motility, extenders, storage, fertilizing ability, larvae.

INTRODUCTION

The Eastern oyster, *Crassostrea virginica* (Gmelin 1791), is an important resource of the Atlantic and Gulf coasts, and many studies have addressed culture of this species (e.g., Loosanoff 1963, Galstoff 1964, Dupuy et al. 1977). Artificial spawning techniques have been developed (Stephano and Gould 1988, Rampersad et al. 1994) and cryopreservation of sperm has been studied (Zell et al. 1979, Yankson and Moyse 1991). Although refrigerated storage of fish sperm is well studied (Stoss et al. 1972, Hara et al. 1982), studies of refrigerated storage of oyster sperm are lacking. Short-term storage of oyster sperm (for a few hours or days) can be useful for aquaculture. Stored samples can be transported from the spawning site to distant facilities for use in genetic studies and artificial breeding programs. Short-term storage offers the possibility of controlling factors that can affect sperm quality such as osmotic pressure and bacterial contamination. Another benefit of short-term storage and dilution of oyster sperm is the possibility of using this information for refinement of cryopreservation techniques.

Sperm quality is ultimately defined by the ability to fertilize eggs (Aas et al. 1991). Some factors that can influence the natural quality of oyster sperm are disease, environmental conditions, and genetic variability among males. In the short-term storage of oyster sperm, factors such as osmotic pressure, sperm concentration, bacterial contamination, temperature, oxygen content, dissolved organic matter, and pH are important. Control of these factors in other aquatic species has improved the success of refrigerated storage (Stoss et al. 1972). Sperm motility is a commonly used indicator of sperm quality and is usually expressed as the percentage of motile sperm observed after activation (Redondo-Muller et al. 1991).

Among invertebrate species, the sea urchin has been used as a model for sperm study demonstrating that factors such as dilution and CO_2 concentration play important roles in motility intensity and duration (Gray 1928a, Gray 1928b). Few studies have examined factors influencing motility of oyster sperm. Humphrey (1950) reported that oyster sperm suspended at less than 6×10^8 cells/mL rapidly lost motility. The effect of amino acids and other nutrients in prolonging the functional life of suspended spermatozoa has been examined. Glycine is utilized for energy production when the normal efficiency of the oxidative process is decreased (Jeffrey 1954a), and copper, zinc, and cadmium at low concentrations can be beneficial to oyster sperm motility (Jeffrey 1954b). Seawater (Yankson and Moyse 1991), Hanks' phosphate-buffered salt solution (Zell et al. 1979), and other buffers such as DCSB4 (Bougrier and Rabenomanana 1986) have been used successfully as extenders for cryopreservation studies for sperm of *C. virginica*, *C. gigas*, *C. tulipa*, *C. iredalei*, and *Saccostrea cucullata* (with storage in buffers for 30 min or less).

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The goal of this work was to evaluate extenders and dilutions used for refrigerated storage of Eastern oyster sperm. Our objectives were to: 1) compare motility of sperm stored undiluted or suspended in artificial seawater (ASW) of five osmotic pressures (22, 203, 403, 601, and 833 mOsm/kg) during 24 h; 2) compare motility of sperm suspended in 833 mOsm/kg solutions of ASW, Hanks' balanced salt solution (HBSS), and DCSB4 during 4 days of refrigerated storage; 3) compare motility of sperm stored for 4 days in ASW or HBSS (833 mOsm/kg) at six ratios of sperm:extender (1:0, 1:1, 1:3, 1:7, 1:15, and 1:31), and 4) compare motility and fertilizing capacity of sperm stored in ASW at 200 or 833 mOsm/kg, ASW with 6% glycine (ASW+G) at 833 mOsm/kg, and HBSS and calcium-free HBSS (C-F HBSS) at 830 mOsm/kg, which might help to decrease acrosome reaction and agglutination, factors that could be in part responsible for loss of fertilizing ability (Gonzalez-Martinez et al. 1992).

MATERIAL AND METHODS

In this paper, solution abbreviations are followed by the osmolality of the solution. For example, an artificial seawater solution of 830 mOsm/kg would be abbreviated as ASW 830.

Oyster Collection

Oysters were collected from a hatchery on Grand Isle, Louisiana, and transported to the Louisiana State University Agricultural Center, Aquaculture Research Laboratory, Baton Rouge. Oysters were opened and inspected visually for the presence of gonad development and prominent genital canals (Supan 1996). A gonad sample was collected with a capillary tube and smeared on a glass microscope slide for examination at 200X. Sex was identified based on the presence of eggs or sperm.

Gamete Preparation

Gamete samples were removed from each oyster by the dry stripping method of Allen and Bushek (1992). The gonad was gently disrupted and gonadal material collected with a Pasteur pipette. A 10-μL sample was removed from the gonad to measure osmolality with a vapor pressure osmometer (Model 5500, Wescor Inc., Logan, Utah, USA). Egg or sperm samples were placed in 50-mL beakers until suspension in an extender. After suspension, eggs sample were washed through a 70-μm screen, collected on a 15-μm screen, and suspended in ASW 601. For sperm, samples were washed through 70-μm and 15-μm nitex screens (Aquacenter, Leland, Mississippi, USA) and motility was estimated as described below.

Motility Estimation

A 10-μL sample was removed from undiluted sperm or suspensions to estimate motility. The sample was mixed with 20-μL ASW 601 on a glass microscope slide. The percentage of sperm exhibiting vigorous forward movement was estimated at 200X using darkfield microscopy (Optiphot 2, Nikon Inc. Garden City, New York). Sperm vibrating in place were not considered to be motile. Only males with actively swimming sperm (>90%) were selected for experimentation.

Throughout the experiments, five extender solutions were used: ASW (Fritz Super Salt, Fritz Industries, Inc. Dallas, Texas, USA), ASW with 6% glycine, HBSS (Tiersch et al. 1994), C-F HBSS (Tiersch et al. 1997), or DCSB4 (Bougrier and Rabenomanana 1986). All chemicals (except ASW) were of reagent grade (Sigma Chemical Corp., St. Louis, Missouri, USA). All the solutions were made fresh, filtered, and adjusted to pH 7.6 (Table 1)

Experiment 1: Sperm Motility in ASW of Different Osmotic Pressures

Sperm samples were collected from three males (initial motility was 99% for all samples). Aliquots of 250 μL from each sample were suspended in ASW of 22, 203, 403, 601, or 833 mOsm/kg. Osmolality of suspensions was measured with a vapor pressure osmometer. The final volume of each sperm suspension and gonadal samples was adjusted to 30 mL. Samples (10 μL) were examined for sperm motility every 4 min for 1 h and once every hour for the following 2 h. To determine if sperm suspended in ASW 22 or ASW 203 could regain motility, 10-μL samples were diluted with 20 μL of fresh ASW 833 and incubated for 5 min. After these observations, sperm were refrigerated at 4°C. Sperm motility was estimated again at 24 h after warming to room temperature (21°C).

Experiment 2: Sperm Motility in ASW, HBSS, and DCSB4

Based on Experiment 1, osmolality was set at 833 mOsm/kg for subsequent experiments. Sperm samples from three males were suspended in ASW, HBSS, and DCSB4 using the procedures described above. Sperm concentrations were estimated with a Makler sperm counting chamber (Sefimedical Instruments, Haifa, Israel). Concentrations were adjusted to 1×10^8 sperm/mL according to Gray (1928a) and the suspensions were refrigerated at 4°C. Motility was monitored daily for 4 days. All suspensions were allowed to warm to room temperature before motility assessment.

Experiment 3: Sperm Motility in ASW and HBSS in Five Dilutions

Sperm samples from three males were stored undiluted, or suspended in ASW 833 or HBSS 833 at six ratios of sperm:extender (1:0, 1:1, 1:3, 1:7, 1:15, 1:31). As in Experiment 2, samples were refrigerated and motility was monitored daily for 4 days.

Experiment 4: Sperm Motility and Fertilizing Capability in Five Extenders

Sperm samples from three males were stored undiluted, or suspended in the following extenders: ASW 200, ASW 833, ASW+G 833, HBSS 830, and C-F HBSS 830. Sperm concentrations were estimated with a Makler counting chamber and the final

TABLE 1.

Ingredients of Hanks' balanced salt solution (HBSS), calcium-free HBSS (C-F HBSS), and DCSB4 solutions (Bougrier and Rabenomanana 1986) used to dilute Easter oyster sperm.

Ingredient	Concentration (g/L)		
	HBSS	C-F HBSS	DCSB4
NaCl	24	24	24
KCl	1.2	1.2	—
$CaCl_2 \cdot 2H_2O$	0.48	—	0.31
$MgSO_4 \cdot 7H_2O$	0.6	0.6	0.31
$Na_2HPO_4 \cdot 7H_2O$	0.36	0.36	—
KH_2PO_4	0.18	0.18	—
$NaHCO_3$	1.05	1.05	—
$C_6H_{12}O_6$ (glucose)	3	3	—
Tris-HCl	—	—	2.98

concentration was set at 5×10^4 sperm/mL. Sperm motility was estimated after suspension for 5 min. Eggs of three ripe females were used to determine the fertilizing ability of sperm suspended in the different extenders. Concentration of eggs was determined by counting of 1-mL aliquots in a Sedgewick-Rafter chamber (Hausser Scientific Parthership, Horsham, Pennsylvania). Seven thousand eggs (35 eggs/mL) were suspended and incubated for 1 h in 200 mL of ASW 830 in 400-mL plastic beakers (VWR Scientific Inc. St. Louis, Missouri, USA). After 12 h of incubation (post-fertilization) at 20°C, the number of trochophore larvae/mL was estimated with a Sedgewick-rafter chamber. All gamete and larvae counts were performed in duplicate.

Data Analysis

Statistical analyses were performed using SAS software for Windows® (SAS Institute, Cary, North Carolina, USA). Data from Experiments 1, 2, and 3 were not distributed normally. For these experiments, a nonparametric Friedman's test was used to test the association among extenders, percent motility, and duration of motility, and to test for significant differences. Data from Experiment 4 were distributed normally and treatment groups were compared using a one-way factorial analysis (ANOVA). Specific differences among treatment groups were identified by the least squares differences test. A value of $p < 0.05$ was chosen as the level for significance.

RESULTS

Experiment 1

Sperm motility in ASW of different osmotic pressures was significantly different ($p = 0.001$). The osmolality of the gonad was 573 ± 45 mOsm/kg (mean ± SD). Sperm suspended in ASW 833 retained 99 ± 0% motility for 2 h (Fig. 1). Sperm suspended in ASW 403 and ASW 601 had an initial motility of less than 30 ± 3% followed by an increase to 90 ± 20% after 8 min. This motility was retained for 2 h. Sperm suspended in ASW 203 showed low motility (~1%) in the first 4 min; the maximum motility after 16 min of incubation was 47 ± 5% and was retained for 1 h. Subsequent dilution of these samples in ASW 833, did not increase motility. No motility was observed in sperm suspended in ASW 22 initially or during the 3-h experiment. After cold storage for 24 h, the highest motilities were 75 ± 7% for sperm suspended in ASW 833 and 80 ± 10% for sperm suspended in ASW 601. Sperm in ASW 403 showed a decreased motility (to 40 ± 15%), and sperm in ASW 203 lost most motility (to <10%).

Experiment 2

Cold storage of oyster sperm in different extenders revealed no significant differences ($p = 0.267$). The highest initial motility was 85 ± 4% for sperm suspended in ASW 833, and the lowest was 73 ± 3% for sperm suspended in DCSB4 833 (Fig. 2). By Day 3, sperm suspensions in each of the three extenders had motility values of <10%. Bacteria were observed during motility estimates after 1 day of storage. Bacterial concentrations increased with time and were associated with decreased sperm motility. Preliminary identification of these bacteria identified the presence of *Vibrio* spp. (A. Camus and R. Siebeling, Louisiana State University, pers. comm.).

Experiment 3

Significant differences in sperm motility were found among different dilutions ($p = 0.001$). For ASW 833, undiluted sperm had the highest motility (96 ± 5%) followed by sperm diluted at 1:1 (86 ± 11%) (Fig. 3). Sperm diluted at 1:31 had the lowest motility (47 ± 15%). After 3 days, sperm samples diluted at 1:31 showed a complete loss of motility, whereas sperm diluted at 1:1, 1:3, or at 1:7 retained <10%. After 4 days, all diluted samples lost motility. For HBSS 833, undiluted sperm had the highest initial motility (95 ± 6%) followed by sperm diluted at 1:7 (90%) (Fig. 4). Sperm diluted at 1:31 had the lowest motility (40 ± 14%). After 1 day of storage, sperm diluted at 1:31 lost motility completely. Motility was maintained best in less dilute samples. For undiluted samples, motility never declined below 50% (Figs. 3 and 4). As in Experi-

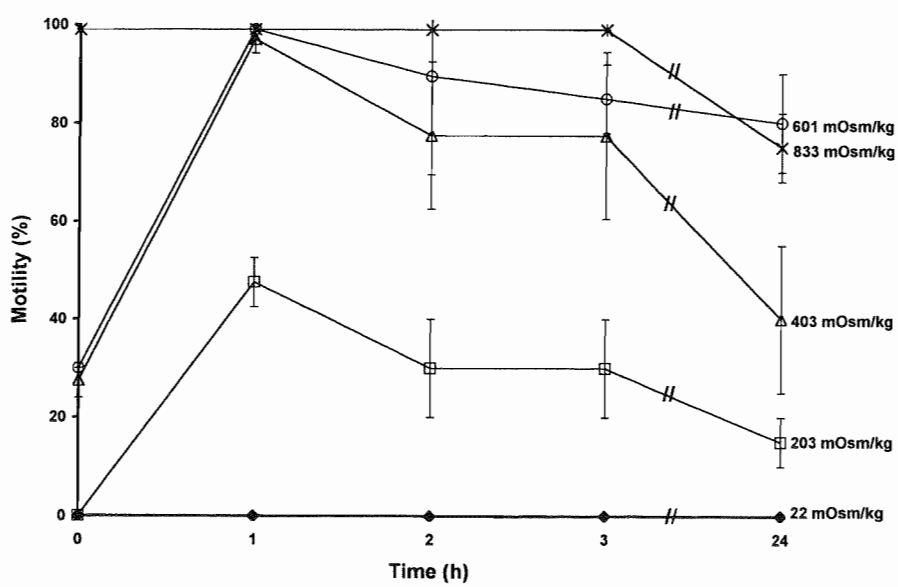

Figure 1. Mean percent motility ± SD of *Crassostrea virginica* sperm suspended in artificial sea water at osmolalities of 22, 203, 403, 601, or 833 mOsm/kg (n = 3 for each treatment).

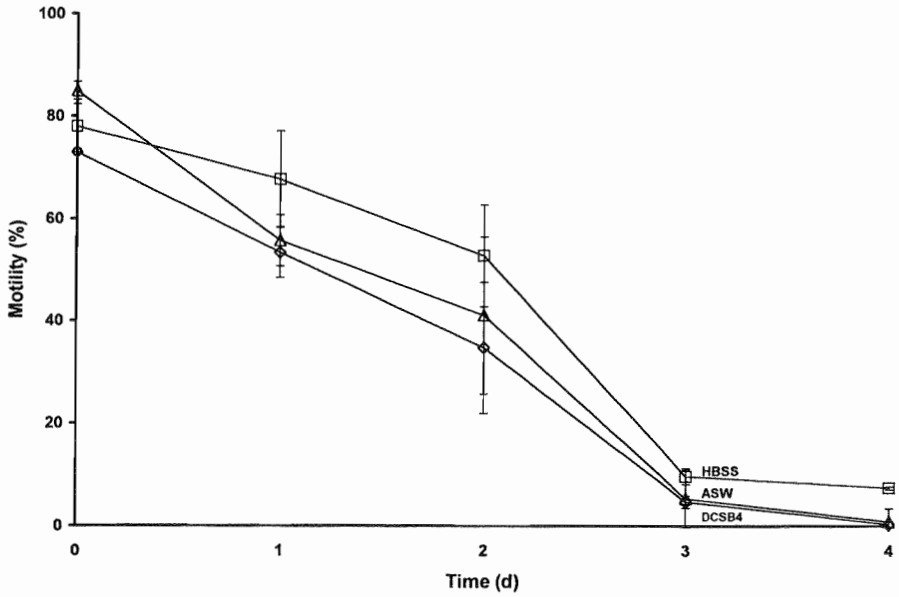

Figure 2. Mean percent motility ± SD of *Crassostrea virginica* sperm stored at 4°C in DCSB4 solution (diamonds), Hanks' balanced salt solution (HBSS; squares), or artificial sea water (ASW; triangles) at 833 mOsm/kg (n = 3 for each treatment).

ment 2, bacteria were observed in all samples, increased with time, and were associated with decreased motility.

Experiment 4

Sperm motility was affected by extender ($p = 0.0001$). The highest motility was observed for sperm suspended in C-F HBSS 830 (96 ± 5%) and the lowest motility was for sperm suspended in ASW 200 (12 ± 8%) (Fig. 5). Agglutination of sperm, causing adherence and sperm immobilization, was evident in samples suspended in ASW 200 and ASW 830. The extender used to dilute sperm had a significant effect on fertilization as measured by larval development at 12 h ($p = 0.0001$). Pair-wise comparison showed significant differences except for ASW 833 and ASW+G 833. Examination of trochophore larvae at 12 h post-fertilization indicated highest fertilization (48%) with sperm diluted in C-F HBSS, and lowest fertilization (<1%) with ASW 200 (Fig. 5).

DISCUSSION

Sperm are active in the gonad of marine invertebrates such as oysters (Loosanoff and Davis 1963). When sperm are released in the water they may accelerate, decelerate or cease moving in response to various stimuli (Nelson 1973). Factors such as salinity,

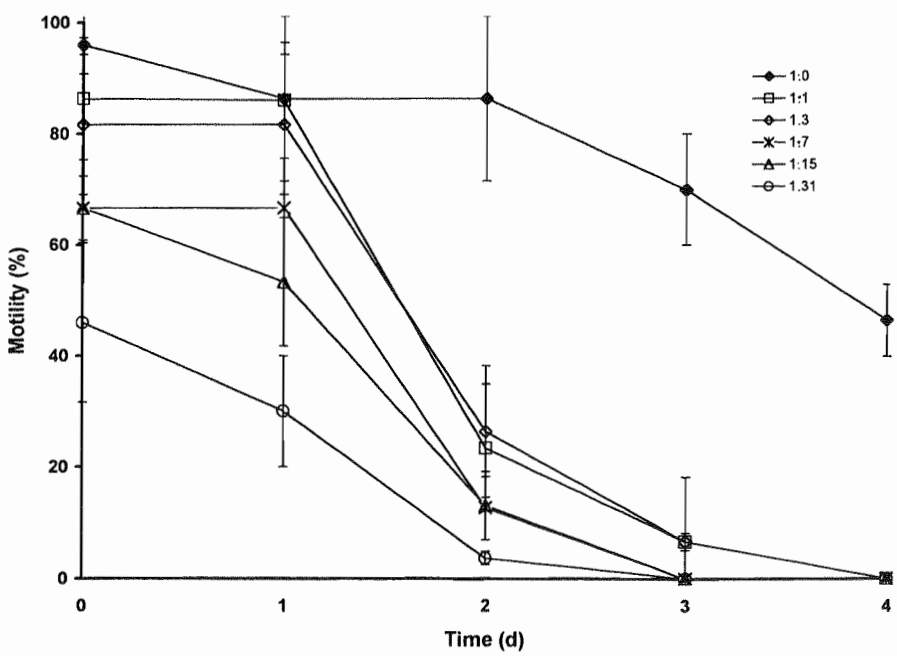

Figure 3. Mean percent motility ± SD of *Crassostrea virginica* sperm suspended in artificial sea water (833 mOsm/kg) at 6 ratios of sperm:extender (n = 3 for each treatment).

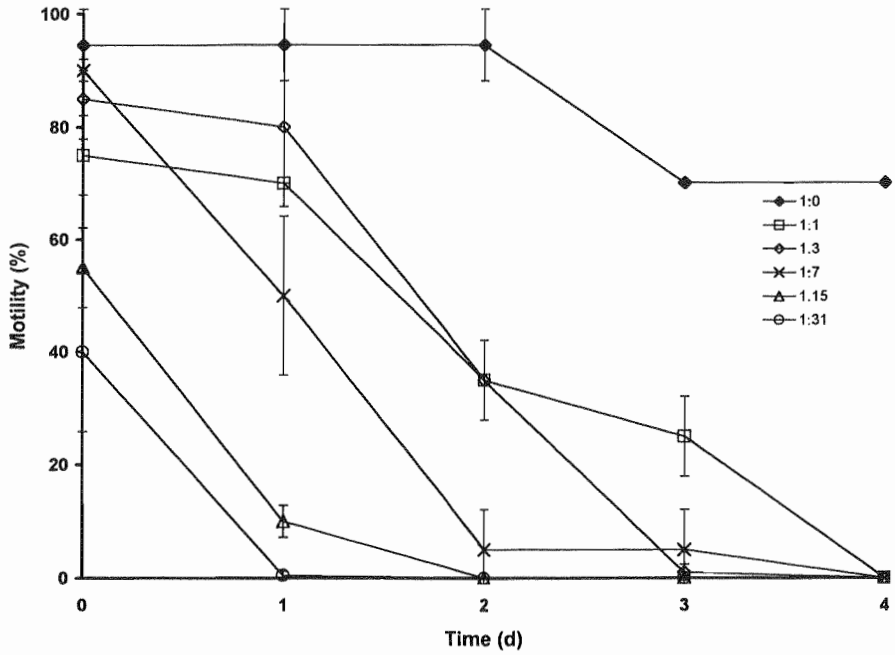

Figure 4. Mean percent motility ± SD of *Crassostrea virginica* sperm suspended in HBSS (833 mOsm/kg) at 6 ratios of sperm:extender (n = 3 for each treatment).

temperature, oxygen content, dissolved organic matter and pH can affect the fertilizing ability and motility of oyster sperm when released in natural sea water (Humphrey 1950). Another factor that can affect motility is osmotic pressure. In this study, sperm motility increased with osmolarity and time of exposure. Sperm suspended in relatively high osmolalities (>400 mOsm/kg) did not lose motility. This result suggests that motility is maintained when oyster sperm are suspended in ASW of high osmotic pressure. After 24 h of storage, the sperm retained a high motility (~85%) suggesting that cold storage of sperm at high osmolalities is possible without significant loss of motility. Some of the factors that could affect sperm motility in dilute ASW are osmotic shock, or imbalance of ions necessary to establish motility. It is possible that sperm suspended in ASW of low osmotic pressure (less than 400 mOsm/kg) require more than 8 min to adjust to equilibrate and acquire maximum motility. The addition of fresh ASW 833 did not

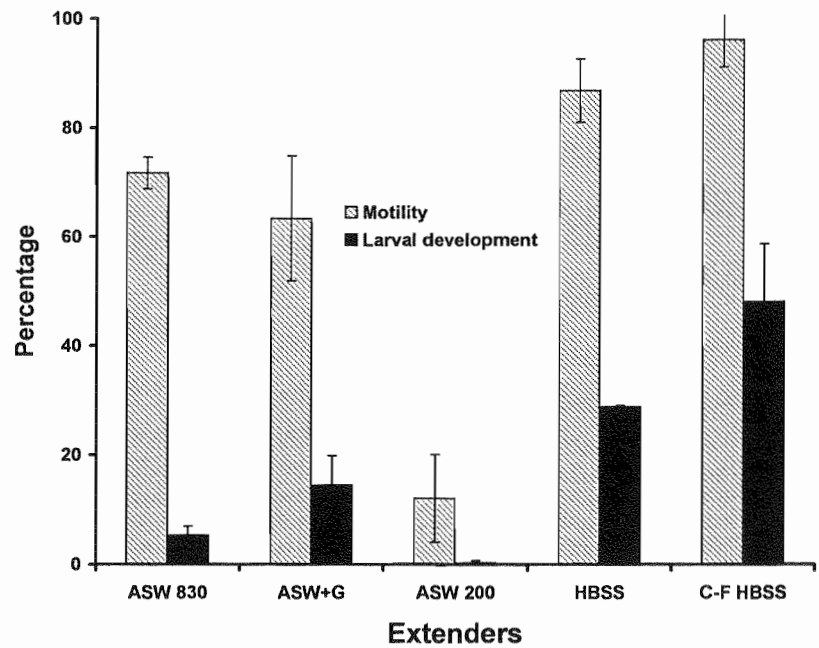

Figure 5. Mean percent of trochophore larvae (12 h post-fertilization) of *Crassostrea virginica* obtained from fertilization by sperm suspended in artificial sea water at 830 mOsm/kg (ASW 830), artificial sea water at 830 mOsm/kg plus glycine (ASW+G), artificial sea water at 200 mOsm/kg (ASW 200), HBSS at 830 mOsm/kg (HBSS), or calcium-free HBSS at 830 mOsm/kg (C-F HBSS) (n = 3 for each treatment).

reactivate sperm initially suspended in ASW 22 suggesting that sperm undergoing strong changes in osmotic pressure are irreversibly damaged. The effect on motility of specific ions such as potassium or sodium remains to be examined.

Sperm motility declined with time when dilutions were made with DCSB4, HBSS, and ASW. This loss of motility could be attributable to exhaustion of metabolic energy reserves with continuous activity after suspension, or detrimental effects caused by bacteria. In refrigerated storage of channel catfish sperm, bacterial growth was a major problem (Jenkins and Tiersch 1997). In this study, declining motility was associated with increased bacterial numbers. Addition of antibiotics could remedy this problem (Scott and Baynes 1980).

Dilution is another possible cause for reduced sperm motility. In the oyster it has been noted that the greater the dilution, the greater the sperm activity in the first minutes (Gray 1928a). The loss of motility during storage because of dilution could be because sperm are less crowded in the suspension and have more free space in which to move, causing a depletion of energy, or because essential factors in the gonad are lost when sperm is diluted.

Studies have shown that when sea urchin sperm are suspended in sea water or ASW, they undergo a spontaneous acrosome reaction consisting of exocytocis of the acrosome granule, which is a lysosome-like vesicle located at the tip of the sperm head (Gonzalez-Martinez et al. 1992). Spontaneous acrosome reaction could in part be responsible for loss of fertilizing ability. In calcium-free artificial sea water, sea urchin sperm showed no morphological changes and swam vigorously (Dan 1955). In the presence of calcium, the sperm underwent the acrosome reaction and became agglutinated.

Oyster sperm, like that of sea urchin, possess an acrosome (Galtsoff and Philpott 1960, Bozzo et al. 1993) that plays an important role in fertilizing capacity. Kurokura et al. (1990) reported that cryopreserved oyster sperm lost fertilizing capacity because of formation of sperm aggregations, a lack of capacity to trigger cleavage, and damage to the acrosome preventing entry of sperm into eggs. In this study, sperm diluted in C-F HBSS reached a high motility (99%) and did not agglutinate, suggesting that the acrosome reaction was minimized. Also trochophore numbers were highest with sperm suspended in C-F HBSS. Glycine has been shown to enhance motility of oyster sperm under certain conditions (Jeffrey 1954a); however, no benefit of glycine was found for sperm motility or larval survival in this study. Sperm had diminished motility when placed in ASW 200, and there was no fertilization, suggesting that the sperm lost fertilizing ability due to damage caused by osmotic shock or a change in ionic composition.

We found that several conditions can influence the motility and fertilizing ability of oyster sperm and these results can be of practical use for refrigerated storage and cryopreservation. Osmotic pressure should be maintained at a high level (>400 mOsm/kg) to ensure good motility, and the use of antibiotics should be investigated. When sperm is to be used within 24 h, a low dilution (1:1 or 1:3) is recommended; however, for more than 1 day of refrigerated storage, samples should be maintained undiluted. In addition, suspension of sperm in C-F HBSS seems to have a positive effect on sperm motility and fertilizing ability, and could enhance larval production.

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