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Changes in motility, ultrastructure, and fertilization capacity of striped bass *Morone saxatilis* spermatozoa following cryopreservation

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Abstract

In the present study, motility, ultrastructure and fertilization capacity of fresh and cryopreserved striped bass spermatozoa were investigated in order to evaluate semen dilution ratio, freezing rate and cryomedia. Four dilution ratios (semen/cryomedia), and four freezing rates were evaluated on the basis of post-thaw sperm motility. The dilution ratio of 1:3 yielded the highest ($P<0.05$) post-thaw motility. Sperm cryopreserved with a freezing rate of $-40\,^{\circ}C\ min^{-1}$ resulted in a higher percentage of motile sperm ($P<0.05$) than other lower freezing rates we examined. Six cryomedia with various dimethyl sulfoxide (DMSO) and glycine concentrations were tested for their influences on ultrastructure, post-thaw motility and fertilizing capacity of cryopreserved sperm. The ultrastructural results revealed that the plasma membranes of spermatozoa were better protected with the higher DMSO concentrations we examined. Two cryomedia containing 5% or 7.5% DMSO, both with glycine added, resulted in the highest ($P<0.05$) post-thaw motility compared with other cryomedia without glycine. The percentage of eggs fertilized with sperm cryopreserved in six cryomedia ranged from $26\pm2.1\%$ (2.5% DMSO without glycine) to $54\pm5.6\%$ (7.5% DMSO with glycine), which were equivalent to 44% and 90% of fresh semen controls. No differences ($P>0.05$) were detected in the percentage of eggs fertilized among DMSO concentrations that did not contain glycine, although post-thaw motility did vary significantly ($P<0.05$) in these treatments. These results suggest that adding glycine to our basic cryomedia containing DMSO increases the fertilization capacity of these cryopreserved spermatozoa.

Keywords: Cryopreservation; Sperm; Fertility; Ultrastructure; Striped bass

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1. Introduction

Striped bass *Morone saxatilis* spermatozoa are used to fertilize the eggs of white bass *Morone chrysops* to produce the hybrid sunshine bass for the rapidly growing striped bass industry (Carlberg et al., 2000). Cryopreservation of striped bass spermatozoa is an effective method to aid the industry, by solving the problems relating to the asynchronous spawning seasons and geographic locations of striped bass and white bass (Harrell et al., 1990). Cryopreserved spermatozoa from striped bass can be easily stored until needed by the culture facilities where white bass females have been conditioned to spawn throughout the year. Similarly, frozen spermatozoa can be easily transported frozen in appropriate shipping dewars to overcome geographic barriers or to avoid the cost and associated risk of having to maintain both white bass and striped bass populations at any given facility.

Dimethyl sulfoxide (DMSO) has been shown to be a more effective cryoprotectant for striped bass spermatozoa when compared with other cryoprotectants commonly utilized for fish species including: methanol and dimethylacetamide, glycerol, ethylene glycol, and propylene glycol (Kerby, 1983; He and Woods, 2003b). In our previously published studies, extender, DMSO concentration and equilibration time has been standardized based on the measurement of post-thaw motility (Jenkins-Keeran and Woods, 2002; He and Woods, 2003b). Also, the addition of glycine to DMSO has been demonstrated to increase the percentage of motile striped bass sperm both in short term refrigerated storage as well as cryopreservation (He and Woods, 2003a). However, other important sperm quality parameters, such as ultrastructure and fertility, were not assessed in those studies. A low correlation between post-thaw motility and fertility has been reported (Kerby, 1983; Billard, 1988; Warnecke and Pluta, 2003). Different types of aberrations of normal spermatozoa morphology have been observed due to cryopreservation, such as ruptured plasma membranes in head, midpiece, and tail regions, swollen mitochondria, loss of mitochondrial cristae, and broken axoneme (Gwo and Arnold, 1992; Lahnsteiner et al., 1992; Conget et al., 1996; Lahnsteiner et al., 1996; Yao et al., 2000). Examination of ultrastructure and fertility would provide more useful information to establish a cryopreservation protocol for striped bass spermatozoa.

In the present study, the effects of semen dilution ratio and freezing rate on post-thaw motility were examined to further standardize the cryopreservation protocol for striped bass spermatozoa. Also, six cryomedia containing various DMSO and glycine concentrations were evaluated on the basis of ultrastructure, post-thaw motility, and fertility.

2. Materials and methods

2.1. Collection of gametes

Domesticated striped bass brood stock were produced at the University of Maryland's Crane Aquaculture Facility (UM-CAF) and then grown to maturity in 8600 l circular tanks, part of a recirculating water system. The fish were fed striped bass brood stock diet twice daily to satiation. The photothermal cycle, similar to that produced on average for the latitudes of Chesapeake Bay, MD, USA, was generated by computer-controlled

(Graphic Eye Model 3000, Lutron Electronics, Coopersburg, PA) lights, chillers and heaters to successfully induce sexual maturation. In the spring of 2003, 3-year-old males were distributed into three 1600-l circular tanks, and held at 15 ± 1 °C until our 5-week study was completed. Each fish was administered a cholesterol cellulose implant (Sherwood et al., 1988) containing 150 μg of mammalian gonadotropin-releasing hormone, [D-Ala^6-Pro^9-NEt]-LHRH (GnRHa, Sigma, St. Louis, MO), inserted into the dorsal lymphatic sinus, as previously described for domestic striped bass (Woods and Sullivan, 1993). To collect fresh semen, males were anesthetized in a 70 mg l^{-1} quinaldine bath (Woods et al., 1992), and urine was removed by applying gentle pressure around the urogenital vent. Semen was expressed directly into 50 ml sterile conical tubes and placed immediately on ice.

Also during the 2003 spring spawning season (April–May), ovarian biopsies were obtained from mature, 4-year-old domestic female striped bass brood fish. Females whose follicle diameters were >900 μm were administered GnRHa implants as described above for the males, placed individually into 1600 l tanks and held at 17 ± 1 °C until ovulation. Once oocytes reached the 10-h stage (Rees and Harrell, 1990), the females were injected with human chorionic gonadotropin (hCG; 150 IU kg^{-1} body weight; Chorulon®; Intervet, Millsboro, DE). Fish ovulated approximately 36 h after the hCG injection and were stripped of their eggs into a 6-l clean, dry, polypropylene pan.

2.2. Motility analysis

2.2.1. Semen dilution ratio

Four semen dilution ratios were evaluated on the basis of post-thaw motility. Fresh semen was diluted 1:1, 1.2, 1:4 or 1:8 (semen/extender) with the extender (14 g l^{-1} NaCl, 0.4 g l^{-1} KCl, 0.25 g l^{-1} $NaHCO_3$, 1 g l^{-1} glucose, pH adjusted to 7.6, osmolality adjusted to 500 mosM kg^{-1}). The extended samples were subsequently diluted 1:1 with the same extender containing 10% DMSO. The final dilution ratios were 1:3, 1:5, 1:9 or 1:17 (semen/cryomedia), and the final DMSO concentration was 5%. Aliquots (150 μl) of the final sperm mixture were quickly pipetted into 500 μl cryo-straws (TS Scientific) and sealed. The equilibrate time was 10 min (He and Woods, 2003b). Cryo-straws containing sperm samples were frozen using a programmable freezer (Planer Kryosave-Model KS30) with a freezing rate of −40 °C min^{-1} until −120 °C was achieved (Jenkins-Keeran and Woods, 2002). Once the straws containing sperm reached −120 °C, the samples were placed into liquid nitrogen. To determine post-thaw motility, straws were thawed in a 35 °C water bath for 8 s (He and Woods, 2003b) after 7 days. A small amount of thawed semen sample containing approximately 200–400 sperm cells was immediately placed into a Makler counting chamber (TS Scientific) and 10 μl filtered deionized water was added and quickly mixed. The loaded Makler chamber was immediately placed under a compound microscope (Zeiss model D-7082) at 400×. The activation of each sample was recorded on videotape using a Hitachi Model KP-140 high contrast, video camera attached to the microscope. By review of the recorded activations, the percentage of motile sperm was determined as the number of motile sperm divided by the total number of sperm. All samples ($n = 5$) were observed and counted by the same individual to avoid bias.

2.2.2. Freezing rate

Four freezing rates were further examined. Fresh semen was extended 1:3 (semen/cryomedia) with the same extender as described above containing 5% DMSO and 50 mM glycine (final concentration). Semen samples were frozen at the rate of -10, -20, -30 or -40 °C min^{-1}. Post-thaw motility was evaluated as described above.

2.2.3. Cryomedia

Based on our previous studies (He and Woods, 2003a,b), six cryomedia with the final concentration of 2.5%, 5%, 7.5%, 10% DMSO, 50 mM glycine with 5% DMSO, or 75 mM glycine with 7.5% DMSO, were chosen to evaluate the effects of DMSO and glycine on cryopreservation of striped bass spermatozoa. Fresh semen collected from three male striped bass in April 2003 was diluted 1:3 (semen/cryomedia) with one of the six cryomedia. The extended samples were cryopreserved as described above with the freezing rate of -40 °C min^{-1}. The cryopreserved semen samples were kept until needed for the fertilization trials. Post-thaw sperm motility and duration were evaluated before fertilization trials. Sperm motility was estimated as described above. The duration of sperm motility was timed beginning with the addition of activation solutions (deionized water) to the sperm sample and ending when the majority (approximately 90%) of sperm in the field of view had stopped moving.

2.3. Fertilization trials

Two fertilization trials were conducted in May 2003. In trial 1, two treatments (5% DMSO with 50 mM glycine, and 7.5% DMSO with 75 mM glycine) were examined. In trial 2, the treatments were expanded to 2.5%, 5%, 7.5%, 10% DMSO, 5% DMSO with 50 mM glycine, and 7.5% DMSO with 75 mM glycine. Fresh striped bass sperm were used as the control in both trials.

Sixteen domesticated striped bass females were selected for fertilization trials. However, due to the difficulties associated with accurately predicting the time of ovulation, eggs from a single female were utilized for each fertilization trial. The freshly stripped eggs and fresh sperm were immediately taken to the lab. Aliquots (1 ml) of eggs (1000–1200) were placed into 300 ml labeled glass dishes. For each treatment, two straws (150 μl cryopreserved sperm each) were thawed in a 35 °C water bath for 8 s, and the contents of both straws were immediately placed directly on the eggs in each dish. This provided a sperm to egg ratio of approximately 4×10^6:1. Each treatment was repeated three times. For the control, 1 ml of undiluted fresh sperm was placed on 1 ml of eggs. This increased quantity of sperm was used establish the maximum fertilization potential of stripped eggs. After the sperm were added and gently mixed with the eggs, 4 ml hatchery water (18 °C) was added to the dish. After 5–10 min, the egg and sperm mixture from each glass dish was poured into labeled beakers containing 600 ml of hatchery water. The beakers were maintained at 18 ± 1 °C and supplied with oxygen. The number of fertilized eggs was determined at early blastula stage as recommended by Harrell et al. (1990) with a dissecting microscope (Nikon SMZ-10) at $40\times$.

2.4. Transmission electron microscope

Sperm samples were fixed with a paraformaldehyde–glutaraldehyde–osmium tetroxide solution, following the method of Lahnsteiner and Patzner (1991). Briefly, sperm samples were mixed with the solution (semen/fixative = 1:2), which consisted of 10% paraformaldehyde, 5% glutaraldehyde and 2% osmium tetroxide. After fixing the sample on ice for 15 min, samples were washed in double distilled water and dehydrated with a series concentration of ethanol. Samples were then embedded in Spurr's resin. Ultrasections were stained with 2% uranyl acetate followed by lead citrate, and were examined under a transmission electron microscope (Zeiss model EM10 CA).

2.5. Statistical analysis

The statistical analysis was performed with SAS version 8.0 (SAS Institute, Cary, NC). Data are presented as means ± standard error of the mean (S.E.M.). A randomized block design was applied for fertilization trials to minimize error due to the variation of time between egg aliquots that were exposed to air before adding the cryopreserved sperm samples. Data were analyzed by one-way analysis of variance (ANOVA), and pairwise contrasts were used to identify significant differences at the 5% level between the means. The correlation procedure was used to estimate the correlation coefficients.

3. Results

3.1. Post-thaw motility

The dilution ratio of 1:3 resulted in the highest post-thaw motility when compared with other dilution ratios (Table 1). However, no significant difference ($P>0.05$) in post-thaw motility was detected between the ratios of 1:3 and 1:5. Post-thaw motility was also significantly ($P<0.01$) affected by freezing rate. The higher freezing rate tested achieved higher post-thaw motility (Table 2). In fertilization trials, effects on post-thaw motility and duration of motility of six cryomedia were assessed (Table 3). Post-thaw motility and duration of motility varied from 3 ± 0.8% (10% DMSO) to 49 ± 2.8% (5% DMSO with 50 mM glycine), and from 10 ± 1.5 to 18 ± 0.3 s, respectively. No differences in post-thaw motility and duration were observed between 2.5% and 5% DMSO. However, further increasing DMSO concentration significantly ($P<0.05$) reduced both post-thaw sperm motility and duration. The adding of glycine increased post-thaw motility, but no effect on duration of motility.

Table 1
Percentage of post-thaw motility (mean ± S.E.M.) of striped bass sperm cryopreserved with different dilution ratios (semen/cryomedia)

Dilution ratio	1:3	1:5	1:9	1:17
Post-thaw motility (%)	38 ± 2.1^{a}	31 ± 2.4^{a}	18 ± 1.6^{b}	18 ± 1.0^{b}

Values with different superscript letters are significantly different ($P<0.05$).

Table 2
Percentage of post-thaw motility (mean ± S.E.M.) of striped bass sperm cryopreserved with different freezing rates

Freezing rate (°C min^{-1})	−10	−20	−30	−40
Post-thaw motility (%)	34 ± 3.2^{a}	37 ± 2.2^{a}	44 ± 2.8^{b}	53 ± 3.8^{c}

Values with different superscript letters are significantly different ($P < 0.05$).

3.2. Ultrastructural alterations

Photomicrographs of samples of spermatozoa cryopreserved with various cryomedia revealed swollen or ruptured membranes in head, midpiece and tail regions (Fig. 1). Spermatozoa cryopreserved with 5 or 10% DMSO exhibited better defined and continuous plasma membranes when compared with 2.5% DMSO (Fig. 1A–C). Glycine did not provide additional protection to the plasma membranes in the presence of 5% DMSO (Fig. 1B and D). Only one or two mitochondria could be observed (Fig. 1C and D). Mitochondria often exhibited vacuolization and disrupted cristae (Fig. 1C). The axoneme was the typical 9 + 2 structure (Fig. 1E). Vacuoles were observed between the flagella membrane and axoneme (Fig. 1E and F).

3.3. Fertilization trials

In fertilization trial 1, sperm cryopreserved in 7.5% DMSO with 75 mM glycine yielded the highest ($P < 0.05$) egg fertilization percentage of 50 ± 2.1% or 89% of the control (Table 3). Sperm cryopreserved in 5% DMSO with 50 mM glycine had an egg fertilization percentage of 39 ± 1.4% or 69% of the control. No difference ($P > 0.05$) in the egg fertilization percentage was observed between the control and the 7.5% DMSO with 75 mM glycine treatment.

In fertilization trial 2, six treatments with various DMSO concentrations with or without glycine were chosen for further evaluation (Table 3). The fertilization percen-

Table 3
Percentage of post-thaw sperm motility, duration of motility, and fertility (mean ± S.E.M.) of fresh and cryopreserved striped bass sperm in fertilization trials

Trial no.	Treatment	Motile sperm (%)	Duration of motility (s)	Fertilization rate (%)
1	Fresh semen	88 ± 1.9^{a}	29 ± 0.6^{a}	57 ± 0.2^{a}
	5% DMSO + 50 mM glycine	53 ± 2.6^{b}	18 ± 0.5^{b}	39 ± 1.4^{b}
	7.5% DMSO + 75 mM glycine	42 ± 2.1^{c}	18 ± 0.8^{b}	50 ± 2.1^{a}
2	Fresh semen	91 ± 2.4^{a}	29 ± 0.5^{a}	60 ± 6.9^{a}
	2.5% DMSO	35 ± 2.9^{c}	18 ± 0.3^{b}	26 ± 2.1^{c}
	5% DMSO	36 ± 4.4^{c}	17 ± 0.5^{b}	33 ± 0.7^{bc}
	5% DMSO + 50 mM glycine	49 ± 3.5^{b}	17 ± 0.3^{b}	45 ± 8.3^{ab}
	7.5% DMSO	7 ± 1.3^{d}	12 ± 0.6^{c}	36 ± 5.9^{bc}
	7.5% DMSO + 75 mM glycine	45 ± 2.0^{b}	18 ± 0.3^{b}	54 ± 5.6^{a}
	10% DMSO	3 ± 0.9^{d}	10 ± 1.5^{c}	28 ± 3.5^{bc}

In each trial, values with different superscript letters within a column are significantly different ($P < 0.05$).

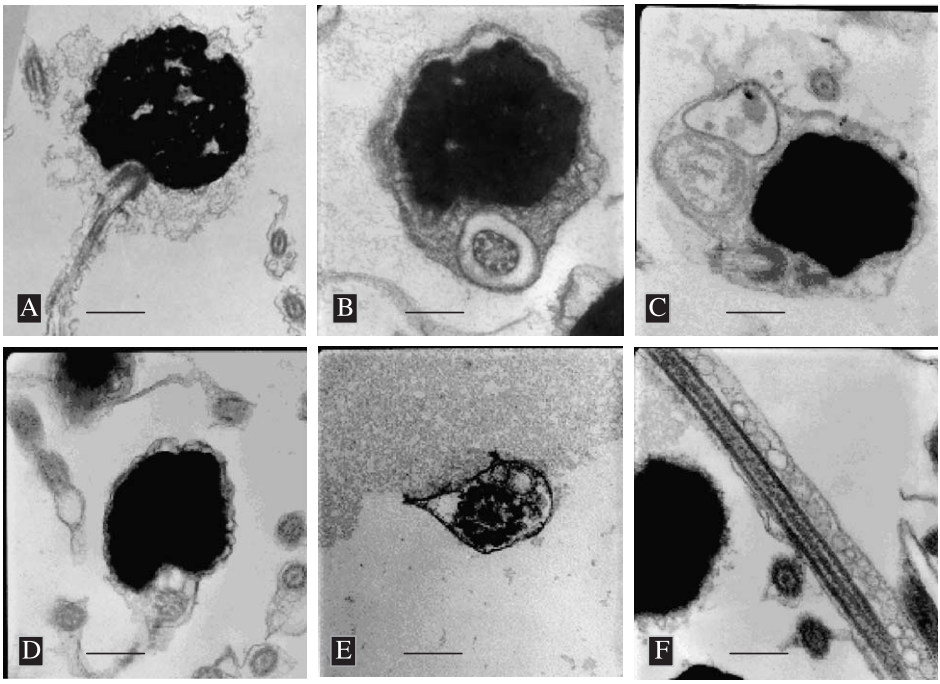

Fig. 1. Transmission electron photomicrographs of post-thaw striped bass spermatozoa. (A) Spermatozoa cryopreserved with 2.5% DMSO (bar = 0.6 μm); (B) spermatozoa cryopreserved with 5% DMSO (bar = 0.4 μm); (C) spermatozoa cryopreserved with 10% DMSO (bar = 0.5 μm); (D) spermatozoa cryopreserved with 5% DMSO and 50 mM glycine (bar = 0.5 μm); (E) axoneme cross-section, spermatozoa cryopreserved with 2.5% DMSO (bar = 0.2 μm); (F) flagellum, spermatozoa cryopreserved with 5% DMSO (bar = 0.5 μm).

tages of six treatments ranged from 26 ± 2.1% (2.5% DMSO) to 54 ± 5.6% (7.5% DMSO with 75 mM glycine), which were 44% and 90% of the fresh control, respectively. Glycine showed a significantly positive ($P < 0.05$) effect on fertilization. Glycine with 7.5% DMSO resulted in a higher ($P < 0.01$) fertilization rate than 7.5% DMSO alone. No differences ($P > 0.05$) were detected in fertilization rates among various DMSO concentrations without added glycine, although there were significant differences in post-thaw motility (Table 3). The fertilization rate was not correlated ($r = 0.45$, $P > 0.05$) with post-thaw motility.

4. Discussion

The optimal semen dilution ratio in cryomedia varied among species. In the present study, the lowest dilution ratio examined obtained the highest post-thaw motility. This may be related to the fact that striped bass sperm can be activated by increasing the dilution ratio in extender. The activation mechanism of striped bass sperm is not clear. It has been demonstrated that neither K^+, H^+, Ca^{2+}, Mg^{2+}, cAMP, nor osmolality is the key factor to

maintain sperm quiescent in seminal plasma, and the dilution of some unknown factor in seminal plasma may trigger activation of sperm motility (He et al., 2004).

In the experiment on freezing rate, glycine was used as a non-permeating cryoprotectant in combination with 5% DMSO. Glycine dehydrates sperm before freezing, therefore freezing points may be decreased and optimal freezing rates may differ from those of cryomedia without glycine. However, our data indicating that the freezing rate of -40 °C min^{-1} yielded the highest post-thaw motility agreed with the result obtained with cryomedia containing 5% DMSO without glycine (Jenkins-Keeran and Woods, 2002). With the increasing of freezing rate we examined, post-thaw motility also increased. This suggests that a freezing rate higher than -40 °C min^{-1} may be more appropriate for striped bass sperm. However, since our programmable freezer can only create consistent freezing rates up to -40 °C min^{-1}, we were unable to examine higher freezing rates.

Glycine has been demonstrated to improve post-thaw motility in a variety of species including: sea urchin (Tyler and Atkinson, 1950), bovine (Roy and Bishop, 1954) and goat (Kundu et al., 2001). In the present study, consistently high fertilization rates were achieved with striped bass spermatozoa cryopreserved with a cryomedia containing moderately high levels of DMSO and glycine. Our fertilization data suggests that glycine has the ability to improve fertilization capacity of striped bass spermatozoa cryopreserved with DMSO as the cryoprotectant. The mechanism by which glycine improves fertilization capacity of cryopreserved spermatozoa is not clear. It has been hypothesized that glycine helps maintain structural stability of the plasma membrane during the freeze–thaw process (Anchordoguy et al., 1988; Lahnsteiner et al., 1992). Our ultrastructure results did not demonstrate that glycine provided any additional protection to the plasma membranes. Glycine was found to increase post-thaw motility; however, our data indicates a very low correlation between post-thaw motility and the percentage of eggs fertilized. The observed ultrastructural damage, mainly in plasma membranes and mitochondria, were similar to those found in cryopreserved spermatozoa of Atlantic croaker *Micropogonias undulates* (Gwo and Arnold, 1992), grayling *Thymallus thymallus* (Lahnsteiner et al., 1992), and rainbow trout *Oncorhynchus mykiss* (Conget et al., 1996; Lahnsteiner et al., 1996). Our findings show that protection to plasma membranes was cryomedia-dependent. This is in agreement with the results obtained by Gwo and Arnold (1992) and Lahnsteiner et al. (1992). It has been proposed that cell damage from cryopreservation might largely be induced by osmotic shock and/or intracellular ice-crystals (Leung, 1991). The present study results indicating that higher DMSO concentrations provided better protection to plasma membranes, suggests that the damage is not due to high DMSO osmolality, but more likely because of the formation of intracellular ice-crystals.

The low correlation between post-thaw motility and fertilizing capacity in this study supports the conclusions drawn by Kerby (1983), who found that immotile post-thaw striped bass spermatozoa were capable of fertilization, albeit with widely varying rates (0–88%). Similar findings have also been reported for other species, such as the common carp *Cyprinus carpio* (Billard, 1988; Warnecke and Pluta, 2003) and Atlantic croaker (Gwo et al., 1991). Because spermatozoa need to possess many attributes (motility, an intact plasma membrane, ability to bind to the oolemma, etc.) in order to fertilize an oocyte, a single attribute measurement of sperm, motility for example, may not provide reproducible results that correlate well with fertility. The more attributes examined and utilized, the

higher correlation with fertility that may be obtained (Graham, 2001). Linhart et al. (2000) found a high correlation coefficient when using two parameters including motility as well as velocity, but not with motility alone. In future studies, establishing a model with multiple parameters, such as motility, plasma membrane integrity, and mitochondrial function, may provide valuable information to better predict fertilization potential of cryopreserved spermatozoa.

It has been reported that fertilization may be affected by the sperm to egg ratio (Den Daas, 1992; Lahnsteiner et al., 1996; Warnecke and Pluta, 2003). As the number of sperm increases, fertility increases until it reaches a maximum (Den Daas, 1992). To achieve the maximum fertilization rate with fresh sperm, sperm to egg ratios can be reduced from 1×10^6:1 to 3×10^3:1 (Warnecke and Pluta, 2003). With cryopreserved rainbow trout spermatozoa, Lahnsteiner et al. (1996) reported that a minimum ratio of 3×10^6 sperm/egg was required in order to yield similar fertilization to that of fresh sperm. In this study, the ratio of 4×10^6 sperm/egg was used. The results suggest that this ratio is high enough for striped bass sperm cryopreserved with 7.5% DMSO and 75 mM glycine to achieve fertilization rates nearly that of the fresh sperm controls.

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