An Evaluation of Extenders for the Short-Term Storage of Striped Bass Milt

KAREN JENKINS-KEERAN AND L. CURRY WOODS III*
Department of Animal and Avian Sciences, 2145 Animal Sciences Center, University of Maryland, College Park, Maryland 20742-2311, USA

Abstract.—Six extenders were evaluated in two experiments for use in the short-term, refrigerated storage of the milt of striped bass Morone saxatilis. In each experiment, milt samples were collected weekly. The percentage of motile sperm obtained from each extended milt sample and each undiluted milt sample (control) were measured after 1, 2, and 7 d of storage at 3 ± 1°C (mean ± SE). In the first experiment, there were significant changes in the percentage of motile sperm obtained across the spawning season with respect to the day of storage, the extender used, and the day × extender combination. Extender 1 yielded the highest percentage of motile sperm after 2 and 7 d of storage but was not significantly different from extender 4 after 1 d of storage. In the second experiment, two modified versions of extender 1 were compared with the original. Extenders 5 and 6 contained the same ingredients as extender 1 except that their sodium chloride concentrations were increased to obtain osmolalities more closely resembling that of striped bass milt. Extender 6 was closest to the osmolality of striped bass milt and yielded the highest percentage of motile sperm after 2 and 7 d of storage but was not significantly different from extender 5 after 1 d of storage. As in experiment 1, there were significant changes in the percentage of motile sperm obtained across the spawning season with respect to the day of storage, the extender used, and the day × extender combination. Sperm motility in the undiluted control samples and the extended milt samples significantly changed over the course of the spawning season, indicating a seasonal change in milt quality. At the beginning and end of the spawning season, milt quality was low and there was little, if any, significant difference in the performance of the various extenders. In the middle of each spawning season, when milt quality was highest, significant differences in the percentages of motile sperm obtained from the extended samples were evident.

The demand for striped bass Morone saxatilis and its hybrids has made it one of the fastest-growing segments of finfish aquaculture in the United States (Striped Bass Growers Association 1998). One of the main problems faced by hybrid striped bass producers is the different spawning times among fish of different sexes, species, and geographical locations. Asynchrony associated with the maturation of gametes and the time of spawning between the sexes has been documented in species such as walleye Stizostedion vitreum (Moore 1987) and salmonids (McNiven et al. 1993). The same is true of the striped bass, with the majority of males being ready to spawn at the beginning of the season before the females reach final oocyte maturation; consequently, when most of the females are ready to spawn, there may be too few flowing males (Parker et al. 1990). This problem can become acute when trying to produce hybrids. The current industry standard, the sunshine bass, is a female white bass Morone chrysops crossed with a male striped bass. Because the peak spawning season of the white bass is earlier than that of the striped bass (Kerby 1984), producing hybrids can be logistically difficult. Spawning fish of different sexes from different regions is equally difficult because striped bass depend on environmental cues, especially photoperiod and temperature, to initiate spawning (Sullivan et al. 1997).

To ameliorate these problems, researchers have examined procedures such as cryopreservation (Kerby 1983, 1984; Brown and Brown 2000; Jenkins-Keeran and Woods 2002), the domestication of broodstock (Leffler 1999; Woods et al. 1999), and control of the reproductive cycle (Woods and Sullivan 1993; Sullivan et al. 1997). Another potential solution is the short-term, refrigerated storage of striped bass milt.

Techniques for the short-term, refrigerated storage of milt have been developed for several teleosts, such as walleye (Moore 1987), red drum Sciaenops ocellatus (Wayman et al. 1998), Atlantic sturgeon Acipenser oxyrinchus (DiLauro et al. 1994), milkfish Chanos chanos (Hara et al. 1982), Mozambique tilapia Tilapia mossambica (Harvey and Kelley 1984), paddlefish Polyodon spathula (Brown and Mims 1995), channel catfish Ictalurus
punctatus (Christensen and Tiersch 1996), and several of the salmonids (Scott and Baynes 1980; Billard 1981; Erdahl et al. 1984; Jensen and Aldercliffe 1984; Erdahl and Graham 1987; McNiven et al. 1993). Although there is evidence of the successful short-term storage of milt from striped bass (Harrell 1997; Brown and Brown 2000; Jenkins-Keeran et al. 2001), current methods need to be improved.

The objective of this study was to evaluate the performance of six different extenders for the short-term storage of striped bass milt.

Methods

Animals.—In the first experiment, milt was collected once a week for 6 weeks beginning on 30 March 1998 from six fish randomly drawn from a population of 142 male striped bass. In the second experiment, milt was collected once a week for 7 weeks beginning on 6 April 1999, from six fish randomly drawn from a population of 115 male striped bass. The fish were housed in a 6-m-diameter circular tank receiving flow-through, ambient water from the Chesapeake Bay. Individual fish were identified by passive integrated transponders embedded just beneath the skin in the dorsal musculature on the left side.

Sample collection.—The striped bass broodfish were anesthetized with quinaldine at a concentration of 70 mg/L (Woods et al. 1992). The urine in the bladder was expressed and the urogenital vent was wiped clean and dried to prevent contamination by water, urine, and feces. The milt was expressed directly into sterile, 50-mL polypropylene conical tubes by applying gentle abdominal pressure. The milt was kept in an ice water bath (2 ± 1°C [mean ± SE]) or under refrigeration (3 ± 1°C) until analysis could be performed.

Extenders.—The formulas for the six extenders evaluated are listed in Table 1. Extenders 1 through 4 were used in the first experiment. Extender 1 has been used successfully in the cryopreservation of milt from rainbow trout Oncorhynchus mykiss and brown trout Salmo trutta (Stein and Bayrle 1978), as well as from Arctic char Salvelinus alpinus (Pillaron 1993). Extender 2 is used by many researchers for the short-term, refrigerated storage and cryopreservation of striped bass milt (Brown and Brown 2000; Jenkins-Keeran et al. 2001; Jenkins-Keeran and Woods 2002). Extender 3 was developed to mimic the mean osmolality, pH, sodium, and potassium measured from milt samples collected from 15 striped bass near the peak of the spawning season in 1997. Osmolality and pH were measured using a Wescor model 5400 vapor pressure osmometer and an Orion model 9810 microcombination pH electrode, respectively. Sodium and potassium concentrations were measured using a Perkin-Elmer model 5100 PC atomic absorption spectrophotometer. Extender 4 was added a week into the experiment as a modification of extender 3. The osmolality was lowered to 315 mmol/kg and the Na:K ratio was raised to 86:1.

In the second experiment, extenders 1 and 2 from experiment 1 were compared with two new extenders, designated 5 and 6. These new extenders were modified versions of extender 1, with the osmolality raised to 310 and 330 mmol/kg, respectively. The amount of NaCl in these two extenders was adjusted to increase the osmolality to more closely resemble that of striped bass milt measured near the peak of the spawning season.

Extender evaluation.—Milt from each fish was activated in three replicate samples immediately after collection by mixing approximately 1 μL of the milt with 10 μL of Fisher’s deionized ultrafiltered water (DIUF) in a Makler counting chamber. Sperm motility was recorded on videotape us-
ing a Hitachi model KP-140 high-contrast video camera attached to a Zeiss model D-7082 compound microscope. Brightfield microscopy was used at a magnification of 400x. Recording began before the milt and DIUF were mixed and continued until all movement had ceased. The percentage of motile sperm was estimated later from the videotapes by the same observer to avoid bias. Spermatozoa that simply vibrated or did not show progressive forward movement were not considered motile (Billard and Cosson 1992).

An aliquot (0.3 mL) of milt from each fish was gently mixed with 0.6 mL of each experimental extender and pipetted into a 1.5-mL polypropylene snap-cap vial. As a control, 0.9 mL of undiluted milt from each fish was pipetted into a snap-cap vial. The samples were stored upright and refrigerated at 3 ± 1°C. After 1, 2, and 7 d of storage, each sample was activated with DIUF in replicates of three according to the methods described above. The average of the three replicates for each sample was used in the statistical analysis.

Statistical analysis.—The data were analyzed using mixed model techniques of SAS version 8.2 (SAS Institute 1996). The fixed portion of the model included the effects of extender, day, and the extender × day interaction. Week was included as a continuous variable, with the initial model containing linear, quadratic, and cubic components and all their interactions with extender, day, and extender × day. The effects of fish, fish × extender, and fish × extender × week were modeled as random effects. Repeated-measures techniques were used to fit the covariance among the residuals from the same fish–extender–week over days. Goodness-of-fit statistics were used to examine the analysis of variance (ANOVA) assumption of variance homogeneity and to select an appropriate variance–covariance structure for the repeated measures. After the variance–covariance structure was selected, the initial model was reduced by removing the highest order, nonsignificant interactions involving week, one at a time. The final model was reached when the highest order terms involving week were all significant. The selected variance–covariance structure included the variance due to fish and fish × extender, while a first-order autoregressive structure was used to fit the repeated measures correlation across days. Although a different variance and correlation was fitted for the undiluted control, it was appropriate to pool the variances and correlations for the extended samples. Since the data were measured as percent motility, the arcsine transformation was used to improve the assumption of normality. Only the extender × day × week cubic was removed from the initial model for the 1998 motility analysis. The extender × week cubic and the extender × day × week quadratic and cubic were removed from the initial model for the 1999 motility analysis. Pairwise comparisons (t-tests) between treatment means within day and week were made at the 0.05 level of significance.

Results

Experiment 1

The mean percentages of motile sperm obtained from fresh, undiluted striped bass milt samples collected each week during the 1998 (experiment 1) and 1999 (experiment 2) spawning seasons (N = 6 for each week). Data are mean estimated percent motility from the polynomial regression equation; whiskers indicate 1 SE.

![Figure 1.](image1.png)
The percentage of motile sperm obtained from undiluted and extended semen samples after 1 d of refrigerated storage in 1998 (experiment 1). Data are mean estimated percent motility from the polynomial regression equation; whiskers indicate 1 SE. Values within a week with identical lowercase letters are not significantly different (N = 6 for each week; P < 0.05). There were no samples for extender 4 in week 1.

During the first 4 weeks, however, significant differences were seen. After 1 d of storage, extenders 1 and 4 contained the highest percentages of motile sperm (Figure 2). After 2 d of storage, extenders 1 and 4 generally contained the highest percentage of motile sperm, although extender 4 was, in general, not significantly different from extender 2 (Figure 3).

Experiment 2

As in experiment 1, the percentage of motile sperm obtained from the initial, freshly collected, undiluted milt samples changed significantly with the progression of the 1999 spawning season (P = 0.0065; Figure 1). As in experiment 1, the actual difference from week to week was rather small but the range (89–96%) was slightly greater than it was in 1998. The percentage of motile sperm obtained from the undiluted and extended semen samples after 1, 2, and 7 d of storage are shown in Figures 4, 5, and 6, respectively. There were significant changes in the percentage of motile sperm obtained across the spawning season with respect to the day of storage (P = 0.0401), the extender used (P = 0.0285), and the day × extender combination (P = 0.0274). The percentage of motile sperm within each extender was also found to decrease as the days of storage increased (Figures 4, 5, 6).

During the first 3 weeks of the spawning season,
The percentage of motile sperm obtained from undiluted and extended semen samples after 7 d of refrigerated storage in 1999 (experiment 2). See the caption to Figure 4 for additional details.

During weeks 4 and 5, extenders 5 and 6 yielded significantly higher sperm motilities than did extenders 1 and 2 (Figure 4). After 2 d of storage, the percentage of motile sperm obtained from extender 2 was significantly lower than that of any of the other extenders for the majority of the spawning season (Figure 5). No motility was observed in extenders 1, 5, and 6 gave similar results after 2 and 7 d of storage, extender 6 consistently yielded higher percentages of motile sperm, followed by extender 5 and then extender 1 (Figures 5, 6).

Discussion

The results of both experiments clearly show that the percentage of motile sperm in striped bass milt changes over the course of a spawning season. This would indicate a change in the milt quality as well. Although milt quality is most accurately measured by fertilization percentages, this method of analysis could not be performed in either of the two experiments because the striped bass females did not produce mature eggs until the third or fourth week of each spawning season. When fertilization trials cannot be performed, sperm motility is used as an indicator of milt quality. It is generally assumed that there is a correlation between motility and fertility (Linhart et al. 2000). The percentage of motile sperm has been positively correlated with the percentage of fertilized eggs obtained in several species, such as common carp Cyprinus carpio (Linhart et al. 2000), rainbow trout (Lahnsteiner 2000), and turbot Psetta maxima (Dreanno et al. 1999). However, evidence to the contrary has also been found. No significant correlation was found between sperm motility and the percentage of fertilized eggs obtained from species such as muskellunge Esox masquinongy (Ciereszko et al. 1999) and striped bass (Kerby 1983). In both cases, high fertilization percentages were obtained with cryopreserved sperm that appeared to be immotile (Kerby 1983). However, Ciereszko et al. (1999) consider it highly unlikely for immotile sperm to fertilize eggs. Instead, they suggest that factors in the eggs or ovarian fluid can stimulate motility in sperm which were previously immotile in the activating solution. These sperm-activating factors have been found in the eggs of rainbow trout (Yanagimachi et al. 1992), coho salmon Oncorhynchus kisutch (Yanagimachi et al. 1992), and Pacific herring Clupea pallasi (Morisawa et al. 1992). Therefore, since Kerby obtained low to no motility in all his samples, it is possible that the sperm were not active until they were exposed to sperm-activating factors present in the striped bass eggs. From Kerby’s results, it is evident that with striped bass, a low percentage of motile sperm does not necessarily indicate an inability to fertilize eggs. However, when comparing striped bass semen samples, it can be assumed that a higher percentage of motile sperm is most likely indicative of a higher ability to fertilize eggs.

Although the percentage of motile sperm obtained from the fresh, undiluted milt changed significantly over the course of each spawning season, the differences observed were rather small. Because of this small range and the subjectivity of the measurement, it would be difficult to determine milt quality based on initial motility values. However, the percentage of motile sperm obtained from the extended samples after 1 d of refrigerated storage appear to give an accurate estimate of milt quality. According to the extended milt samples, striped bass milt quality appeared to be highest during the first 3 weeks of the 1998 spawning season. In 1999, motility was observed in some of the undiluted control samples after 1 d of storage. In the weeks where sperm motility was observed in the undiluted milt samples, the extended milt samples contained their highest percentage of motile sperm for that spawning season. According to both the undiluted milt and the extended milt samples, striped bass milt quality appeared to be highest during weeks 3, 4, and 5 of the 1999 spawning season. As the quality of the striped bass milt changed with the progression of the spawning season, so did the performance in each extender. The
biggest differences in the extenders were seen during the weeks when the milt quality was highest. In the beginning and towards the end of each spawning season, the milt quality was lower and little, if any, significant difference was seen in most of the extenders. Similar changes in sperm motility have been observed in ocean pout Macrourus americanus (Wang and Crim 1997) and rainbow trout (Büyükhatipoglu and Holtz 1984). The change in milt quality during the spawning season is important to striped bass hatcheries since most of the highest quality should be used for cryopreservation.

In experiment 1, extender 1 proved to be the best. Since extender 1 and extender 2 were similar in pH and osmolality, the differences observed in sperm motility between these two extenders must have been due to one or more of the individual components of the extenders. The presence of egg yolk in extender 1 may account for the higher percentage of motile sperm. The lecithin, lipoproteins, and lipids contained in egg yolk have been shown to aid in preserving the motility and fertility of mammalian spermatozoa stored at 5°C (Watson 1995; Society for Marine Mammalogy 1998). Egg yolk was also shown to increase the motility and fertility of tilapia sperm stored at 5°C (Harvey and Kelley 1984).

Although egg yolk may have aided in maintaining striped bass sperm motility, other factors must have been involved as well. Extender 4 contained the same amount of egg yolk as extender 1, but its performance declined in comparison to extender 1 after 2 and 7 d of storage. One major difference between these two extenders was the concentration of potassium. Potassium was shown to inhibit sperm motility in species such as rainbow trout (Benau and Terner 1980; Morisawa et al. 1983a) and chum salmon Oncorhynchus keta (Morisawa and Suzuki 1980) but to enhance sperm motility in goldfish Carassius auratus, common carp, crucian carp Carassius carassius, and dace Tribolodon hakonensis (also known as Japanese dace) and T. tazacronisii (also known as Pacific redfin) (Morisawa et al. 1983b). Although both extenders contained potassium, extender 1 contained a concentration 10 times higher than extender 4. The higher concentration of potassium in extender 1 may have inhibited striped bass sperm motility during storage, thereby increasing the percentage of motile sperm upon activation with DIUF. If the sperm in extender 4 were motile during storage, their energy reserves would have been depleted faster which would, in turn, account for the percentage of motile sperm decreasing faster in extender 4 than in extender 1. While this explanation seems plausible, extender 3 contained a potassium concentration in between the levels found in extenders 1 and 4 but yielded the lowest percentage of motile sperm in experiment 1 (aside from the undiluted milt). Therefore, the concentration of potassium alone must not inhibit sperm motility. Kurokura (1979) reported that the ratio of Na:K ions in the extender was an important factor for the successful cryopreservation of rainbow trout sperm (Hara et al. 1982). The duration of sperm motility in summer sand whiting (also known as sand sillage) Sillago ciliata was found to significantly increase with a Na:K ratio greater than 2:1 (Goodall et al. 1989). The inhibiting action of potassium is counteracted by calcium and magnesium ions and, to a lesser extent, sodium ions (Morisawa et al. 1983a; Billard and Cosson 1992). The Na:K ratio was approximately 19:1 in extender 1 and 86:1 in extender 4, whereas the Na:K ratio of extender 3 was approximately 8:1. Since DIUF was used to activate the samples, the initial Na:K ratios were not altered upon activation. Therefore, the amount of sodium in extender 3 may have been too low to completely overcome the inhibitory action of potassium.

Another major difference between extenders 1, 3, and 4 was the concentration of glucose. Glucose levels were highest in extender 3 and lowest in extender 1. The uptake of extracellular glucose by fish sperm for use as a source of energy has been documented in species such as rainbow trout (Lahnsteiner et al. 1997, 1999), the cyprinid Danube bleak Chalcalburnus chalcoides (Lahnsteiner et al. 1999), shiner perch Cymatogaster aggregata (Gardiner 1978), and the guppy Poecilia reticulata (Gardiner 1978). However, the amount of glucose utilized by fish sperm is limited (Terner and Korsh 1963) and the ability of fish sperm to oxidize glucose is significantly low in comparison to mammalian sperm (Minassian and Terner 1966). Therefore, it is unlikely that the large amounts of glucose in extenders 3 and 4 provided any more energy than the small amount of glucose in extender 1. In fact, the larger amounts of glucose in extenders 3 and 4 may have had adverse effects in terms of osmotic damage. Because glucose does not pass through the cell membrane as easily as water, the initial dilution of milt with Extenders 3 and 4 may have dehydrated the sperm cells for some period of time until equilibrium was reached. Hypertonic-induced cell shrinkage may cause an alteration in the phospholipid bilayer structure or a loss of membrane components (Morris 1981).
Since extender 1 proved to be the best one for striped bass in experiment 1, simple modifications of the osmolality of extender 1 were made in experiment 2 to see if the resulting sperm motility could be improved. Extender 2 from experiment 1 was also reevaluated in experiment 2 because at the time of the experiment, it was the only extender known to be in current use with striped bass milt. The results of experiment 2 clearly show that raising the osmolality of extender 1 to levels more closely resembling the osmolality of striped bass milt improves the percentage of motile sperm obtained after 1, 2, and 7 d of refrigerated storage. According to measurements made during the 1998 spawning season, the average milt osmolality of striped bass ranged between 330 and 350 mmol/kg (Jenkins 1999). A clear trend can be seen with respect to extenders 1, 5, and 6, in which the percentage of motile sperm increased as the osmolality of the extender increased. However, as shown in experiment 1, osmolality alone does not determine how successful an extender will be. Other factors (such as the sodium, potassium, egg yolk, and glucose concentrations) contribute to the effectiveness of an extender as well.

Although extender 6 proved to be the best extender for the short-term storage of striped bass milt in this study, further research is needed to evaluate its effectiveness in relation to Brown and Brown's (2000) extender #4H and extender #7. Also, the effect that the individual components of extender 6 have on striped bass sperm motility need to be evaluated thoroughly. It is interesting to note that the best extender for the cryopreservation of striped bass milt was not the best extender for the short-term preservation of striped bass milt. When extenders 1, 2, and 5 were used to cryopreserve striped bass milt, extender 2 yielded significantly higher sperm motility and fertilization percentages than did extenders 1 and 5 (Jenkins-Keeran and Woods 2002).

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References


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