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[Communication]

Preliminary Assessment of Refrigerated and Frozen Storage of Sperm of the Coppernose Bluegill

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Abstract.—The coppernose bluegill (CBG) *Lepomis macrochirus purpurescens* is a bluegill subspecies that is native to Florida. The CBGs and their hybrids are of interest as potential food fish. Our objectives were to (1) develop an osmolality activation curve for CBG sperm, (2) determine suitable cryoprotectants for the cryopreservation of CBG sperm, and (3) demonstrate the ability of cryopreserved CBG sperm to fertilize eggs. Sperm were stripped from mature CBGs (mean ± SD = 113 ± 35 g, n = 10), diluted in Hanks' balanced salt solution (HBSS; osmolality = 300 mosmols/kg), and stored at 4°C. An activation curve was generated by exposing the sperm to solutions prepared by serial dilution of HBSS with de-ionized water. The sperm remained inactive at osmolalities above 265 mosmols/kg. Dilution below 115 mosmols/kg was required for complete activation. The motility of sperm stored in these solutions was estimated at 24-h intervals. Stripped sperm motility (mean ± SD) declined from 90 ± 7% initially to 26 ± 11% after 6 d of storage at 4°C. Motility of sperm from crushed testes declined from 80 ± 8% at collection to 28 ± 10% at 1 d of storage. By day 5, motility of sperm from crushed testes was 9 ± 9%; no motility was observed at 6 d. Sperm were exposed to 10% concentrations of the cryoprotectants dimethyl acetamide (DMA), dimethyl sulfoxide (DMSO), and methanol (MeOH), and motility was evaluated at 15-min intervals for 60 min. Initially, motility was 81 ± 11% for the control sperm. At 60 min, motility was 0% for DMA, 30 ± 24% for DMSO, 78 ± 10% for MeOH, and unchanged for the control. For samples frozen in a controlled-rate freezer (CRF), post-thaw motility was 26 ± 10% for samples with 10% DMSO and 51 ± 18% for samples with 10% MeOH. For samples frozen by means of dairy industry technology (DIT), post-thaw motility was 46 ± 21% for samples with 10% DMSO and 56 ± 13% for samples with 10% MeOH. Overall, samples frozen in 10% MeOH had significantly higher post-thaw motility (P = 0.043) than did those frozen in 10% DMSO. However, post-thaw motility did not differ between samples frozen by use of the CRF and DIT (P = 0.13). For sperm frozen in the CRF, fertilization was 15% for samples with 10% DMSO and 50% for samples with 10% MeOH. For sperm frozen by means of DIT, fertilization was 50% for samples with 10% DMSO and 75% for samples with 10% MeOH.

Artificial propagation of sunfish hybrids has been of interest to North American fish culturists for decades (Smitherman and Hester 1962; Childers 1967). While these species will hybridize in nature, development of methods to collect and store sperm could facilitate commercial production of sunfish hybrids (Mischke and Morris 1997, 1998). Current events, such as a less-than-stable market for channel catfish *Ictalurus punctatus* and a relaxation of laws concerning commercial culture of sunfish and sunfish hybrids in some states, have led to increased interest by commercial producers. For example, in Louisiana, a change of state regulations in 1999 legalized production of certain sunfish hybrids as food fish (Act 1022, 1999). The key change in the Louisiana statute was the specific allowance of production of coppernose bluegills (CBG) *Lepomis macrochirus purpurescens* or certain hybrids of the coppernose bluegill. The principal hybrid of interest is the female green sunfish *L. cyanellus* × male bluegill (Heidinger 1975; Tidwell et al. 1994). If cryopreservation of sunfish sperm is possible, sperm could be collected from males and evaluated for quality prior to stripping of eggs from female broodstock. Fertilization and incubation of eggs in the hatchery would allow enhanced control of water quality and temperature to maximize hatching, survival, and growth rate. Cryopreservation of sperm can be useful in future

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experiments and also in the optimization of hatchery production of sunfish and sunfish hybrids.

Fish maintain their internal fluidic and ionic balance by osmoregulation of water and electrolytes. At physiological osmolality, sperm cells are maintained in an inactive state. Thus, blood plasma osmolality can be used as a starting point for the preparation of a sperm storage extender solution (Bates et al. 1996). When a male freshwater fish spawns, the osmolality experienced by the sperm immediately declines due to the dilution effect caused by the lower osmolality of the surrounding water. This decrease in osmolality triggers the sperm cells to become active (motile). Once activated, the sperm have a limited period of motility. Methods must be developed on a species-by-species basis to determine a suitable solution that will maintain sperm cells in an inactive state until the time of fertilization.

The collection and temporary storage of sperm from fish is required regardless of whether the fish culturist wishes to use the sperm relatively quickly (i.e., within hours or days) or to place the sperm into long-term storage by cryopreservation (Stoss 1983). Here we employ methods that others have used to gain the preliminary data required for the storage of fish sperm from freshwater species such as the channel catfish (Bates et al. 1996), the black drum *Pogonias cromis* and freshwater drum *Aplodinotus grunniens* (Wayman 1996), and the endangered razorback sucker *Xyrauchen texanus* (Tiersch et al. 1996).

Sperm cryopreservation protocols developed for dairy bulls have been applied to aquaculture purposes. Sperm of blue catfish *I. furcatus* frozen by means of dairy technology were effective for producing the hybrid cross of channel catfish × blue catfish (Lang et al. 2003). To gain the benefits of the technology employed by dairy cryopreservation, we evaluated its use in the cryopreservation of CBG sperm.

While the production of sunfish hybrids from stripped gametes has been reported (Childers 1967), the storage and cryopreservation of sunfish sperm have not been addressed. Our objectives were to (1) develop an osmolality activation curve for sperm of CBG, (2) determine suitable cryoprotectants for cryopreservation of CBG sperm, and (3) demonstrate the ability of cryopreserved CBG sperm to fertilize eggs.

Methods

The animals used in this study were collected from farm ponds in Laurel, Mississippi (Suttle Fish Farms; www.suttlefish.com), and Natchitoches, Louisiana, by hook-and-line or seining and were maintained at the Aquaculture Research Center of Northwestern State University (NSU), Lena, Louisiana, or at the Louisiana State University Agricultural Center Aquaculture Research Station (ARS), Baton Rouge, Louisiana. Fish were collected from April to August during their natural spawning season. Fish ranged in size from 50 g (green sunfish female) to 300 g (the largest spawning CBG male). Males exhibiting spawning characteristics like aggressive behavior and dark body coloration were selected for use.

Blood was collected from mature CBG males ($n = 3$) by use of a syringe (25-gauge needle, 1-mL tuberculin syringe) and caudal vein puncture. Blood was drawn without anticoagulant additives since their use alters the osmolality of a sample. The blood was immediately injected into sterile 5-mL tubes and was allowed to clot. Blood plasma was carefully removed from above the clot, and osmolality was determined by vapor pressure osmometry (Wescor, Inc., Logan, Utah; model 5520). The extender, Hanks' balanced salt solution (HBSS), was prepared with reagent-grade chemicals (Sigma Chemical Corp., St. Louis, Missouri) as follows: 8.00 g of NaCl, 0.40 g of KCl, 0.20 g of $CaCl_2 \cdot 2H_2O$, 0.20 g of $MgSO_4 \cdot 7H_2O$, 0.06 g of Na_2HPO_4, 0.06 g of KH_2PO_4, 0.35 g of $NaHCO_3$, 1.00 g of glucose, and sufficient de-ionized water to yield the desired osmolality. The final volume based on this formula was approximately 1 L. To reduce fish stress during handling, we anesthetized the fish with tricaine methanesulfonate (MS-222; Argent Laboratories, Inc., Redmond, Washington), a fish anesthetic approved by the U.S. Food and Drug Administration; fish that were euthanatized for this study were overdosed with MS-222.

Gamete collection, activation curve, and short-term storage (NSU).—For collection of sperm, gentle pressure was applied to the abdominal area of male CBGs ($n = 10$) and the semen expressed was drawn into a sterile pipette. The semen was diluted in 200 μL of HBSS and was stored at 4°C until motility estimation. For estimating motility, aliquots (2 μL) of the sample from each male were placed on a glass slide, mixed with 20 μL of de-ionized water to activate the sperm, and evaluated at 200× magnification by use of dark-field microscopy (Nikon Optiphot2). Percent motility took into account initial movement and duration of motility within each activated sample. Sperm vibrating in place were not considered to

be motile. Initial motility was recorded for each sperm sample. Motility was reported as mean ± SD.

For the sperm activation curve, 10 solutions were prepared from a stock HBSS solution (osmolality = 300 mosmols/kg) by serial dilution in increments of approximately 10%. This yielded 10 HBSS solutions with osmolalities equally spaced from 300 to 30 mosmols/kg. Motility was estimated as previously described and was scored as follows: 0 = 0% to less than 10%, 1 = 10% to less than 25%, 2 = 25% to less than 50%, 3 = 50% to less than 75%, and 4 = 75–100%. Osmolality was determined from a 10-μL sample taken directly from the microscope slide. For short-term storage, the sperm samples were held at 4°C and sperm motility was estimated initially and every 24 h for 6 d.

Preparation of sperm samples, cryoprotectant toxicity, and cryopreservation (ARS).—The scant volumes attainable by hand stripping necessitated the collection of sperm samples from crushed testes. For collection of sperm, testes of CBGs (n = 4) were surgically removed, crushed in 1–2 mL of HBSS per 2 g of testis, and strained through a 70-μm screen to remove testicular tissue. Initial motility was determined for each sample before dilution with HBSS (300 mosmols/kg) at 1:7 (sperm sample: HBSS) and storage in 50-mL centrifuge tubes at 4°C. Final volumes of sperm were variable but equaled less than 0.5 mL. Each tube was inverted at 24-h intervals to re-suspend sperm cells, and motility was assessed daily until sperm in all samples would no longer activate.

To determine acute cryoprotectant toxicity, we exposed testicular sperm of CBGs (n = 4) to 10% concentrations of three cryoprotectants: methanol (MeOH), dimethyl acetamide (DMA), and dimethyl sulfoxide (DMSO). Cryoprotectants were diluted 1:1 with HBSS (300 mOsm/kg) before being added to sperm solutions. The final volume for each sample was 5 mL. Motility was evaluated at 15-min intervals for 60 min.

Based on the results of the acute toxicity study, two cryoprotectants were evaluated for cryopreservation of CBG sperm. Testicular sperm (n = 4 males for each freezing method) were frozen in 10% solutions of the appropriate cryoprotectant by use of a controlled-rate freezer (CRF) or the commercial dairy protocols developed for the cryopreservation of bull sperm (T. E. Patrick Dairy Improvement Center, Baton Rouge, Louisiana). For the CRF (Kryo-10, Planer Products, Ltd., England), sperm were frozen in 0.5-mL French straws at a rate of −45°C per min to −80°C and were plunged into liquid nitrogen for storage. For cryopreservation with dairy industry technology (DIT), straws were filled by means of an automated straw-filler (model MRS 1, IMV International Corp., Minneapolis, Minnesota) in a walk-in cooler at 5°C. Straws were placed in a chamber held at −140°C. After an initial warming period caused by the release of heat from the samples, the chamber was cooled to −140°C at a rate of 16°C per min. Samples were plunged into liquid nitrogen for storage. For estimation of motility after thawing, two straws from each male were thawed at 40°C for 7 s. Thawed sperm were evaluated for motility as described above.

Fertilization of green sunfish eggs.—Cryopreserved CBG sperm were transported from the ARS to NSU in a liquid nitrogen shipping container (Taylor Wharton, CP-65). A green sunfish female (68 g) was injected with 50 μg of luteinizing-hormone releasing-hormone analog (Argent Laboratories, Redmond, Washington) per kilogram of body weight at 1530 hours; eggs were stripped at 1130 hours the following morning. The eggs were stripped into five plastic dishes (6.3 × 6.3 cm), and enough HBSS (300 mosmols/kg) was added to cover the eggs. Sperm were stripped from a male CBG, motility was estimated, and the sperm were added to one of the dishes. For the other four egg dishes, sperm samples were chosen to represent both cryoprotectants and both freezing methods. Straws were removed from the liquid nitrogen container and were thawed as previously described. Two straws (0.5-mL) were thawed for each dish. Freshwater was added to the samples to activate the gametes. All fertilization trials were performed within 20 min of egg collection. The eggs were incubated at 28°C in the fertilization dishes, and water was decanted and replaced every 60 min. Eggs were counted for fertilization by viewing with a dissecting microscope (Nikon SMZ-U). Eggs were considered fertilized when they developed to the morula stage (3–4 h).

Statistical analysis.—All percentage data were arcsine–square root transformed prior to analysis. Differences in the motility of thawed samples between the CRF and DIT freezing methods and between MeOH and DMSO were analyzed by use of chi-square tests (Statistical Analysis System version 8.1e for Windows; SAS Institute, Inc., Cary, North Carolina). Results were considered significant at P-values less than or equal to 0.05.

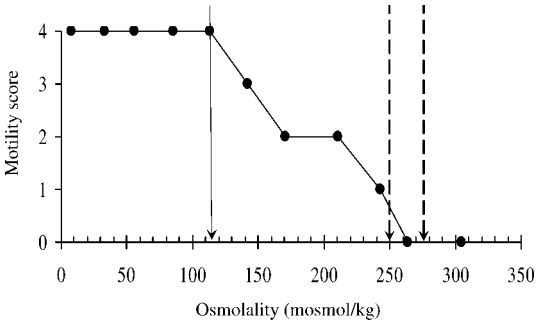

FIGURE 1.—Motility of coppernose bluegill sperm activated in diluted Hanks' balanced salt solution. Each point represents the mean value of samples from 10 fish. The solid arrow indicates the osmolality of complete activation. The dashed arrows indicate the range in which threshold activation occurs.

Results

Activation Curve and Short-Term Storage of Stripped Sperm

The mean osmolality of the blood plasma of CBGs was 288 ± 5 mosmols/kg. At osmolalities above 265 mosmols/kg no activation of sperm was observed. Between approximately 245 and 265 mosmols/kg, low levels of motility (<10%) were observed. Below 245 mosmol/kg, vigorous linear motility was observed and the percentage of actively motile sperm increased as osmolality decreased to 115 mosmol/kg. Below 115 mosmol/kg, no significant increase in sperm activation was observed (Figure 1). The initial motility of stripped sperm (mean ± SD) was 90 ± 7%. Motility after the first 24 h of storage at 4°C decreased significantly to 69 ± 9% ($P = 1.4 \times 10^{-8}$), and motility decreased again significantly to 44 ± 7% at 2 d of storage ($P = 2.7 \times 10^{-18}$). Motility decreased nonsignificantly to 42% ± 6% at 3 d of storage ($P = 0.60$) and 36% ± 7% at 4 d ($P = 0.13$). Motility was 31 ± 8% at 5 d and 26 ± 11% at 6 d (Figure 2).

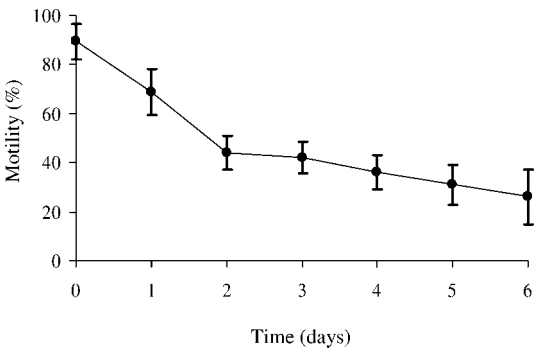

FIGURE 2.—Motility (mean ± SD) of coppernose bluegill sperm collected by hand stripping and stored in Hanks' balanced salt solution (300 mosmols/kg) for 6 d.

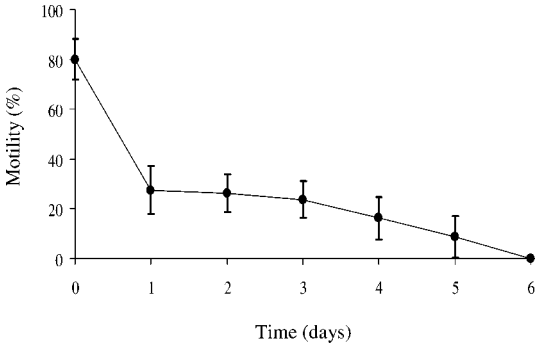

FIGURE 3.—Motility (mean ± SD) of coppernose bluegill sperm obtained from crushed testes and stored in Hanks' balanced salt solution (300 mosmols/kg) for 6 d.

Short-Term Storage of Sperm Solutions Prepared from Crushed Testes

Sperm collected from crushed testes had an initial motility (mean ± SD) of 80 ± 8% ($n = 4$). Motility decreased significantly to 28 ± 10% at 24 h of storage, but did not decrease over the next 2 d (26 ± 8% at 2 d and 24 ± 8% at 3 d). Motility over the final 3 d decreased significantly to 16 ± 9% at 4 d of storage, 9 ± 9% at 5 d, and 0% at 6 d (Figure 3).

Cryoprotectant Toxicity

Methanol had no significant effect on sperm motility over the 1-h exposure period (Figure 4). Dimethyl sulfoxide reduced motility from 80% ini-

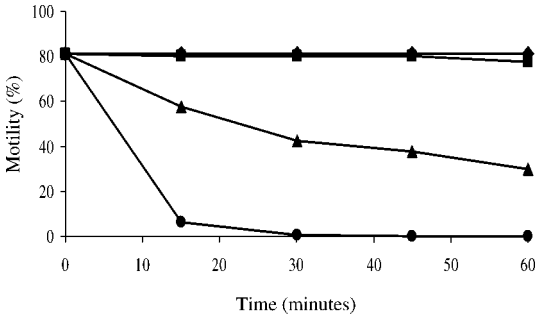

FIGURE 4.—Motility over time of coppernose bluegill sperm exposed to the cryoprotectants methanol (squares), dimethyl sulfoxide (triangles), and dimethyl acetamide (circles) (10% in each case). The motility of control cells unexposed to cryoprotectants (diamonds) remained unchanged during the experiment.

TABLE 1.—Initial motility (mean ± SD; $n = 4$ fish), post-thaw motility (mean ± SD), and fertilization capability of coppernose bluegill sperm cryoprotected with 10% methanol or 10% dimethyl sulfoxide and frozen by use of the controlled-rate freezer method or the dairy industry technology method. Green sunfish eggs were used to determine fertilization rate. Eggs fertilized with fresh sperm had a fertilization rate of 95%.

		Methanol		Dimethyl sulfoxide	
Freezing method	Initial motility (%)	Post-thaw motility (%)	Fertilization (%)	Post-thaw motility (%)	Fertilization (%)
Controlled-rate freezer	83 ± 10	51 ± 18	50	26 ± 10	15
Dairy industry technology	78 ± 5	56 ± 13	75	46 ± 21	50

tially to 30% at 1 h of exposure. Dimethyl acetamide reduced motility to 6% at 15 min of exposure and to 0% within 30 min.

Cryopreservation

Cryopreserved CBG sperm were motile after thawing and were able to fertilize green sunfish eggs (Table 1). Overall, samples frozen in 10% MeOH had significantly higher motility after thawing ($P = 0.043$) than did those frozen in 10% DMSO. There was no significant difference in the post-thaw motility of sperm cryopreserved with CRF versus DIT ($P = 0.13$). Eggs fertilized with fresh-stripped sperm had a fertilization rate of 95% at 4 h postfertilization. Due to the small sample size, no direct fertilization comparisons were made between the two freezing methods.

Discussion

This study demonstrates techniques that allow CBG sperm cells to be held in refrigerated storage for several days prior to use in fertilization of eggs or to be held in long-term storage by cryopreservation. By manipulating osmolality, we were able to activate CBG sperm cells at 10–235 mosmol/kg. At osmolalities between 245 and 265 mosmols/kg, there were low levels of activation and sluggish swimming by a few isolated sperm cells, which has been termed threshold activation (Bates et al. 1996). The findings of this study have additional implications in defining dilutions required for fertilization with CBG sperm cells. The study further demonstrates the viability of CBG sperm cells cryoprotected by either 10% MeOH or 10% DMSO and frozen by means of two different cryopreservation methods. However, the relatively nontoxic effect of MeOH combined with higher post-thaw motility and higher fertilization rates indicates that MeOH should be the cryoprotectant of choice for CBG sperm cells. The mechanism of CBG sperm activation appears to be chiefly modulated by osmolality, similar to observations made for channel catfish (Bates et al. 1996) and common carp *Cyprinus carpio* (Morisawa et al. 1983).

The method of collection appears to have a direct effect on sperm storage at 4°C. No direct comparison was possible between the sperm that were obtained by hand stripping and those obtained from crushed testes. However, it seems logical that the sperm obtained by straining crushed testes would contain cellular debris that would in turn increase the likelihood of bacterial contamination, as was noted for channel catfish (Jenkins and Tiersch 1997). While stripped sperm remained motile longer in this study than did sperm obtained from crushed testes, the volumes obtainable for stripped sperm were insufficient for use in artificial spawning. Furthermore, hand stripping of sperm increases the likelihood of contamination with urine, which can prematurely activate samples. Therefore, we conclude that males should be killed and sperm should be collected by straining crushed testes as described in this study. While the killing of males is an undesirable prospect to many producers, this approach could be easily adapted into an artificial spawning production design. The culturist would only need to hold females in the hatchery and could replace the sperm reserves each season if needed. In the case of cryopreserved sperm from superior individual males, repeated use could be controlled through proper breeding designs. Linebreeding, for instance, could maintain genetic attributes of a population with the possibility of collecting sperm from replacement donor males in future generations.

Although there was no significant difference in post-thaw sperm motility between the CRF and DIT, the DIT method offers many advantages. Methods for cryopreserving bull semen are standardized and have quality control guidelines promoted by groups such as Certified Semen Services, a subsidiary of the National Association of Animal Breeders (www.naab-css.org). Automated filling of straws reduces the potential for human error

and provides increased flexibility in allowing for sperm exposure (equilibration) with cryoprotective agents. Storage of sperm in commercial collection repositories would make the entire sperm volume of a male available for use and would allow greater access by breeders to superior genetic material for use in artificial spawning. Furthermore, sperm can be frozen in bulk quantities (>600 straws per freezing), whereas most CRF systems have capacities limited to 100 straws or fewer.

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