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The Cryopreservation of Striped Bass *Morone saxatilis* Semen

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Abstract.—Two experiments were designed to improve upon existing methods for cryopreserving striped bass *Morone saxatilis*, semen. In the first experiment, two extenders, two cryoprotectant concentrations, and two freezing rates were evaluated on the basis of post-thaw semen motility after 1, 7, and 30 d of storage at −196 C. Semen samples cryopreserved at a freezing rate of −40 C/min resulted in a significantly higher percentage of motile sperm ($P < 0.001$) and longer duration of spermatozoa motility ($P < 0.001$) than samples cryopreserved at a freezing rate of −30 C/min. Also, the cryoprotectant dimethyl-sulfoxide yielded a significantly higher percentage of motile sperm ($P < 0.001$) and longer duration of spermatozoa motility ($P < 0.001$) when a 5% concentration was used instead of 7.5%. In the second experiment, the two extenders from Experiment 1 were re-evaluated and a new extender, which was a modified version of Extender 1, was tested. The samples were cryopreserved at −40 C/min with 5% DMSO and thawed in a 25 C water bath. Spermatozoa motility and fertilization ability were evaluated, and semen cryopreserved in Extender 2 yielded the longest duration of spermatozoa motility ($P < 0.001$), the highest percentage of motile sperm ($P < 0.001$), and the highest percentage of fertilized eggs ($P < 0.002$) in comparison to Extenders 1 and 3.

Cryopreservation of spermatozoa is a widely used practice in animal husbandry that is now being intensively studied for use in aquaculture. Successful cryopreservation techniques are needed to enhance and broaden the seasonal period of seedstock production as well as to establish gene banks. Gene banks would allow commercial production facilities to reduce the number of male broodstock maintained on site, expedite shipping of sperm to other facilities, preserve superior genomes (Kerby 1983), increase the genetic diversity of a population (Piironen 1993), and possibly aid in the conservation of important genetic information and/or the protection of threatened and endangered species (Gwo et al. 1991).

The striped bass *Morone saxatilis* industry is one of the fastest growing segments of finfish aquaculture in the United States (Striped Bass Growers Association 1998). The major limitation to this industry's growth is its reliance on wild broodstock for seedstock. To overcome this, domesticated broodstocks and methods for their controlled reproduction must be developed (Smith 1989; Harrell and Webster 1997). Although progress has been made (Woods et al. 1999), researchers are still trying to find solutions to many of the reproductive problems associated with striped bass held in captivity (Zohar 1989; Sullivan et al. 1991, 1997; Woods and Sullivan 1993; Mylonas et al. 1998). One of the more significant problems faced by striped bass hatchery biologists is the different spawning times for fish of different sex and geographical location, as well as the current inability to spawn fish year round. The problem of differing spawning times becomes especially acute when trying to produce hybrids such as the current industry standard, the sunshine bass (a white bass female *Morone chrysops* crossed with a striped bass male). Successful cryopreservation of striped bass semen could help mitigate these problems.

There has been limited research on the cryopreservation of striped bass semen. The only peer-reviewed publications to date are two papers presenting quantitative data by Kerby (1983, 1984) and a qualitative description of striped bass and white bass semen cryopreservation by Brown and Brown (2000). Kerby had limited success in cryo-

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preserving striped bass sperm. Most of his cryopreserved samples exhibited little or no post-thaw motility, and there was a wide range of variability between individual samples in each trial (from 0 to 88% fertilization). Kerby found when comparing the cryoprotectants glycerol, methanol, and dimethyl sulfoxide (DMSO), that DMSO was the only cryoprotectant that yielded any motility or fertilization post-thaw. Brown and Brown detailed a cryopreservation procedure for striped bass semen but referred only to unpublished data in their article. In this study, two experiments were performed to improve upon existing methods for the cryopreservation of striped bass semen. Three extenders, two DMSO concentrations, and two freezing rates were evaluated with performance based on post-thaw semen fertility and/or motility.

Materials and Methods

Experiment 1

Semen was pooled from six striped bass in the middle of the spawning season on 28 April 1998. To obtain the initial pre-freezing motility, a sub-sample of the pooled undiluted semen was activated immediately after collection in replicate samples of three, using deionized ultra-filtered water (DIUF). Spermatozoa motility was recorded on videotape using a Hitachi model KP-140 video camera attached to a Zeiss model D-7082 compound microscope at a magnification of 400×.

After initial motility was recorded, half of the pooled semen was extended with Extender 1 and half with Extender 2 in a 2:1 ratio of extender:semen (Table 1). The cryoprotectant DMSO was added in concentrations of 5 and 7.5% to semen of each extender. To obtain a 5% DMSO concentration, 0.6 mL of DMSO was added to 11.4 mL of extended semen. To obtain a 7.5% DMSO concentration, 0.9 mL of DMSO was added to 11.1 mL of extended semen.

The extended samples were added in aliquots of 0.35 mL into 0.5-mL straws and sealed. The total time the extended semen and cryoprotectant were allowed to equilibrate before freezing was 36 min (the amount of time it took to fill the straws). Sixteen straws from each extender and cryoprotectant combination were frozen at a rate of −30 C/min or −40 C/min using a Planer, Kryosave Model KS30 controlled rate freezer. Once the samples reached −120 C, they were immediately plunged into liquid nitrogen.

TABLE 1. *Extender formulas for experiments 1 and 2.*

	Extender		
	1[a]	2[b]	3
Glucose (mg)	100	—	100
NaCl (mg)	750	860	870
$NaHCO_3$ (mg)	200	—	200
KCl (mg)	40	—	40
Water[c] (mL)	100	100	100
Egg yolk (mL)	20	—	20
Osmolality (mmol/kg)	290	270	310
pH[d]	7.6	7.6	7.6

[a] Formula taken from Stein and Bayrle (1978).

[b] Formula modified from Brown and Brown (2000).

[c] Water used was Fisher's deionized ultra-filtered water (DIUF).

[d] Extenders were mixed and then pH adjusted with HCl or NaOH.

A total of 16 straws were made for each treatment to allow three replicate straws to be thawed after 1, 7, and 30 d of storage. Seven extra straws for each treatment were made in case physical damage occurred to the straws during the freezing or thawing process. The straws were thawed by direct plunge into a 25-C water bath for 12 sec. The thawed semen was immediately poured out of the straw and activated with DIUF. As with the undiluted semen samples, the motility obtained from cryopreserved semen samples was recorded on videotape. The percentage of motile sperm and the amount of time the spermatozoa remained active were determined from the videotapes. The percentage of motile sperm was estimated for all samples by the same observer to avoid bias. Duration of spermatozoa motility was timed beginning the in-

stant the DIUF was mixed with semen and ending when the majority (approximately 90%) of the sperm in the field of view had stopped moving. Spermatozoa that simply vibrated or did not show progressive forward movement were not considered motile. Duration of spermatozoa motility for each straw was measured three times with a digital stopwatch and recorded to the nearest 1/100 sec.

Experiment 2

Semen was collected from six striped bass on 13 April 1999. Motility for the undiluted semen from each fish was recorded on videotape, and the percentage of motile sperm and the duration of spermatozoa motility were measured using the same procedures as those described in Experiment 1. The formulas for the extenders used in Experiment 2 are listed in Table 1. Extenders 1 and 2 were carried over from the first experiment and evaluated against Extender 3. Extender 3 is a modification of Extender 1. Striped bass semen diluted with Extender 3 yielded a higher percentage of motile sperm after 1, 2, and 7 d of storage at 4 C than semen diluted with Extender 1 or Extender 2 (Jenkins 1999).

Semen from each fish was divided into three portions of 1.5 mL each and diluted with 3 mL of Extender 1, Extender 2, or Extender 3. An additional 3 mL of each extender containing 0.375 mL of DMSO was then added to each 4.5-mL portion of semen diluted with the same extender. The final concentration of DMSO was 5% and the final dilution of extender + cryoprotectant: semen was 4:1.

The extended samples with cryoprotectant were added in aliquots of 0.25 mL into 0.5-mL straws and sealed. The total time the extended semen and cryoprotectant were allowed to equilibrate before freezing was 25 min. This was the amount of time needed to fill all the straws for one fish. Straws were frozen at a rate of −40 C/min to a temperature of −120 C using a controlled rate freezer. The samples were then plunged directly into liquid nitrogen. A total of 30 straws were made for each fish and extender combination.

After 48 h of storage, three straws from each fish and extender combination were thawed in a 25-C water bath and activated with DIUF. Motility for each sample was recorded on videotape and the percentage of motile sperm and duration of spermatozoa motility was measured using the same procedures as those described in Experiment 1. The six fish from which semen was cryopreserved were moved to individual 2-m diameter tanks with recirculating water. Mature female striped bass were anesthetized in a 70 mg/L quinaldine bath (Woods et al. 1992) and carefully catheterized to collect a sample of their oocytes. The oocytes were examined with a Nikon model SMZ-10 dissecting microscope to determine their stage of development. The stage of oocyte maturation was determined according to the methods outlined by Rees and Harrell (1990). Six females with oocytes staged at 10 h from ovulation were injected intramuscularly with 330 IU/kg of human chorionic gonadotropin (hCG), and each was placed into a 2-m diameter tank containing a male striped bass. Beginning 24 h after the hCG injection, oocyte samples were periodically collected by catheterization of the ovary from each female to re-estimate time until ovulation. Fish are sampled between 20 and 28 h after injection because it normally takes between 15–20 h for hCG hormone to have an effect and samples taken before 20 h will probably result in a false reading (Rees and Harrell 1990). At ovulation, each female was removed from the tank and anesthetized. The urogenital vent was wiped clean and dry and the eggs were stripped into a clean, dry pan. The male striped bass from the same tank was then removed, anesthetized, and semen was collected into a separate container in the same manner as the female.

Samples of approximately 900 eggs (1 mL) were placed into four 300-mL glass dishes. Due to the limited volume of cryo-

preserved semen from each fish, only the semen from one male could be used to fertilize the eggs from one female. Eighteen straws (six samples of each extender) of cryopreserved semen from the same fish that the fresh, undiluted semen was collected from were thawed in a 25-C water bath for approximately 12 sec. Immediately after thawing, six straws of cryopreserved semen from each extender were placed directly on top of 1 mL of eggs. To insure that each treatment contained approximately the same sperm/egg ratio, only 0.3 mL of undiluted semen was added to the control eggs. About 300 mL of hatchery water was then added to each dish, and the eggs and semen were gently mixed. After 5 min, the entire contents of the glass dishes were poured into labeled beakers containing 600 mL of clean hatchery water. The beakers were maintained at a temperature of 24 ± 1 C and supplied with pure oxygen. After 12 h, all eggs were examined for the presence of a germ ring to confirm fertilization and estimate its percentage.

Statistical Analysis

Percentage of motile sperm, duration of spermatozoa motility, and in the second experiment, percentage of eggs fertilized from cryopreserved semen were analyzed by mixed model analysis of variance techniques using SAS version 8.2 (SAS Institute, Inc., Cary, North Carolina, USA). Percent fertile and percent motile were arcsine transformed to improve the analysis of variance assumption of normality. In the first experiment, the fixed portion of the model included the effects of storage time, extender, DMSO concentration, freezing rate, and all possible two, three, and four factor interactions. Only the residual variance was defined as random. In the second experiment, the fixed portion of the model included the effects of the extender, and the random portion of the model included the effect of the individual fish and the residual variance. In both experiments, goodness of fit statistics were used to examine the assumption of variance homogeneity. When variance heterogeneity was identified, residual variances were partitioned to satisfy the assumption. Pairwise comparisons between means were tested at the 5% level using t probabilities. Results are reported as means ± SEM and when data were transformed, the inverse transformed mean follows in parentheses.

Results

Experiment 1

The average percentage of motile sperm obtained from the freshly collected and pooled undiluted semen was 74 ± 3.3 (92%) with duration of motility of 26 ± 1.2 sec. Significant differences in the percentage of motile sperm were found between the freezing rates ($P < 0.001$), DMSO concentrations ($P < 0.001$), and days of storage ($P < 0.02$). Semen frozen at a rate of −40 C/min had a percentage of motile sperm approximately 7 times higher than semen frozen at a rate of −30 C/min. Semen frozen with 5% DMSO had a percentage of motile sperm approximately 7 times higher than semen frozen with 7.5% DMSO. The percentage of motile sperm increased as the days of storage increased but the only significant difference was between 1 and 30 d of storage where the percentage of motile sperm doubled. Significant two factor interactions were found between the day of storage and freezing rate ($P < 0.003$) and the freezing rate and DMSO concentration ($P < 0.001$). After 7 and 30 d of storage, the percentage of motile sperm obtained from semen frozen at −40 C/min was significantly higher than the percentage of motile sperm obtained from semen frozen at −30 C/min. No significant difference was seen between the two freezing rates after 1 d of storage. The percentage of motile sperm from semen frozen at −40 C/min with 5% DMSO was significantly higher (8–27 times higher) than the percentage of motile sperm from the other three freezing rate/DMSO concentration combinations. Significant three factor interactions

TABLE 2. *Sperm motility and duration of sperm motility obtained for undiluted and cryopreserved striped bass semen in Experiment 1. Within each day and column, means with different letters are significantly different at the 5% level by* t *probabilities.*

Day	DMSO concentration (%)	Freezing rate (C/min)	Extender	Motile sperm (%)[1]	Duration of sperm motility (sec)
0	0	None	1	74 ± 3.3 (92)	26.1 ± 1.2
1	5	−30	1	4 ± 3.8 b (1)	4.1 ± 3.6 b
1	5	−40	1	4 ± 3.8 b (1)	5.4 ± 3.6 ab
1	5	−30	2	4 ± 3.8 b (1)	3.9 ± 3.6 b
1	5	−40	2	19 ± 3.8 a (11)	15.6 ± 3.6 a
1	7.5	−30	1	0 ± 0.0 b (0)	0.0 ± 0.0 b
1	7.5	−40	1	0 ± 0.0 b (0)	0.0 ± 0.0 b
1	7.5	−30	2	0 ± 0.0 b (0)	0.0 ± 0.0 b
1	7.5	−40	2	0 ± 0.0 b (0)	0.0 ± 0.0 b
7	5	−30	1	0 ± 0.0 b (0)	0.0 ± 0.0 b
7	5	−40	1	24 ± 3.8 a (16)	19.3 ± 3.6 a
7	5	−30	2	2 ± 3.8 b (0)	4.5 ± 3.6 b
7	5	−40	2	21 ± 3.8 a (13)	16.2 ± 3.6 a
7	7.5	−30	1	4 ± 3.8 b (0)	4.6 ± 3.6 b
7	7.5	−40	1	0 ± 0.0 b (0)	0.0 ± 0.0 b
7	7.5	−30	2	0 ± 0.0 b (0)	0.0 ± 0.0 b
7	7.5	−40	2	0 ± 0.0 b (0)	0.0 ± 0.0 b
30	5	−30	1	0 ± 0 d (0)	0.0 ± 0.0 d
30	5	−40	1	29 ± 3.8 a (23)	17.0 ± 3.6 a
30	5	−30	2	4 ± 3.8 cd (0)	3.4 ± 3.6 cd
30	5	−40	2	18 ± 3.8 ab (10)	15.9 ± 3.6 ab
30	7.5	−30	1	0 ± 0.0 d (0)	0.0 ± 0.0 d
30	7.5	−40	1	9 ± 3.8 bc (2)	8.4 ± 3.6 abc
30	7.5	−30	2	0 ± 0.0 d (0)	0.0 ± 0.0 d
30	7.5	−40	2	6 ± 3.8 cd (1)	5.6 ± 3.6 bc

[1] Values are arcsine transformed means ± SEM (inverse transformed, mean percent).

were found between the day of storage, freezing rate, and DMSO concentration ($P < 0.02$) and the day of storage, extender, and freezing rate ($P < 0.03$). After 1 d of storage, the percentage of motile sperm obtained from semen frozen at −40 C/min with 5% DMSO was significantly higher than the percentage of motile sperm obtained from semen frozen at −40 C/min or −30 C/min with 7.5% DMSO but was not significantly different from semen frozen at −30 C/min with 5% DMSO. For 7 and 30 d of storage, the percentage of motile sperm obtained from semen frozen at −40 C/min with 5% DMSO was significantly higher than all other freezing rate/DMSO concentration combinations. After 1 d of storage, the percentage of motile sperm obtained from semen frozen at −40 C/min with Extender 2 was significantly higher than all other freezing rate/extender combinations. After 7 and 30 d of storage, there was no significant difference between extenders, but the percentage of motile sperm was significantly higher in semen frozen at −40 C/min than in semen frozen at −30 C/min. There was a significant four-factor interaction between day of storage, extender, freezing rate, and DMSO concentration ($P < 0.04$, Table 2).

For duration of spermatozoa motility, significant differences were found between the freezing rates ($P < 0.001$) and DMSO concentrations ($P < 0.001$) used. The duration of motility for semen cryopreserved using 5% DMSO or a freezing rate of −40 C/min was approximately 5 times greater

TABLE 3. *Spermatozoa motility, duration of sperm motility, and fertilization ability for undiluted and cryopreserved striped bass semen in Experiment 2. Means with different letters within a column are significantly different at the 5% level by* t *probabilities.*

Extender	Motile sperm (%)[1]	Duration of sperm motility (sec)	Fertility (%)[1]
Undiluted	72 ± 9.0 a (90)	26.0 ± 0.6 a	28 ± 3.3 a (22)
1	5 ± 3.9 c (1)	2.9 ± 3.6 c	10 ± 3.3 b (3)
2	22 ± 3.9 b (14)	15.5 ± 0.6 b	21 ± 4.5 a (13)
3	3 ± 3.9 c (0)	4.2 ± 3.6 c	9 ± 4.5 b (3)

[1] Values are arcsine transformed means ± SEM (inverse transformed, mean percent).

than the duration of motility for semen cryopreserved using 7.5% DMSO or a freezing rate of −30C/min. The only significant two factor interactions were between the day of storage and freezing rate ($P < 0.03$) and the freezing rate and DMSO concentration ($P < 0.001$). For 1 and 7 d of storage, the duration of spermatozoa motility was 2.5 to 4 times longer for the freezing rate of −40 C/min as compared to −30 C/min. For 30 d of storage, the duration of spermatozoa motility was 13 times longer for −40 C/min than for −30 C/min. Semen samples frozen with 5% DMSO at −40 C/min had a significantly longer duration of spermatozoa motility (5.6–19 times longer) than the semen frozen with the other DMSO/freezing rate combinations. There were no significant three or four factor interactions.

Experiment 2

There were significant differences in the percentage of motile sperm ($P < 0.001$), the duration of spermatozoa motility ($P < 0.001$), and the percentage of fertilized eggs ($P < 0.002$) obtained from fresh undiluted semen and semen cryopreserved with Extenders 1, 2, and 3 (Table 3). Due to the difficulty associated with estimating the time of ovulation in striped bass, the eggs collected from two of the six fish were overripe. Therefore, only the eggs from four females could be used in the fertilization portion of Experiment 2.

Discussion

In the first experiment, 5% DMSO yielded the highest post-thaw sperm motility ($P < 0.001$). These results support previous work where Kerby (1983) reported that 5% DMSO produced the highest fertilization percentages. However, Kerby's experiments and our experiments did not examine DMSO concentrations below 5%. Brown and Brown (2000) anecdotally reported obtaining their best results using 4% DMSO. Further controlled experimental research to examine the effectiveness of DMSO concentrations below 5% for the cryopreservation of striped bass semen is warranted.

In order for a cryopreservation procedure to be successful, the optimal freezing rate needs to be determined. An optimal freezing rate is fast enough to minimize exposure to harmful concentrated solutes and slow enough to allow water to leave the cell, thus preventing intracellular ice formation (Leung 1991). When Kerby (1983) cryopreserved striped bass semen using freezing rates ranging from −2.4 to −18.8 C/min, he found that freezing rates greater than −5 C/min resulted in higher fertilization percentages. In Experiment 1, the faster freezing rate of −40 C/min proved to be significantly better than the freezing rate of −30 C/min in terms of striped bass sperm motility post-thaw.

After the freezing process, semen samples should be stored at −130 C or below (Mazur 1984; Leung 1991). At these temperatures, biochemical reactions do not occur; therefore, samples can theoretically be stored for an infinite period of time (Mazur 1984; Leung 1991). However, in Experiment 1, the percentage of motile sperm in semen samples stored at −196 C was found

to significantly increase between 1 and 30 d. The percentage of motile sperm would not be expected to increase as storage time increased. This difference is most likely due to an inconsistency in the procedure. The majority of the cryopreserved semen samples were motile for only a few seconds. Also, the highest percentage of motile sperm in any given sample was seen immediately after activation and decreased dramatically within seconds. The amount of time required to mix the semen and activating medium before being placed under the microscope decreased with practice and repetition of the procedure. Therefore, a decrease of as little as 2 sec, in the amount of time to prepare a sample, could potentially result in what would appear to be an increase in the percentage of motile sperm.

Extender 2 proved to be the best extender for the cryopreservation of striped bass semen. The results of Experiment 1 showed only a small difference between the two extenders with the only significant difference occurring in the three factor interaction of the day of storage, extender, and freezing rate, and the four factor interaction of the day of storage, extender, DMSO concentration, and freezing rate. In the second experiment, a much larger difference could be seen between the extenders. Semen cryopreserved in Extender 2 yielded significantly higher post-thaw sperm motility and fertility than semen cryopreserved in Extenders 1 and 3 (Table 3).

In conclusion, the best results in terms of post-thaw sperm motility and fertility were obtained by cryopreserving striped bass semen in Extender 2 with 5% DMSO at a rate of −40 C/min. Possible areas of future research to improve upon this cryopreservation protocol could include the examination of DMSO concentrations less than 5%, cryoprotectant equilibration times less than 25 min, and straws larger than 0.5 mL.

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