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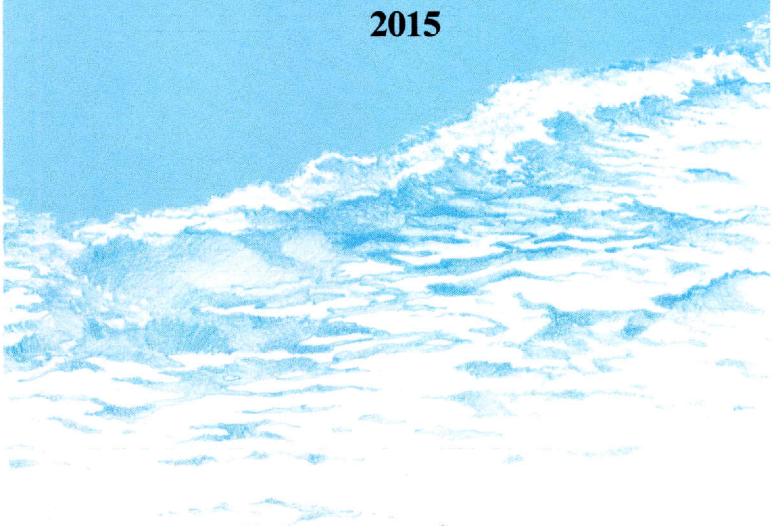

TEXAS PARKS & WILDLIFE
INLAND FISHERIES DIVISION
4200 Smith School Road
Austin, Texas 78744

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ABSTRACT

Two experiments were conducted to test the viability of extended milt for mass production of Striped Bass fry. We crossed each female with two male pairs to account for gender and treatment effects in Experiment 1 and each female with a single male pair to examine only treatment effects in Experiment 2. Each male pair supplied both extended and fresh milt for fertilizing eggs from a female. Fertilized eggs were incubated in McDonald egg hatching jars. Egg fertilization and hatch rates were estimated approximately 4 h after fertilization and 8 h before hatch, respectively. Overall, fresh milt was no better than extended milt. In the first experiment, fertilization rates were similar between extended (72.3%) and fresh milt (68.0%) ($P = 0.325$) whereas hatch rate was significantly higher ($P = 0.002$) with extended milt (36.2%) than with fresh milt (19.6%). In the second experiment, egg fertilization rate was higher ($P = 0.038$) using extended milt (94.7%) than when using fresh milt (88.9%), but hatch rates were statistically similar between extended (36.0%) and fresh milt (22.0%) ($P = 0.182$). The extender protocol used in this study was effective in maintaining sperm viability and suitable for mass production of Striped Bass fry.

INTRODUCTION

Most state natural resource agencies that produce fingerling Striped Bass *Morone saxatilis* rely on wild brood fish for their production. Male and female brood fish are transported from collection site(s) to hatcheries and held separately in tanks until females are ready to spawn. Because males produce large volumes of semen, they are traditionally strip-spawned directly into a pan of water containing eggs from a female where fertilization occurs (Rees and Harrell 1990). One male may be used to fertilize eggs from one or more females. However, states with genome conservation programs (e.g., South Carolina, Maryland, and Texas) aimed at maintaining or improving the genetic diversity of Striped Bass fisheries may use two or more males to fertilize eggs from a female (Kerby and Harrell 1990).

Inclement weather, high water flows, or other constraints can prevent sufficient collection of brood fish, which results in inadequate production of fingerlings to meet management needs. To improve efficiencies of fingerling production programs, some agencies (e.g., Kansas Department of Wildlife, Parks and Tourism, and Texas Parks and Wildlife Department) have developed, or are developing, captive brood stocks for either routine production or contingencies. Because captivity can diminish milt production in Striped Bass (Mylonas et al. 1998) and maintaining captive brood stock indoors can incur high costs (in terms of labor, space, water use, feed, disease control, etc.), an alternative strategy is development of captive female brood stock and use of preserved milt for fry production. Success of this strategy would depend, at least partially, on the availability of functionally reliable preserved milt.

As preservation techniques for fish semen, cryopreservation and refrigeration have been investigated for aquaculture applications (e.g., Kerby 1983; Bart et al. 1998; Glogowski et al. 1999; Brown and Brown 2000; Jenkins-Keeran and Woods III 2002; He and Woods III 2003). Cryopreservation is more suitable if storage of milt would exceed 2 weeks (Brown and Brown 2000) whereas refrigeration is appropriate for short-term (1-7 d) preservation (Jenkins-Keeran and Woods III 2002). The cryopreservation technique is complicated and tedious, involving cooling, freezing, and thawing of semen. A misstep during any of these tasks can result in poor sperm quality by decreasing the number of viable sperms capable of fertilizing eggs. Further, equipment and materials required for cryopreservation (e.g., controlled-rate freezer, freezing straws, dry ice, and liquid nitrogen storage dewars) make the technique expensive. Conversely, refrigeration storage is relatively simple and involves cooling the semen to 3-4°C and allowing it to attain room temperature before egg fertilization.

Techniques for short-term preservation of Striped Bass milt exist (Jenkins-Keeran et al. 2001; Jenkins-Keeran and Woods III 2002; He and Woods III 2003), but to our knowledge, are not currently used in mass production of fry for commercial or natural resource management purposes. Jenkins-Keeran and Woods III (2002) noted that the techniques need improvements to become suitable for application in most fish production programs. Reporting effectiveness of these techniques in terms of fertilization rate, hatch rate, or fry production, rather than sperm motility, which is not routinely measured in fish hatcheries, might also facilitate incorporation of these techniques into typical hatchery operations.

We conducted this study to evaluate what appears to be the most effective extender and handling protocol available for short-term ($\leq$ 7 d) refrigerated storage of Striped Bass milt,

hereafter called the "Keeran-He-Woods" protocol (Jenkins-Keeran et al. 2001; Jenkins-Keeran and Woods III 2002; He and Woods III 2003). This protocol can extend sperm viability for up to 7 d, though the best results occur when milt is used within 48 h of preservation (He and Woods III 2003). Thus, it would be suitable for Striped Bass production programs that practice strip-spawning of hormone-injected fish because fertilization of eggs is routinely completed 40 h or less after injections. The objective of this study was to determine if fresh milt and milt preserved for up to 48 h yield the same egg fertilization and hatch rates.

MATERIALS AND METHODS

Study Design

This study was conducted during routine spring spawning of brood fish for mass production of *Morone* fingerlings at the A. E. Wood Fish Hatchery, San Marcos, Hays County, Texas. The study comprised two experiments. Experiment 1 was a hierarchical, randomized block design with males nested within females (i.e., each female "block" was crossed with two male pairs) to account for gender and treatment effects (Figure 1a). A male pair per female was used because most natural resource agency hatcheries use this model to improve or maintain the genetic diversity of their Striped Bass fisheries. Because Experiment 1 results suggested no increase in power from such a complex design, we used a simple randomized design (i.e., each female crossed with a single male pair) to examine treatment effects in Experiment 2 (Figure 1b).

Brood Fish Collection

Brood fish were collected by electrofishing from the Lake Livingston tailrace, Polk County, Texas in April 2009 (Experiment 1) and 2010 (Experiment 2). Six females and 24 males were collected for Experiment 1, and three females and six males were collected for Experiment 2. Fewer fish were used in 2010 due to inadequate available females for the Texas state *Morone* production program. At capture, females were 10-13 h from ovulation, and each was large enough (≥6.5 kg) to produce a sufficient number of eggs to allow egg incubation rates to be similar to those of routine production (i.e., 100,000-120,000 eggs/jar) and thereby not adversely affect program production goals. Females were injected intramuscularly, above the lateral line, with human chorionic gonadotropin hormone (HCG) at a dose of 68 IU/kg body weight. Each fish was tagged with a unique color and number for identification, and fish were held together in a fish-hauling tank, along with non-experimental fish at approximately 79.3 g/L of water. Upon capture, males were held in cages submersed in lake water until sufficient numbers were collected for each experiment. This was done so that all milt samples would be preserved at about the same time, thereby minimizing differences in milt preservation periods.

Field Collection and Preservation of Milt

Milt collection was performed under a tent to prevent any potential adverse effect of sunlight on sperm cells. Care was taken to avoid contamination of milt by feces, urine, or mucus, or activation of sperm cells by dilution. This was accomplished by manually expressing feces and urine, and cleaning the ventral side of each male with a damp towel before the milt was expressed (Jenkins-Keeran and Woods III 2002). Males freely released milt with gentle palpation of the abdomen, and each was large enough (≥ 2 kg) to release approximately 25 mL of milt. Milt was collected from each fish by hand-stripping into a 50-mL graduated centrifuge tube via a 127-mm-

diameter, short-stem (35 mm) funnel. Males were randomly paired, and the milt samples from each pair were pooled as one sample with both contributing approximately equal amounts. This yielded 12 milt samples for Experiment 1 and three samples for Experiment 2. Immediately after collection, each pooled milt sample was mixed with an extender (NaCl 8.0 g/L, KCl 0.4 g/L, $NaHCO_3$ 0.25 g/L, glucose 1.0 g/L, glycine 50.0 mM, osmolarity 350 mmol/kg, and pH 7.6; He and Woods III 2003) in a 500-mL high density polypropylene screw-cap container. We used a ratio of one part milt to two parts extender, by volume. Prior to capping the container, the air above each milt-extender mixture was displaced with oxygen (Jenkins-Keeran et al. 2001). The container was then stored vertically in crushed ice (Jenkins-Keeran and Woods III 2002; He and Woods III 2003). After milt collection, males were injected intramuscularly, above the lateral line, with HCG (34 IU/kg body weight) to enhance milt flow without adversely impacting sperms' ability to fertilize eggs (J. H. Kerby in Rees and Harrell 1990). These fish were each tagged with a unique color and number for identification and then held in a fish-hauling tank along with production fish at approximately 14.6 and 58.5 g/L of water in 2009 and 2010, respectively.

Brood fish and extended milt were then transported to the A. E. Wood Fish Hatchery. At the hatchery, the extended milt samples were transferred from ice to refrigeration at 4°C, and the male and female brood fish were separated into 1,600-L circular tanks along with production fish at densities of 4-5 fish per tank. Water temperature and dissolved oxygen concentration were maintained at 18-19°C and >6 mg/L, respectively. Females were monitored for ovulation by egg staging (Rees and Harrell 1990; Lyon et al. 2006).

Spawning and Egg Handling

Each ovulating female was strip-spawned into a dry 3-L graduated plastic pitcher. The eggs were gently mixed with a feather, the volume recorded, and samples were taken to estimate number of eggs in the spawn. Then, the eggs were distributed approximately equally among labeled 250-L plastic bowls (eight bowls in Experiment 1 and four bowls in Experiment 2). The depth of eggs in each bowl was measured with a ruler for egg volume determination. Egg handling from post-spawning to initiation of fertilization lasted about 1 min for each female (Rees and Harrell 1990). For each female, the number of eggs per mL was estimated by counting 2-3 samples (1 mL each) taken from the spawn in the pitcher separately with a Jensorter counter (model FC 2; Jensorter, LLC, Bend, Oregon) and calculating the average (720-912 for Experiment 1 and 691 for Experiment 2). The latter was multiplied by the volume of eggs in the pitcher or in each bowl to get the number of eggs. The volume of eggs in each bowl was estimated by filling the bowl with water to the same height as was the eggs and transferring the water into a measuring cylinder for volume measurement.

Fresh Milt Collection and Egg Fertilization

To avoid premature activation of sperm cells by contamination or dilution, males were handled as they were at the collection site. In addition, dry centrifuge tubes and graduated cylinders were used for fresh milt collection and for holding of aliquots of preserved milt. The preserved milt samples were used for egg fertilization in the order they were collected in the field. This was part of the strategy to minimize the differences in preservation period among samples. Preserved milt was taken out of refrigeration, to allow it to attain room temperature, about 10 min before use. Each preserved milt sample was divided into two equal aliquots in 100-mL graduated cylinders; each aliquot then was used to fertilize a bowl of eggs. Simultaneously during spawning

of female, fresh milt samples from each corresponding male pair were collected by hand-stripping into a 50-mL graduated centrifuge tube, each contributing about the same amount. This pooled milt was divided into two aliquots to fertilize replicate bowls of eggs. Both preserved and fresh milt aliquots, all of similar volumes in terms of actual milt content (~25 mL each), were added to their respective replicate bowls of egg immediately following the addition of about 100 mL of incubation water to each bowl of eggs. The eggs and milt were mixed by stirring for about 1 min with a feather. The eggs in each bowl were allowed to settle for about 2 min, excess water decanted; then transferred into a McDonald hatching jar, labeled as the bowl, for incubation.

Fertilization Rate

Egg fertilization rate was estimated about 4 h after fertilization. With the eggs in each McDonald hatching jar gently moving with water circulation, two samples (27-97 eggs for Experiment 1 and 729-1904 eggs for Experiment 2) were taken with a clear, rigid plastic tube (6-mm inside diameter and 450-mm long). Each sample was placed on a counting wheel and examined under a dissecting microscope. Viable (eggs with normal development) and non-viable (dead) eggs were counted to estimate fertilization rate, expressed as a percentage of number of eggs.

Hatch Rate

Hatch rate is routinely estimated by the fry method at production hatcheries. However, space constraints caused by conducting this study alongside the annual production of Palmetto bass (female *M. saxatilis* × male *M. chrysops*) fry required us to combine fry from multiple hatching jars into fewer fry-collection aquaria by treatment (e.g., fry from 24 fresh-treatment jars into four aquaria and from 24 extended-treatment jars into three aquaria in Experiment 1), thereby reducing statistical power to detect a difference between treatments. Therefore, we estimated hatch rate by the embryo method (Rees and Harrell 1990). Based on water temperatures, we estimated eggs would hatch approximately 48-49 h after fertilization. Approximately 8 h before anticipated hatch and with embryos gently moving with water circulation, two samples of embryos (approximately 4-8 mL each) were taken with a plastic tube from each jar and transferred into separate graduated centrifuge tubes. Embryos were allowed to completely settle before volumes were recorded. All embryos in each sample were examined under a dissecting microscope on a counting wheel and counted into viable and non-viable groups, which totaled 526-1,679 embryos per sample. Immediately following embryo sampling from each hatching jar, water flow into the jar was turned off to allow the embryos to completely settle. The volume of embryos was estimated from graduations on the jar, and the water flow was quickly turned back on. The data (number of embryos/mL, volume of embryos per jar, and percent viable embryos) were used to calculate hatch rate (total number of viable embryos/total number of eggs × 100) for each jar.

Data Analysis

SAS 9.2 (SAS Institute 2010) was used for all data analyses. We used mixed models regression (PROC MIXED) to test whether fertilization rate or hatch rate (response variable) differed between fresh and extended milt. The data for each response variable were normalized using arcsin square-root transformation (Zar 1984) before analysis. Treatment effect was considered fixed, male and female effects as random factors, and repeated counts as subsamples. For each analysis, we started with a full model containing all fixed and random effects. Random effects that were estimated to be zero were removed from the model, because leaving them in the

model resulted in a non-positive definite Hessian (i.e., failure of covariance convergence). We examined several different covariance models to account for possible correlation between measures from the same fish. The final model selected for each dataset was the one with the lowest Akaike's Information Criterion (AIC). Based on the AIC, the best model for each set of data was slightly different. However, in all cases the variance components covariance structure was the best fit for the data. The different models, along with the different designs for the various components of the experiments, resulted in different denominator degrees of freedoms for the *F*-tests associated with the effectiveness of milt. For all analyses, differences were considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

All six females of Experiment 1 were spawned within approximately 7 h, and the three females of Experiment 2 were spawned within about 3 h. Refrigeration storage periods for extended milt before use in egg fertilization were 27-34 h in Experiment 1 and 21-23 h in Experiment 2. Eggs samples for fertilization rate estimation were taken 4.0-4.2 h and 4.0-5.0 h after fertilization in Experiments 1 and 2, respectively. Embryos were sampled for hatch rate estimation about 40 h after egg fertilization in Experiment 1 and 43 h after egg fertilization in Experiment 2. For both experiments, eggs hatched in about 48-49 h and incubation water temperatures, dissolved oxygen concentrations, and pH were 18.9-19.2°C, 9.23-9.35 mg/L and 8.01-8.05, respectively.

Egg production averaged 133,330 eggs/kg of female in Experiment 1 and 125,002 eggs/kg of female in Experiment 2 (Table 1). These numbers compare favorably with the 125,439 eggs/kg of female in 2009 and 146,264 eggs/kg of female in 2010 for the production fish, as well as the 5-year (2006 -2010) average of 134,018 eggs/kg of female for the Striped Bass production program (D. Smith, Dundee State Fish Hatchery, Electra, Texas, personal communication).

In Experiment 1, fertilization rate did not significantly differ among females or the majority of the males (Figure 2). Among males, fertilization rate significantly differed only between male pairs 10 and 11, and the reason is unclear. The mean fertilization rate was 72.3% for extended milt and 68.0% for fresh milt, and the difference was not statistically significant ($P(F_{1,41} > 0.99) = 0.325$). Conversely, the mean hatch rate was significantly higher ($P(F_{1,17} > 13.2) = 0.002$) with extended milt than with fresh milt; the mean values were 36.2 and 19.6%, respectively (Table 2).

In Experiment 2, the mean fertilization rate was significantly higher ($P(F_{1,10} > 5.69) = 0.038$) with the extended milt (94.7%) than with fresh milt (88.9%). However, the difference was not biologically or economically important. Mean hatch rate did not significantly differ between extended and fresh milt ($P(F_{1,2} > 4.05) = 0.182$). The mean hatch rate for extended milt was 35.8% whereas for fresh milt the average was 21.9%. These proportions are similar to those in Experiment 1; however, in this experiment the difference was not statistically significant. The small sample size ($n = 3$) may explain the lack of significant difference. Overall, these results suggest that using extended milt throughout the rearing process can produce results comparable to using fresh milt. Further, our results suggest that the "Keeran-He-Woods" technique for short-term preservation of Striped Bass milt is suitable for mass production of fry. Therefore, incorporating this technique into typical hatchery operations would allow elimination of the annual harvest of males for production purposes and reduce the cost associated with

transportation of brood fish from collection sites to hatcheries. Further, this technique could allow exclusion of males from captive brood stock programs and result in more space, labor, and other resources to be dedicated to a captive female brood stock program and thus achieve a higher capacity program.

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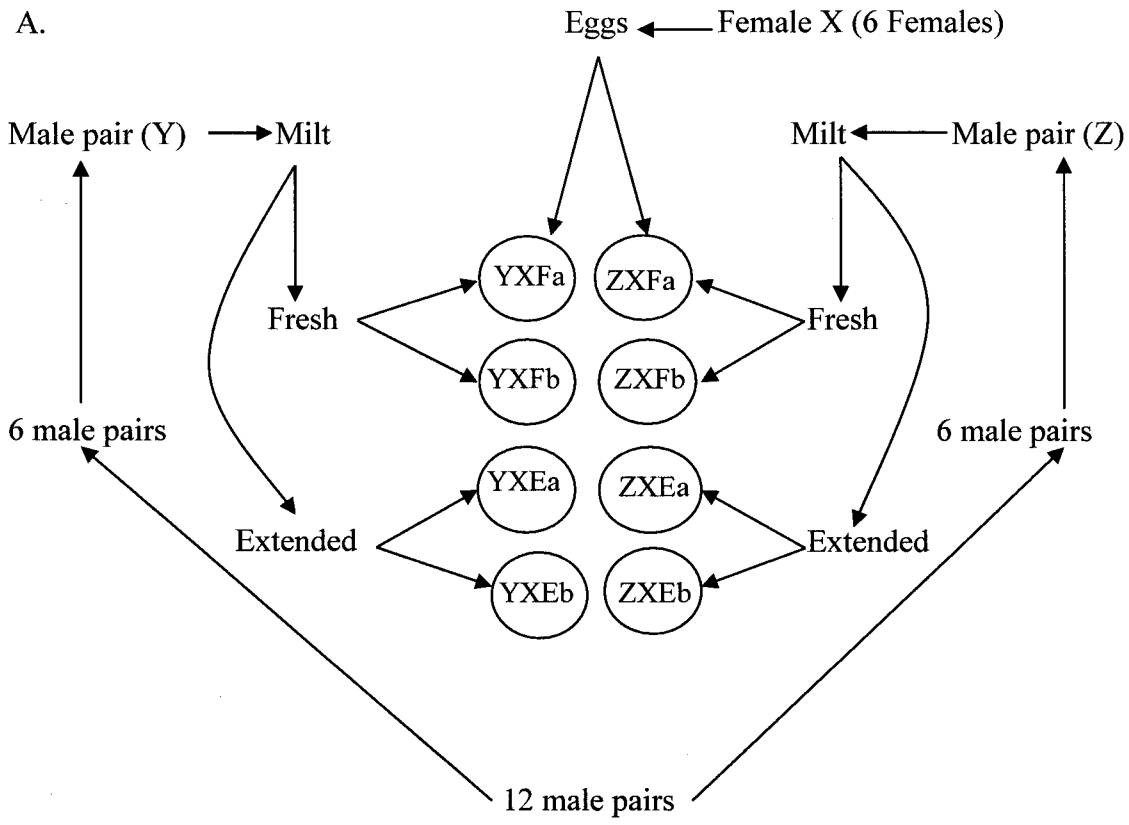
TABLE 1.—Summary statistics (mean ± SD; range in parentheses) for fish used to evaluate viability of short-term preserved Striped Bass milt for mass production of fry.

Gender	Number	Weight (kg)	Length (mm)	Milt volume (mL)	Eggs/kg body weight	Eggs/jar
			Experiment 1 (2009)			
Male	24	3.56 ± 1.37 (1.8 - 7.01)	603.17 ± 57.25 (522 - 696)	24.25 ± 2.72 (15 - 28)	N/A	N/A
Female	6	7.11 ± 1.05 (6.34 - 8.82)	764 ± 31.16 (733 - 810)	N/A	133,330 ± 33,157 (78,509 - 168,899)	115,836 ± 21,095 (79,000 - 133,852)
			Experiment 2 (2010)			
Male	6	4.72 ± 0.83 (3.2 - 5.5)	679 ± 43.31 (602 - 723)	24.17 ± 2.04 (20 - 25)	N/A	N/A
Female	3	7.40 ± 1.11 (6.20 - 8.40)	772 ± 14.47 (756 - 782)	N/A	125,002 ± 49,079 (71,009 - 166,909)	232,637 ± 98,069 (125,762 - 380,050)

TABLE 2.—Mean fertilization and hatch rates (± SE; range in parentheses) of Striped Bass eggs fertilized with fresh or preserved milt. For each experiment values within a row bearing the same letter are not significantly different ($P > 0.05$).

	Experiment 1 (2009)		Experiment 2 (2010)	
Variable	Fresh	Extended	Fresh	Extended
Fertilization rate	68.0 ± 3.0 x (18.6 - 100.0)	72.3 ± 2.6 x (26.7 - 100.0)	88.9 ± 1.0 x (87.2 - 90.7)	94.7 ± 1.4 y (92.6 - 97.2)
Hatch rate	19.6 ± 3.0 x (5.8 - 55.9)	36.2 ± 3.2 y (8.7 - 69.4)	21.9 ± 2.8 x (7.5 - 37.0)	35.8 ± 5.3 x (6.4 - 53.2)



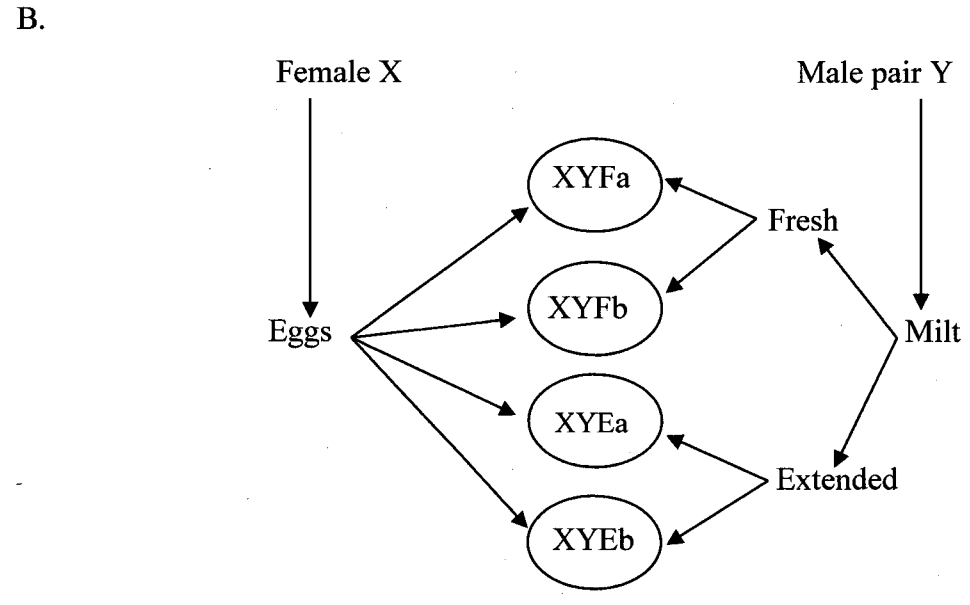


FIGURE 1.—Experiment 1 was a hierarchical, randomized block design with males nested within female blocks (i.e., each female "block" was crossed with two male pairs) to account for gender and treatment effects (A) on Striped Bass egg fertilization and hatch rates and Experiment 2 was a completely randomized design to test treatment effects on the same variables (B). Symbols: Y and Z are male pairs, X is female, F is fresh milt, E is extended milt, and a and b are replicates.

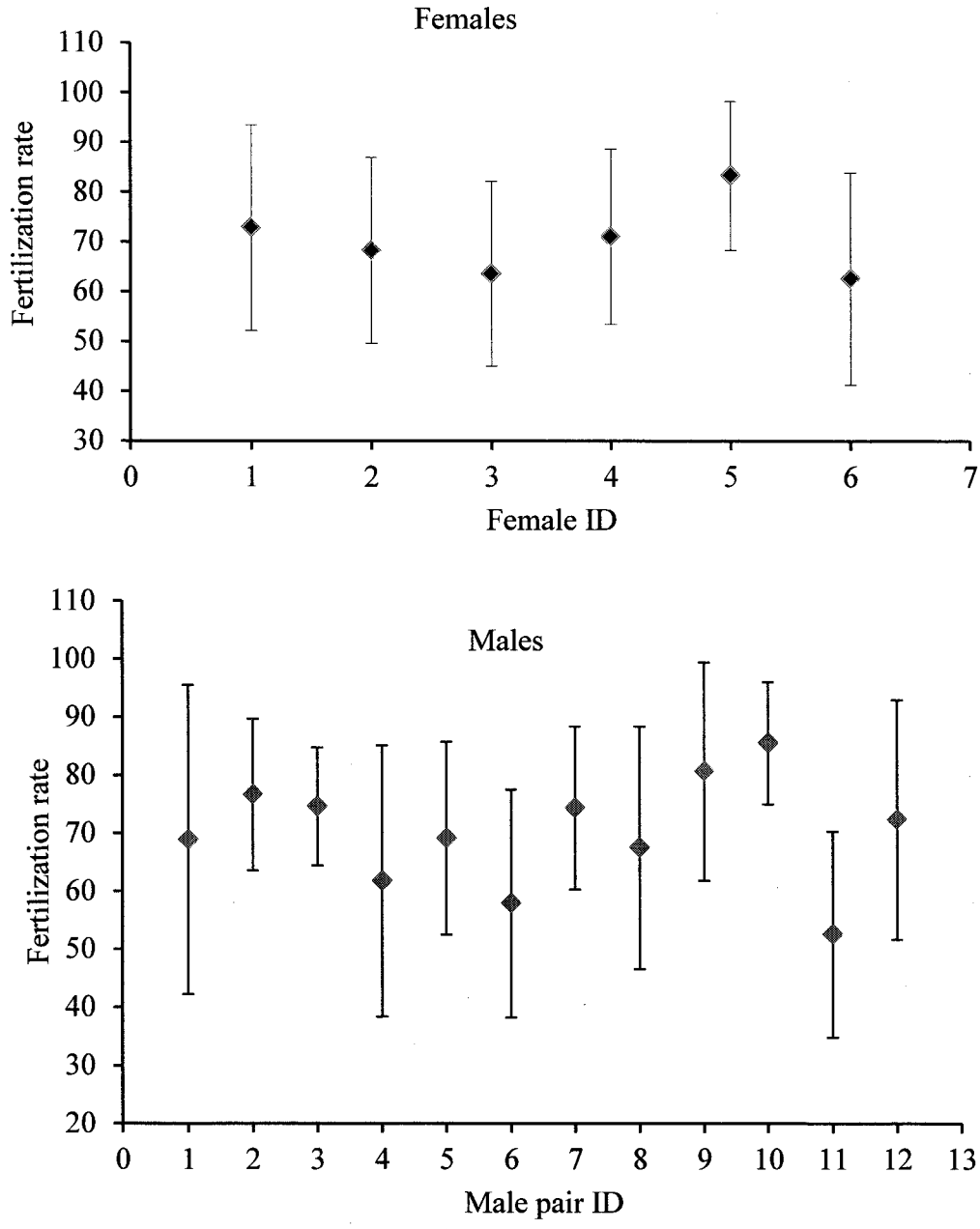


FIGURE 2.—Fertilization rates (mean ± SD) of individual females and pairs of males of Experiment 1 that evaluated a short-term milt preservation protocol for mass production of Striped Bass fry. Overall, there was no gender effect on fertilization rate ($P > 0.05$).

Texas Parks and Wildlife Department
4200 Smith School Road, Austin, Texas 78744

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