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FACTORS AFFECTING SPERM MOTILITY OF TETRAPLOID PACIFIC OYSTERS

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ABSTRACT Factors such as osmotic pressure, extender solution, addition of caffeine, and pH have been shown to affect sperm motility in aquatic species. We evaluated the effects of 18 osmotic pressures, two extender solutions, seven caffeine concentrations, and a pH range of 3 to 14 on motility of sperm from tetraploid Pacific oysters, *Crassostrea gigas*. Motility was highest at 1000 mOsmol/kg (mean ± SD: 83 ± 14%). Calcium-free Hanks' balanced salt solution yielded significantly higher sperm motility than did artificial seawater. Sperm motility increased with caffeine concentrations to 20 mM (81 ± 12%) and decreased when concentrations were higher than 50 mM (55 ± 20%). Highest motility was obtained at a pH range of from 4 to 12; values outside this range yielded no motility. Addition of 10 mM caffeine to the different pH treatments also enhanced motility significantly. Overall, calcium-free Hanks' balanced salt solution at 1,000 mOsmol/kg, the addition of 10 mM caffeine, and a pH of around 10 could be used to enhance sperm motility of tetraploid Pacific oysters. Our findings would assist the use of motility assays to evaluate the effectiveness of various refrigeration or cryopreservation procedures, especially outside of the peak spawning season, when sperm motility can be low and variable.

KEY WORDS: *Crassostrea gigas*, sperm motility, pH, caffeine, osmotic pressure, tetraploid, cryopreservation

INTRODUCTION

Factors such as osmotic pressure (Bates et al. 1996), extender composition, pH, temperature (Lahnsteiner et al. 1997, Sunitha and Jayaprakas 1997), and additives such as caffeine (Scheerer and Thorgaard 1989, Tiersch et al. 1998) have been shown to affect sperm motility in aquatic species. Most studies have been conducted on teleosts (e.g., Morisawa et al. 1983a, Morisawa 1983b), and studies on invertebrates mainly focus on sea urchin (Morisawa et al. 1990), polychaetes (Pacey et al. 1994), and ascidians (Yoshida et al. 1992, Yoshida et al. 1994). Little is known about the effects of these factors on oyster sperm, especially on sperm from tetraploid oysters, which possess four sets of chromosomes instead of the normal diploid two sets.

Tetraploid oysters create opportunities for genetic improvement, including direct production of triploid (sterile) seedstocks by crossing with normal diploids. Refrigerated and frozen storage of tetraploid oyster sperm will be a critical tool for commercial-scale application of tetraploid stocks and for developing tetraploid breeding programs. Although subjective, motility estimation is the technique used most commonly to assess sperm quality of fish and shellfish (Piironen 1993, Tiersch et al. 1994, Koupal et al. 1995). Motility has been used to assess the sperm quality of oysters (Paniagua-Chavez et al. 1998), but its application with tetraploid oyster sperm is unexplored. The objective of this study was to develop procedures for evaluation of sperm quality to assist the overall goal of sperm storage for tetraploid Pacific oysters, *Crassostrea gigas*. Specifically, we evaluated the effects on sperm motility of: (1) osmotic pressure; (2) extender solution; (3) caffeine, and (4) pH. Our findings indicate that these factors can alter the motility of tetraploid oyster sperm collected late in the spawning season. To our knowledge, this is the first study to systematically characterize sperm motility of tetraploids of an aquatic species.

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MATERIALS AND METHODS

Tetraploid oysters were obtained in September and October 2001 from Whiskey Creek Shellfish Hatchery (WCSH) (Tillamook, Oregon) and were shipped chilled by overnight delivery to the Louisiana State University Agricultural Center, Aquaculture Research Station (ARS). Water samples from WCSH had an osmolality of 873 mOsmol/kg as measured by vapor pressure osmometry (model 5500, Wescor Inc., Logan, UT) at the ARS. Sperm were collected by dry stripping of the gonad (Allen & Bushek 1992). Undiluted nonmotile sperm were equilibrated in 30 µL of test solutions at 23°C for 2 min before assessment of motility. Sperm motility was estimated at 200× magnification using darkfield microscopy (Optiphot 2, Nikon Inc., Garden City, NY) and was expressed as the percentage of cells actively moving in a forward direction.

Throughout the experiments, two extender solutions were used: artificial sea water (ASW) (Fritz Super Salt, Fritz Industries, Inc. Dallas, TX) and calcium-free Hanks' balanced salt solution (C-F HBSS) (Paniagua-Chavez et al. 1998). All chemicals (except ASW) were of reagent grade (Sigma Chemical Corporation., St. Louis, MO). Osmolality was measured with a vapor pressure osmometer.

In our first study, the effect on sperm motility of ASW of 18 different osmolalities ranging from 30 to 1400 mOsmol/kg was evaluated with a total of 20 oysters in four trials, for which oysters were received on August 24, August 30, September 19, and September 26. The second study compared ASW and C-F HBSS at 13 different osmolalities with five oysters, which were received on October 16. In the third study, the effect of caffeine was evaluated at seven concentrations (2 to 100 mM) with eight oysters from two shipments received on October 10 and October 16. In the fourth study, a pH range of from 3 to 14, with and without caffeine, was evaluated with three oysters that were received on October 10. Sperm from individual oysters was used for all studies (samples were not pooled). Within this manuscript, extender solutions at

specific osmolalities such as ASW at 1,000 mOsmol/kg are abbreviated as ASW1000.

Data were analyzed using one-way or two-way analysis of variance. Tukey's honestly significant difference procedure was used to test for differences ($\alpha = 0.05$) among results for osmolalities, caffeine concentrations and pH levels (SAS Institute 1991).

RESULTS

Among the osmolalities tested, sperm from tetraploid Pacific oysters remained immotile when diluted with ASW below 500 mOsmol/kg (Fig. 1). Motility increased from 12 ± 12% (mean ± SD) in ASW500 to 50 ± 11% in ASW670. There was no significant difference among sperm motilities activated with ASW at 670, 700, 750, and 800 mOsmol/kg ($P > 0.05$). Although sperm motility was not significantly different among ASW at 900, 950, 1,000, and 1,100 mOsmol/kg, ASW1000 elicited the highest motility 83 ± 14% (Fig. 1). Motility decreased significantly when osmolality was greater than 1,100 mOsmol/kg.

Motility in C-F HBSS was significantly higher than that in ASW ($P < 0.0001$) (Fig. 2). Each extender showed the highest motility at 1,000 mOsmol/kg, which agreed with the results of the previous experiment. But motility in C-F HBSS1000 (81 ± 9%) was double that in ASW1000 (40 ± 22%). Other osmolalities of C-F HBSS also enhanced sperm motility. For example, motility in C-F HBSS670 (50 ± 10%) was six times greater than that in ASW670 (8 ± 13%) (Fig. 2). Because the oysters used in this experiment were received later in the spawning season than those used for the first study, lower motilities overall were observed for ASW.

Based on the results of the second experiment, C-F HBSS at 1,000 mOsmol/kg was used for caffeine assessments. Motility increased with caffeine concentrations of from 2 mM (60 ± 13%) to 20 mM (81 ± 12%) and decreased when concentrations were higher than 50 mM (55 ± 20%) (Fig. 3). The lowest motility (49 ± 10%) was in the control treatment: C-F HBSS1000 without the addition of caffeine, but additions of 2, 4, and 6 mM caffeine were not significantly different from the control ($P > 0.05$). Motilities in 10 and 20 mM caffeine were significantly higher than in other concentrations ($P < 0.05$), but they were not different from each other ($P > 0.05$) (Fig. 3).

Based on these results, C-F HBSS1000 with and without 10 mM caffeine were used for pH assessment. Motility was highest at a pH range of from 4 to 12; values outside this range yielded no motility (Fig. 4). The highest motility was in pH 10.5 (67 ± 6%), followed by pH 10 (63 ± 6%). The addition of 10 mM caffeine to these pH treatments enhanced motility significantly ($P < 0.001$)

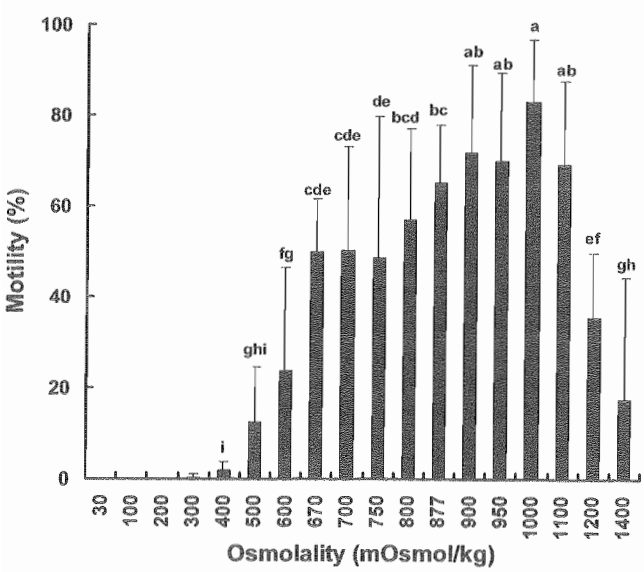

Figure 1. Percent motility (mean ± SD) of tetraploid Pacific oyster sperm activated in artificial seawater at 18 different osmotic pressures. Bars sharing a letter were not significantly different ($P > 0.05$).

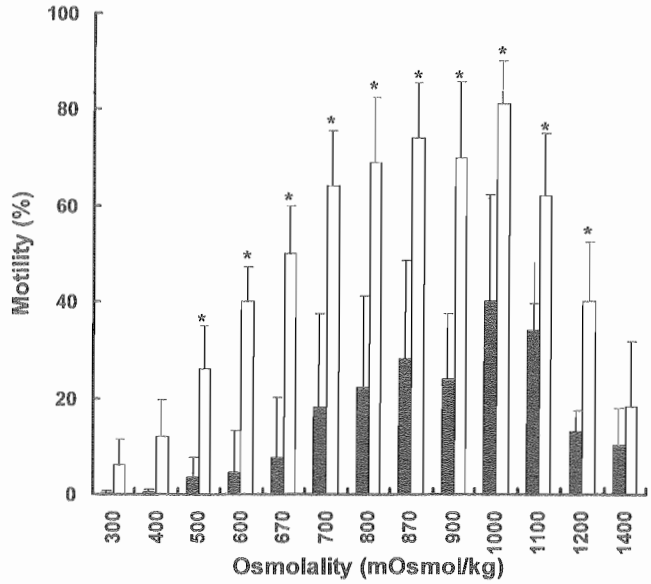

Figure 2. Percent motility (mean ± SD) of tetraploid Pacific oyster sperm activated in ASW (filled bars) and C-F HBSS (open bars). Bars with an asterisk indicate a significant difference ($P > 0.05$) between ASW and C-F HBSS.

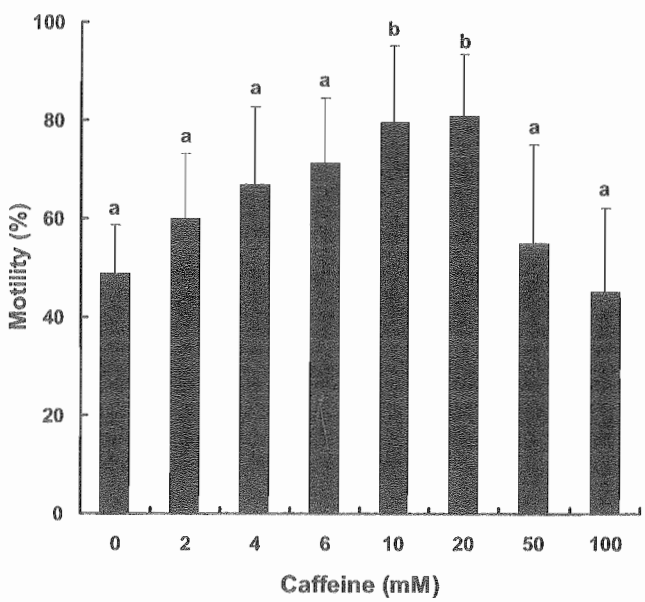

Figure 3. Percent motility (mean ± SD) of tetraploid Pacific oyster sperm activated with the addition of caffeine into C-F HBSS at 1,000 mOsmol/kg. Bars sharing a letter were not significantly different ($P > 0.05$).

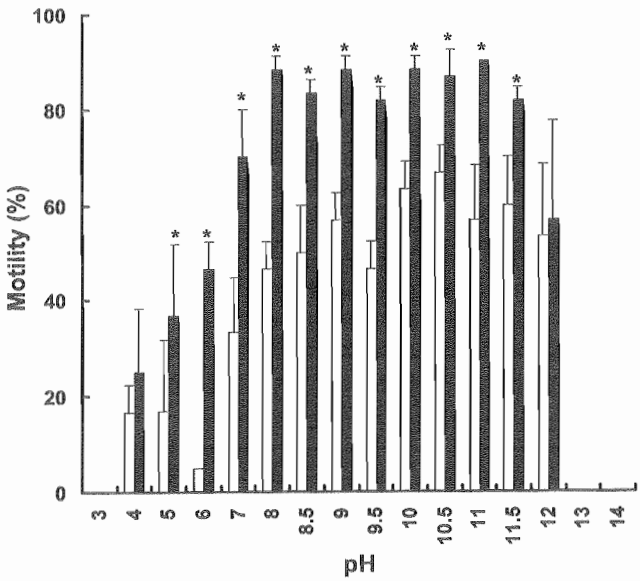

Figure 4. Percent motility (mean ± SD) of tetraploid Pacific oyster sperm in C-F HBSS at 1,000 mOsmol/kg with different pH treatments. Open bars, C-F HBSS without addition of caffeine; filled bars, C-F HBSS with 10 mM caffeine. Bars with an asterisk indicate a significant difference ($P > 0.05$) between presence and absence of caffeine.

(Fig. 4). Motility was 90 ± 0% in pH 11, 87 ± 6% in pH 10.5, and 88 ± 3% in pH 10. However, there was no significant difference in sperm motility across the pH range of from 7 to 12 ($P > 0.05$).

DISCUSSION

Sperm activation is a complex process in which roles are played by many factors, including osmotic pressure, extender composition, membrane structural changes, and extracellular and intracellular pH. Previous studies have suggested that different species have different sperm activation mechanisms. Basically, in fish there seem to be two major factors influencing sperm activation: changes of osmotic pressure and changes of ionic concentration. For instance, studies on muskellunge *Esox masquinongy* (Lin & Dabrwoski 1996), channel catfish *Ictalurus punctatus* (Bates et al. 1996), and Asian catfish *Clarias macrocephalus* (Tan-Fermin et al. 1999) suggested that sperm motility was initiated by a reduction of osmotic pressure. However, activation of sperm from salmonids and cyprinids was caused by changes in concentrations of ions such as potassium and calcium (Morisawa et al. 1983a, Billard & Cosson 1992). The mechanism of initiation of sperm motility has been most studied in salmonids although much remains unknown, especially at the intracellular level.

Motility studies in other taxa such as invertebrates are limited and the associated mechanisms remain largely unknown. There are less than 40 references addressing oyster sperm motility in any way since 1970. Most use motility as a criterion to evaluate fertilization or in toxicological assays to evaluate waste effluents or heavy metals. Factor that affect sperm motility such as osmolality, extender composition, pH, and temperature are briefly mentioned in these studies and others on sperm cryopreservation, and therefore the information is fragmented and dispersed. The present study was designed to address factors affecting the sperm motility of tetraploid Pacific oysters and provided a more detailed and systematic approach.

Osmolality, as mentioned above, plays an important role in the activation of fish sperm. Osmolalities of ~1000 mOsmol/kg (the full-strength salinity of sea water, 32 ppt) and 670 mOsmol/kg were previously used for sperm activation and extender solutions for *C. gigas* (Yankson & Moyse 1991, Kurokura et al. 1990). Other species were studied with sea water of ambient environmental salinity. The first investigation of sperm motility across a wide range of osmolalities was with the diploid eastern oyster, *C. virginica* (Paniagua-Chavez et al. 1998). High sperm motility (90%) was observed across a range of 600 to 1,500 mOsmol/kg (Paniagua-Chavez & Tiersch 2001). In the present study, sperm motility of tetraploid Pacific oysters was also observed across a wide range of osmotic pressures (500–1400 mOsmol/kg), but the highest motility was limited to 1,000 mOsmol/kg, although the oysters were conditioned in seawater at 873 mOsmol/kg. Whether osmotic pressure plays the major role in oyster sperm activation remains unknown; however, motility was suppressed in tetraploid sperm of *C. gigas* at osmolalities of lower than 500 mOsmol/kg. Suppression was reported to occur at less than 22 mOsmol/kg in *C. virginica* (Paniagua-Chavez et al. 1998). This might correspond to the habitat of these species in natural environments. *Crassostrea gigas* prefers higher and more stable salinities (15 to 33 ppt) (Kusuki 1991) than does *C. virginica*, which normally occurs from 5 to 40 ppt (Galtsoff 1964, Wallace 1966).

Natural seawater and ASW are most commonly used as extender solutions for sperm of marine organisms including estuarine organisms like oysters. Other extenders such as DCSB4 (Bougrier & Rabenomanana 1986), HBSS1990 (Zell et al. 1979), and C-F HBSS640 (Paniagua-Chavez et al. 1998) with specific ionic compositions have also been successfully used for oyster sperm. The removal of calcium from HBSS was found to enhance motility in sperm of diploid eastern oysters (Paniagua-Chavez et al. 1998) and tetraploid Pacific oysters (this study) and was also superior to ASW. Contrary to the situation in salmonids, in which sperm motility is initiated by a decrease in potassium concentration upon release into fresh water (Morisawa et al. 1983a), an increase of potassium concentration (200 mM) was found to have an activating effect on diploid Pacific oyster sperm (Faure et al. 1995). The same study showed no effect of the increase of potassium on sperm of the king scallop *Pecten maximus*, but an increase in motility was observed with media lacking sodium. These results may indicate a species-specific response for ionic effects on bivalve sperm motility. Also, changes in ion concentration, rather than absolute concentration, may act as the trigger of initiation of sperm motility.

Caffeine has been used as motility stimulant to optimize the recovery and quality of thawed spermatozoa in mammalian species (Correa & Zavos 1996, Park & Sirard 1996). Few studies have addressed use of this chemical in sperm of aquatic species and there are no reports for oysters. The present experiment showed a significant increase in sperm motility with the addition of 10 mM caffeine, while concentrations above 50 mM reduced motility. A previous study in the razorback sucker *Xyrauchen texanus* showed increased motility in refrigerated sperm after the addition of 5 mM caffeine, but not in thawed sperm (Tiersch et al. 1998). We did not evaluate the addition of caffeine with thawed sperm in this study, but fertilizing capacity of cryopreserved semen of rainbow trout *Oncorhynchus mykiss* was improved when eggs were fertilized in a buffered saline solution containing 5 mM theophylline, a chemical relative to caffeine used to prolong and intensify sperm motility (Scheerer & Thorgaard 1989).

Alkaline pH has been found to be conducive to sperm activation in aquatic species (Thorogood & Blackshaw 1992, Sunitha &

Jayaprakas 1997, Ciereszko et al. 2001), and pH values between 7 and 9 have been used in most studies for oyster sperm (e.g., Zell et al. 1979, Paniagua-Chavez et al. 1998). Study of the king scallop (Faure 1996) found that gonadal pH was more acidic than seawater pH and suggested that the acidity of the genital tract maintained the spermatozoa in a quiescent state. A reduction of sperm motility was reported in *P. maximus* and *C. gigas* upon decrease of pH in seawater (Faure 1996). In the present study, pH values below 7 induced a significant reduction in sperm motility although there was variation among individual oysters. The highest sperm motility was observed at a pH of 10, which agrees with findings for the Japanese pearl oyster *Pinctada fucata* (Yu et al. 1998), although that study found an interaction between salinity and pH. In that study, sperm motility could not be activated by seawater at a salinity of 30 ppt and a pH of 8.0, but motility was greater than 80% when pH ranged from 9.0 to 11.5. Sperm from tetraploid Pacific oysters showed motility over a wider pH range in the present study. It appears that relative influence of most factors affecting sperm motility of bivalves are species-specific.

Finally, the main practical interest in tetraploidy is for the production of triploids by mating with diploids. The induction of tetraploidy in oysters was first reported in 1994 (Guo and Allen, 1994). Studies of the factors affecting sperm motility of tetraploid oysters have only now begun as reported here. Our experiments indicate that use of C-F HBSS at 1,000 mOsmol/kg as an extender, the addition of 10 mM caffeine, and a pH of around 10 can be used to enhance sperm motility of tetraploid Pacific oysters. This would assist the use of motility assays to evaluate the effectiveness of various refrigeration or cryopreservation procedures, especially outside of the peak spawning season when sperm motility can be low and variable such as in this study. The effectiveness of these conditions in improving fertilization rates requires further study. Future research is required to evaluate differences between sperm of diploid and tetraploids within and among aquatic species.

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LITERATURE CITED

Allen, S. K. & D. Bushek. 1992. Large scale production of triploid *Crassostrea virginica* (Gmelin) using "stripped" gametes. *Aquaculture* 103: 241–251.

Bates, M. C., W. R. Wayman & T. R. Tiersch. 1996. Effect of osmotic pressure on the activation and storage of channel catfish sperm. *Trans. Am. Fish. Soc.* 125:798–802.

Billard, R. & M. P. Cosson. 1992. Some problems related to the assessment of sperm motility in freshwater fish. *J. Exp. Zool.* 261:122–131.

Bougrier, S. & L. D. Rabenomanana. 1986. Cryopreservation of spermatozoa of the Japanese oyster, *Crassostrea gigas*. *Aquaculture* 58:277–280.

Ciereszko, A., K. Dabrowski, B. Piros, M. Kwasnik & J. Glogowski. 2001. Characterization of zebra mussel (*Dreissena polymorpha*) sperm motility: duration of movement, effects of cations, pH and gossypol. *Hydrobiologia* 452:225–232.

Correa, J. R. & P. M. Zavos. 1996. Preparation and recovery of frozen-thawed bovine spermatozoa via various sperm selection techniques employed in assisted reproductive technologies. *Theriogenology* 46: 1225–1232.

Faure, C. 1996. Physiological parameters of spawning and activation of the male gametes in two bivalve mollusks the scallop *Pecten maximus* (L.) and the oyster *Crassostrea gigas* (Thunberg). Dissertation. Paris: Univ. Paris 7 Denis Diderot, 255 pp.

Faure, C., N. Devauchelle, J. P. Girard & J. Cossson. 1995. Gametes as physiological references and parameters of sperm activation in scallop *Pecten maximus* and Japanese oysters *Crassostrea gigas*. The Natural and Controlled Reproduction of Cultivated Bivalves in France: Symposium Report IFREMER/Nantes/France, November 14–15, 1995. 61–67.

Galtsoff, P. S. 1964. The American oyster *Crassostrea virginica* Gmelin. *Fish. Bull.* 64:480.

Guo, X. & S. K. Allen Jr. 1994. Viable tetraploids in the Pacific oyster (*Crassostrea gigas* Thunberg) produced by inhibition of polar body I in eggs from triploids. *Mol. Mar. Biol. Biotechnol.* 3:42–50.

Koupal, K. D., J. R. Satterfield, Jr. & S. A. Flickinger. 1995. Evaluation of walleye semen: Extender ratios in relation to hatching percentage. *Prog. Fish-cult.* 57:188–191.

Kurokura, H., K. Namba & T. Ishikawa. 1990. Lesions of spermatozoa by cryopreservation in oyster *Crassostrea gigas*. *Bull. Japan Soc. Sci. Fish.* 56:1803–1806.

Kusuki, Y. 1991. Oyster culture in Japan and adjacent countries: *Crassostrea gigas* (Thunberg). In: W. Menzel, editor. Estuarine and marine bivalve mollusk culture. Boca Raton, FL: CRC Press. pp. 245–260.

Lahnsteiner, F., B. Berger, T. Weismann & R. Patzner. 1997. Sperm motility and seminal fluid composition in the burbot, *Lota lota*. *J. Appl. Ichthyol.* 13:113–119.

Lin, F. & K. Dabrwoski. 1996. Characteristics of muskellunge spermatozoa 2: Effects of ions and osmolality on sperm motility. *Trans. Am. Fish. Soc.* 125:195–202.

Morisawa, M., K. Suzuki & S. Morisawa. 1983a. Effects of potassium and osmolality on spermatozoan motility of salmonid fishes. *J. Exp. Biol.* 107:105–113.

Morisawa, M., M. Okuno, K. Suzuki, S. Morisawa & K. Ishida. 1983b. Initiation of sperm motility in teleosts. *J. Submicrosc. Cytol.* 15:61–65.

Morisawa, M., N. Funayama & H. Michibata. 1990. Involvement of zinc in the regulation of sperm motility in starfish and sea urchin. Proceedings of the sixty-first meeting of the zoological society of Japan, October 3-5, 1990, Niigata. *Zool. Sci.* 7:1045.

Pacey, A. A., J. C. Cosson & M. G. Bentley. 1994. The acquisition of forward motility in the spermatozoa of the polychaete *Arenicola marina*. *J. Exp. Biol.* 195:259–280.

Paniagua-Chavez & T.R. Tiersch. 2001. Laboratory studies of cryopreservation of sperm and trochophore larvae of the eastern oyster. *Cryobiology* 43:211–223.

Paniagua-Chavez, C. G., J. T. Buchanam & T. R. Tiersch. 1998. Effect of extender solutions and dilution on motility and fertilizing ability of eastern oyster sperm. *J. Shellfish Res.* 17:231–237.

Park, C. K. & M. A. Sirard. 1996. The effect of preincubation of frozen thawed spermatozoa with oviductal cells on the in vitro penetration of porcine oocytes. *Theriogenology* 46:1181–1189.

Piironen, J. 1993. Cryopreservation of sperm from brown trout (*Salmo trutta* m. lacustris L.) and Arctic charr (*Salvelinus alpinus* L.). *Aquaculture* 116:275–285.

SAS Institute. 1991. SAS system for linear models, 3rd ed. Cary, NC: SAS Institute, 327 pp.

Scheerer, P. D. & G. H. Thorgaard. 1989. Improved fertilization by cryopreserved rainbow trout semen treated with theophylline. *Prog. Fish-cult* 51:179–182.

Sunitha, M. S. & V. Jayaprakas. 1997. Influence of pH, temperature, salinity and media on activation of motility and short term preservation of

spermatozoa of an estuarine fish, *Mystus gulio* (Hamilton) (Siluridae-Pisces). *Indian J. Mar. Sci.* 26:361–365.

Tan-Fermin, J. D., T. Miura, S. Adachi & K. Yamauchi. 1999. Seminal plasma composition, sperm motility and milt dilution in the Asian catfish *Clarias marcrocephalus* (Gunther). *Aquaculture* 171:323–338.

Thorogood, J. & A. Blackshaw. 1992. Factors affecting the activation, motility and cryopreservation of the spermatozoa of the yellowfin bream, *Acanthopagrus australis* (Guenther). *Aquacult. Fish Manage.* 23:337–344.

Tiersch, T. R., C. A. Goudie & G. J. Carmichael. 1994. Cryopreservation of channel catfish sperm: Storage in cryoprotectants, fertilization trials and growth of channel catfish produced with cryopreserved sperm. *Trans. Am. Fish. Soc.* 123:580–586.

Tiersch, T. R., C. R. Figiel Jr, W. R. Wayman, J. H. Carmichael & O. T. Gorman. 1998. Cryopreservation of sperm of the endangered razorback sucker. *Trans. Am. Fish. Soc.* 127:95–104.

Wallace, D. H. 1966. Oysters in the estuarine environment. A symposium of estuarine fisheries. *Am. Fish. Soc. Spec. Publ.* 3:68–73.

Yankson, K. & J. Moyse. 1991. Cryopreservation of the spermatozoa of *Crassostrea tulipa* and three other oysters. *Aquaculture* 97:259–267.

Yoshida, N., K. Inaba & M. Morisawa. 1992. Partial purification of sperm-activating and sperm-attracting substance in the ascidian, *Ciona intestinalis*. *Zool. Sci.* 9:1206.

Yoshida, N., K. Inaba, K. Ishida & M. Morisawa. 1994. Calcium and cyclic AMP mediate sperm activation, but Ca^{2+} alone contribute sperm chemotaxis in the ascidian, *Ciona savignyi*. *Dev. Growth Differ.* 36:589–595.

Yu, D., J. Chen, T. Su, H. Zhu & S. Jiang. 1998. A primary study on experimental biology of spermatozoa of *Pinctada fucata* (Gould). *Trop. Oceanol./Redai Haiyang* 17:83–87.

Zell, S. R., M. H. Bamford & H. Hidu. 1979. Cryopreservation of spermatozoa of the American oyster *Crassostrea virginica* Gmelin. *Cryobiology* 16:448–460.