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Evaluation of Commercial-scale Approaches for Cryopreservation of White Crappie, *Pomoxis annularis*, Sperm

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Abstract

Crappie, *Pomoxis* spp., are popular game fish throughout North America and are produced by public and private hatcheries. However, production is limited by a lack of information on tank culture and induced spawning methods. Development of techniques for storage of sperm and *in vitro* fertilization would increase flexibility in spawning. Therefore, techniques for sperm cryopreservation were examined in white crappie, *Pomoxis annularis*. Sperm from adult wild white crappie were used to evaluate sperm extender, cryoprotectant agent and concentration, and cooling technique based on post-thaw sperm motility. Percent egg fertilization was also compared between sperm stored in the two best cryopreservation protocols and two different osmotic activator solutions. Sperm were cryopreserved using treatment combinations of two extenders (350 mOsmol/kg Hanks' balanced salt solution [HBSS] and 350 mOsmol/kg Ca^{2+}free HBSS) and two cryoprotectants (dimethyl sulfoxide [DMSO] and methanol) at concentrations of 5, 10, and 15% that were cooled at four different rates: 5, 10, 20, and 40 C/min. Post-thaw sperm motility and fertilization rates indicated white crappie sperm can be cryopreserved using either extender, cryoprotectants of either 5% DMSO or 10% methanol, and cooling at 40 C/min. A follow-up experiment demonstrated sperm in suspensions on ice retained viability after overnight transport.

KEYWORDS

crappie, cryopreservation, Pomoxis annularis, shipping, sperm

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The artificial spawning of crappie, *Pomoxis* spp., currently requires sperm from freshly excised testes for egg fertilization, obligating culturists to collect live males (Gomelsky et al. 2000, 2002). Similarly, culture of many fishes utilizes fresh sperm stripped or from excised testes, which are activated osmotically on contact with water (Brown and Brown 2011). However, sperm typically remain viable for only 1–5 min after activation, which constrains spawning and fertilization and limits genetic diversification techniques (Alavi and Cosson 2005). The use of systematic germplasm cryopreservation provides benefits to fish reproductive efforts such as: increasing the availability of spermatozoa; decreasing impacts on endemic, wild populations by providing sperm without requiring live male mortality at each spawn; reducing facility costs associated with maintaining brood stock; increasing reproductive success by reducing human error and increasing consistency in gamete maturity; and reducing interbreeding though maintenance of large gene pools (Hagedorn and Kleinhans 2011). Furthermore, the use of commercial-scale or practical techniques (Tiersch 2011), such as those previously developed for cryopreservation of bull sperm (Chandler and Godke 2011), would provide opportunities for wide adoption of techniques through establishment of reproducible protocols and minimization of costs.

In crappie, one beneficial application of sperm cryopreservation would be for the culture of triploid hybrid crappie (female white crappie, *Pomoxis annularis* × male black crappie, *P. nigromaculatus*). Hybrids are currently produced by several public and private hatcheries in the southeastern USA to stock small impoundments (ca. 0.4–2.0 ha) (Culpepper and Allen 2016a), as either parental species can quickly overpopulate smaller aquatic systems (Busack and Baldwin 1988; Guy and Willis 1995). A morphological variant of black crappie commonly referred to as "black-stripe crappie," endemic to the Arkansas River and currently present in several large reservoirs in the southeastern USA (e.g., Grenada Lake, Grenada, MS, USA; 33.8194 N, 89.7736 W), possess a black stripe running along the dorsal surface of the head. This phenotypic marker is preferred by hatcheries to produce triploid hybrid crappie that can be easily identified following stocking (Parsons 1996; Gomelsky et al. 2005). However, acquiring adequate numbers of male black crappie with this phenotypic marker is often problematic during the short, natural spawning season and can limit crappie production (C. Silkwood, pers. commun., 2016). The cryopreservation and long-term storage of crappie sperm possessing this morphological trait would aid in the production of phenotypically marked, triploid hybrid crappie in the southeastern USA. Importantly, methods for cryopreservation and fertilization need to be developed in white crappie, before methods can be evaluated for hybrid crappie.

Currently, no information is available regarding the refrigerated storage of crappie sperm cells. However, sperm of other, more intensively cultured fishes, such as coppernose bluegill, *Lepomis macrochirus purpurescens* (Bates et al. 2005); rainbow trout, *Oncorhynchus mykiss* (Perez-Cerezales et al. 2010); striped bass, *Morone saxatilis* (Brown and Brown 2011); and white bass, *Morone crysops* (Brown and Brown 2011), have been cryopreserved using extenders and cryoprotectant agents. For coppernose bluegill, striped bass, and white bass, Hanks' balanced salt solution (HBSS) has been used as an extender (Bates et al. 2005; Brown and Brown 2011). Cryopreservation techniques are variable among species in terms of extender solution composition, sperm : extender dilution ratios, cryoprotectant agents and concentrations, and cooling and thawing regimes (Hagedorn and Kleinhans 2011). Determining commercial-scale or practical procedures for cryopreservation of a given fish species requires several experimental phases that gradually narrow down the optimal technique. Optimization consists of fresh sperm concentration calculation and initial sperm motility estimations, buffer agent analysis, examination of acute toxicity of cryoprotectants, sperm motility estimations following various cooling rates, sperm motility estimation following thawing, and direct comparisons of percent fertilization between fresh and thawed samples (Tiersch 2011). At

each of these phases, computer-assisted sperm analysis (CASA), which quantifies single-sperm movement using computer-calculated motility characteristics, is currently the most objective and comprehensive method available for quality assessment (Wilson-Leedy and Ingermann 2007).

Developing a method to preserve and extend the viability of crappie sperm by use of extender solutions and cryopreservation for long-term frozen storage offers important benefits. Therefore, the objectives of this study were to: (1) determine an extender that prevents sperm cell activation, (2) identify suitable cryoprotectant agents for sperm cryopreservation, (3) identify a suitable cooling rate that maximizes percent motility following thawing, (4) compare fertilization rates following the use of different cryoprotectants, and (5) examine the effect of short-term storage and transport.

Materials and Methods

Fish Collection

All methods followed Mississippi State University institutional animal care and use committee protocol #13–111. Male white crappie were collected from Enid Reservoir, in northern Mississippi, USA (34.1489 N, 89.9061 W). Fish were collected by electroshocking using a sampling boat and a 7.5 hp, generator-powered shocking unit set at 60 Hz direct current (Smith-Root Inc., Vancouver, WA, USA), with 3-m shocking poles extended beyond the front of the boat. For the extender and cryopreservation experiments, seven male white crappie were transferred within 1 h after collection to a hauling tank supplied with compressed oxygen and transported to the Aquaculture Research Station of the Louisiana State University Agricultural Center, Baton Rouge, LA, USA. The hauling tank was filled with fresh reservoir water, and 3 ppt NaCl was used to reduce stress during transport. No mortality occurred during the 6-h transport. Upon arrival at the Aquaculture Research Station, male white crappie were placed into a 5600-L recirculating system (15 C) until they were needed for sampling (ca. 13 h).

Initial Sperm Concentration Calculation

A 500-µL sperm suspension was prepared for each male by diluting sperm, excised from testes and filtered through a 0.8-mm screen, at 1:50 with 350 mOsmol/kg HBSS350. This suspension was lightly vortexed to ensure proper mixing. A 10-µL sample was removed from the sperm suspension and placed on a Makler® counting chamber (SEFI Medical Instruments Ltd, Irvine Scientific, Santa Ana, CA, USA) viewed at 200× magnification (Olympus CX41RF, Tokyo, Japan). Each sample was counted in triplicate using the standard Makler counting protocol. The counts were averaged to obtain the sperm concentration per mL for each male (Tiersch et al. 2011).

Motility Estimation

Motility was estimated by placing 5 µL of sperm on the Makler counting chamber and adding 20 µL of hatchery water (filtered and ultraviolet [UV]-treated reservoir water) for activation. The chamber was immediately placed on a microscope (Olympus CX41RF) at 200× magnification and quantified using CASA (CEROS model; Hamilton Thorne, Inc., Beverly, MA, USA). The instrument settings were: minimum contrast, 60; minimum cell size, 2; number of frames for recording, 100; average path velocity cutoff, 25 µ/s; straight line velocity cutoff, 1 µ/s. For each sample, at least three measurements of different viewing fields were performed, and the average was used as the motility for that sample. These variables were verified using the "playback" function of the software.

Acute Toxicity

Sperm samples from three white crappie males were used in the acute toxicity trials to evaluate extenders, cryoprotectants, and their concentrations. Extenders and cryoprotectants were chosen for ease of standardization, replication, and practical application and demonstrated efficacy with many other fishes (Horvath et al. 2005; Yang et al. 2007; Cabrita et al. 2010) including centrarchids (Bates et al. 2005). From each male, two sperm suspensions were made by diluting sperm with the appropriate extender

(1:1) (HBSS350 [Tiersch et al. 1994] or 350 mOsmol/kg Ca^{2+} free HBSS350) and concentrations were adjusted to 5.0×10^8 sperm per mL. Two cryoprotectants (methanol and dimethyl sulfoxide [DMSO]) were diluted with HBSS350 or Ca-free HBSS350 to concentrations of 10, 20, and 30% (v/v). These cryodiluents were then mixed 1:1 (v : v) with sperm suspension to achieve the final cryoprotectant concentrations of 5, 10, and 15% (v/v) and a cell concentration of 2.5×10^8 sperm/mL. This concentration was used to provide a high density of sperm in cryopreserved straws, while maintaining viability of sperm. The high density provides an economic benefit for subsequent users by reducing the number of straws needed to be stored.

Cryopreservation

For sperm samples from all male white crappie, upon mixing with the cryoprotectant as described above, the sperm samples were placed in an automated packaging system (Quattro Minitube, Verona, WI, USA) and straws were filled, sealed, and labeled using a proprietary computer program (Minijet ver. 4.00.01, Verona, WI, USA). Samples were drawn into 0.5-mL French straws by vacuum applied to the cotton end of the straw. The straws were continuously transferred to the sealing platform and sealed on one end by application of a 158 C heat clamp. Afterward, straws were dropped onto a conveyor belt and labeled with alphanumeric information and a barcode by an ink jet printer (A-series plus, Domino, IL, USA) before being transferred to the collection area for label verification. Straws were arrayed on horizontal racks (40 straws/rack) and placed in a commercial-scale programmable freezer (Micro Digitcool; IMV, France) with a capacity of 280 straws per cycle. Thermal mass was not equalized at every cooling cycle (e.g., by adding "dummy" straws to fill the freezer) and the number of straws in each freezing cycle ranged from 113 to 221 straws. Between 15 and 19 min after the cryoprotectant was added to the sperm suspension ("equilibration time"), the cooling program was initiated. Based on results from the acute toxicity trials, three cryoprotectant concentrations (5% methanol, 5% DMSO, and 10% methanol) were selected for cooling experiments because these treatments yielded the highest percent of motile sperm after 30 min at equilibration. Four cooling rates (5, 10, 20, and 40 C/min), with 27 straws per cooling rate, were used to determine the rate that maximized sperm survival.

Thawing and Motility Assessment

Straws were thawed by immersion in a water bath at 40 C for 8 sec. Excess water was wiped from the outside of the straw before opening. For each sample, the sperm suspension was transferred into a 1.5-mL centrifuge tube. Motility of the thawed sperm was estimated within 30 sec after thawing using CASA as outlined above. Two straws for each male at each treatment were thawed and assessed for motility.

Fertilization Assessment

Fertilization rates were compared between cryoprotectants (5% DMSO and 10% methanol) after long-term (1 yr) storage in liquid nitrogen. Cryoprotectants were also compared using two osmotic activators for sperm: hatchery water (filtered and UV-treated reservoir water; 4 mmol/kg) or 4.0 g/L (70 mM) NaCl with 3.0 g/L (50 mM) urea (salt/urea; 165 mmol/kg), yielding four treatment groups. Eight female white crappie were induced to spawn using intramuscular injection of gonadotropin-releasing hormone (0.5 mL/kg; Ovaprim; Western Chemical, Ferndale, WA, USA) at 10% priming and 90% resolving doses, and ovulated eggs were stripped from females by use of established methods (Culpepper 2015; Culpepper and Allen 2016a). Approximately 750 eggs were pipetted (0.25 mL) into each of three replicate petri dishes per treatment for a total of 96 petri dishes (four treatments × three replicates × eight females). For all females, egg fertility was verified using separate aliquots of eggs combined with fresh sperm collected from male white crappie following Culpepper (2015) and Culpepper and Allen (2016b). Fresh sperm was also tested for fertilization using the same osmotic activation solutions of water or salt/urea with eggs from nine females with three petri dish replicates

per treatment, per female. Aliquots of fresh sperm (0.2 mL) to petri dishes were similar for each male and motility was verified, but concentration of fresh sperm was not able to be determined, therefore, comparisons were not made with cryopreserved sperm. Previously sampled white crappie ($n = 7$) had mean ($\pm$SE) fresh sperm concentrations of 2.24×0^9 ($\pm 2.27 \times 10^8$) cells/mL, which were higher than cryopreserved sperm concentrations (2.5×10^8 cells/mL) that had been diluted prior to freezing.

Eggs, sperm (two straws), and osmotic activator solution (10 mL of water or salt/urea) were sequentially added to petri dishes, swirled twice, and left stationary for 10 min to allow for fertilization and egg attachment to the petri dish. Afterward, petri dishes were carefully rinsed with fresh water to remove sperm and placed in a shallow, flow-through tank under approximately 2.5 cm of slowly flowing hatchery water from the nearby reservoir (Enid Reservoir), following sand filtering and UV sterilization. Fertilization percentages were calculated 24 h afterward by placing each petri dish under a stereomicroscope and making three counts of fertilized and unfertilized eggs (following Culpepper (2015)) from at least 30 eggs from randomly selected locations on the petri dish.

Short-term Storage and Transport

A second experiment was conducted to assess the effect of short-term storage and transport (24 h) on sperm motility following cryoprotection and thawing using different male crappie from the first experiment. Seven white crappie males were collected using previously described methods and held in a recirculating tank system (15 C; 3–5 ppt salinity) for 4 wk. Fish were placed in a fiberglass trough tank within the recirculating system (2460 L) that was divided into four compartments (125 cm length $\times$ 91 cm width $\times$ 54 cm depth; 0.61 m^3), using prefabricated metal dividers. Water quality was maintained using a 120-W high-output UV sterilizer (Emperor Aquatics, Pottstown, PA, USA) and a bead filter (DF3, 85 L media volume; Aquaculture System Technologies, New Orleans, LA, USA). Temperature was maintained at 17.1 ± 0.4 C (mean $\pm$ SE) using a chiller (1/2 HP, 115 volt Cyclone water chiller; Aqua Logic Inc., San Diego, CA, USA). Water quality variables (dissolved oxygen [6.7 ± 0.8 mg/L], total ammonia nitrogen [0.4 ± 0.2 mg/L], salinity [4.8 ± 1.1 g/L], and pH [7.8 ± 0.4]) were measured daily throughout acclimation using a YSI Professional Plus multiparameter probe (Model #10102030; YSI Inc., Yellow Springs, OH, USA).

After 4 wk, males were netted and placed in a portable electroshocking unit (Portable Electroshocking System; Smith-Root Inc., Vancouver, WA, USA) and stunned using a 60-Hz, 300 V shock for 3–4 sec. The testes were excised from each male by dissection, placed in a sterile petri dish, weighed, and minced with a scalpel. A small sample (ca. 100 µL) of fresh sperm was activated by the addition of 1–2 mL of water and observed under a stereomicroscope (Model 162-P; National, Schertz, TX, USA) at 100× magnification to confirm sperm motility before buffering. Testes samples were pushed through a standard aquarium net (ca. 0.5 mm mesh; Tetra, Blacksburg, VA, USA) into a 50-mL sample vial to remove large tissue pieces prior to dilution. Sperm samples were diluted 1 g testes : 10 mL HBSS350, which was within the dilution range of 1:1–1:15 shown to improve sperm motility in several other freshwater species (Glenn et al. 2011). The samples were gently mixed and vial caps were loosely tightened to allow for oxygen exchange (Tiersch et al. 2011). The samples were placed in an insulated container with ice (4 C) and shipped overnight to the Aquaculture Research Station of The Louisiana State University Agricultural Center (Baton Rouge, LA, USA) for motility assessments after transport, cryoprotection, and thawing. The cooling rate was programmed at 40 C/min based on results from cooling trials. After the final target temperature of −80 C was reached, the frozen samples were held for 5 min and placed into liquid nitrogen (−196 C). Individual straws were sorted in liquid nitrogen and placed into storage containers (Daisy goblets, reference number: 015144; Cryo Bio Systems, Maple Grove, MN) for long-term storage in liquid nitrogen.

Thawing and motility assessment methods were those described previously.

Statistical Analysis

All data were analyzed using program R (R Foundation for Statistical Computing, Vienna, Austria). A Shapiro–Wilk normality test was used to test normality of data and a Bartlett test was used to determine equality of variance between treatments. All percentage data were logit transformed. A three-way ANOVA was used to test for differences among extender buffer (HBSS350 and Ca-free HBSS350), cryoprotectant type (methanol and DMSO), and concentration of cryoprotectant in sperm motility. A two-way ANOVA was used to determine if a difference existed between cooling rate (5, 10, 20, 40 C/min) and cryoprotectant type. If differences were identified by ANOVA, Tukey's honestly significant difference *post hoc* test was used to identify significant differences between treatment means. One-way ANOVAs were run for post-thaw sperm motility and motility following short-term storage. For fertilization percentages a two-way ANOVA was conducted with factors of cryoprotectant (5% DMSO, 10% methanol) and osmotic activator solution (water, salt/urea). Fresh sperm was compared for osmotic activator solution using a student's *t* test. For all tests, $\alpha = 0.05$.

Results

Sperm Concentration in Excised Testes

White crappie ($n = 7$) had mean (±SE) fresh sperm concentrations of 2.24×10^9 ($\pm 2.27 \times 10^8$) cells/mL in excised testes.

Acute Toxicity

There was no difference between extenders (HBSS350 and Ca-free HBSS350), indicating either could be effectively used as a short-term sperm extender solution (Fig. 1). There were differences between cryoprotectant type (methanol or DMSO) and concentration (5, 10, or 15%) from 10 to 30 min after mixing (significant interaction of cryoprotectant × concentration) (Fig. 1). Based on these results, three treatments

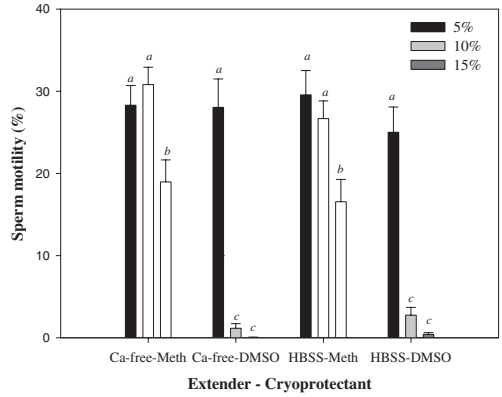

FIGURE 1. *Percent sperm motility (mean ± SE) of white crappie,* Pomoxis annularis*, following 10–30 min acute exposure to 5, 10, and 15% methanol (Meth) or dimethyl sulfoxide (DMSO) mixed with either of two sperm extender solutions: 350 mOsmol/kg Ca-free Hanks' balanced salt solution (HBSS) or 350 mOsmol/kg HBSS (*n = *10 fish/treatment). Different letters above each treatment represent significant differences (*$P < 0.05$*) using a three-way ANOVA and Tukey's honest significant difference test.*

(5% methanol, 10% methanol, and 5% DMSO) with the highest motility were selected to be used for the next phase of cryopreservation experiments on determination of cooling rate.

Cooling Rate

There was a difference in sperm motility among cooling rates (5, 10, 20, and 40 C/min), with 40 C/min having the highest overall motility after thawing, while the lowest motility was observed at 5 C/min (Fig. 2). There was no difference among 5% methanol, 10% methanol, and 5% DMSO at a cooling rate of 40 C/min.

Thawing

There was a difference among post-thaw sperm in motility (mean ± SE: 5% methanol = 5 ± 3%, 5% DMSO = 24 ± 10%, and 10% methanol = 28 ± 13%), where 5% methanol resulted in lower percent sperm motility following thawing. No difference was found in motility between 5% DMSO and 10% methanol.

Fertilization

There were no differences in fertilization between 5% DMSO and 10% methanol,

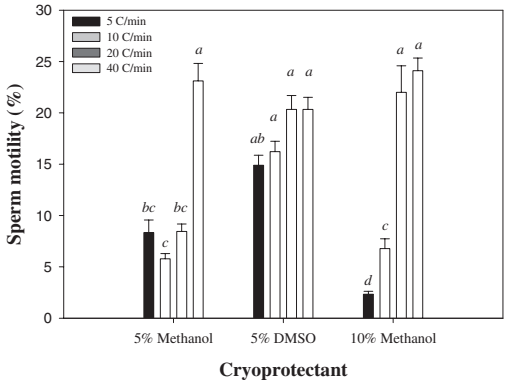

FIGURE 2. *Comparison of percent motility (mean ± SE) of white crappie,* Pomoxis annularis*, sperm following four different cooling rates (5, 10, 20, or 40 C/min) (*n = *27 sperm sample straws/cooling rate) for three cryoprotectant treatments (5% methanol, 5% DMSO, and 10% methanol) after being thawed at 40 C for 8 sec. Different letters above each treatment represent significant differences (*P < *0.05) using a two-way ANOVA and Tukey's honest significant difference test.*

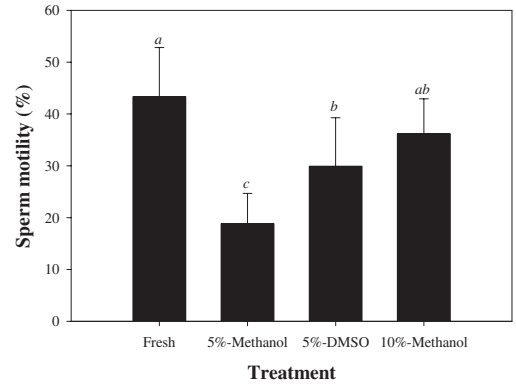

FIGURE 3. *Percent sperm motility (mean ± SE) of white crappie,* Pomoxis annularis*, unfrozen sperm (fresh), and sperm transported overnight, cryopreserved in one of the three cryoprotectants (5% methanol, 5% DMSO, and 10% methanol) and thawed at 40 C for 8 sec. Different letters above each treatment represent significant differences (*P < *0.05) using a one-way ANOVA and Tukey's honest significant difference test.*

water and salt/urea, nor their interaction. Treatment means ± SE were: 5% DMSO + water, 14.2 ± 3.3%; 5% DMSO ± salt/urea, 13.7 ± 3.9%; 10% methanol + water, 10.7 ± 4.8%; and 10% methanol + salt/urea, 14.5 ± 4.8%. Fresh sperm fertilization rates were similar between osmotic activator solutions, with 52.7 ± 6.9% in water and 46.7 ± 7.9% in salt/urea, but were not directly compared to cryopreserved sperm due to presumably higher sperm concentrations. Despite the filtration and UV sterilization of hatchery water, parasites were found in a number of eggs (ca. 10%), with similar numbers of parasitized eggs among treatments in petri dishes, which may have reduced fertilization percentages.

Short-term Storage

After short-term storage, sperm cryopreserved in 10% methanol recovered the same motility (ca. 40%) as that of fresh sperm, with 5% DMSO recovering approximately 30% motility and 5% methanol only approximately 20% (Fig. 3). Also notable was that 5% methanol has the lowest sperm motility, lower than 5% DMSO and 10% methanol, which were not different from each other.

Discussion

Although cryopreservation techniques are increasingly being developed for a number of fishes, only a limited number of studies have been conducted on centrarchid species. This study describes the first work with crappie, investigating extender buffers, cryoprotectants, and thaw rate on white crappie sperm motility and egg fertilization percentage. In addition, these methods are evaluated for their efficacy with short-term storage and transport of sperm in a field collection scenario.

For white crappie sperm, effective extenders and corresponding dilution ratios were similar to those utilized with several other fish species. Osmolality requirements for inhibiting sperm cell activation in extenders were 300–350 mOsmol/kg and utilized HBSS, which were similar to those for coppernose bluegill (Bates et al. 2005). In terms of sperm dilution ratios, the 1:1 sperm : extender ratio used for white crappie in these experiments has also been recommended for white bass (Brown and Brown 2011), and a 1:1 ratio with Ca-free HBSS350 was recommended for razorback sucker, *Xyrauchen texanus* (Tiersch et al. 2011). Greater dilutions of sperm with sperm : extender ratios of 1:3 have

been recommended for striped bass (Woods 2011) and rainbow trout (Lahnsteiner 2011). For commercial-scale or practical approaches, sperm is maintained at as high of a concentration as possible to reduce storage costs for sperm. However, low dilution ratios and resulting high concentrations of sperm may have diminished post-thaw motility, possibly due to limited intercellular space or cell compression during storage (Lahnsteiner 2011). Therefore, in salmonid fishes, diluted sperm has been suggested to not exceed 2.5×10^9 sperm cell/mL (Lahnsteiner 2011). Further dilutions of white crappie sperm (e.g., 1:3, 1:5, and 1:7) may increase sperm motility and fertilization capacity of individual storage straws.

Cryoprotectant concentrations similar to those identified in this study (i.e., 5% DMSO and 10% methanol) have been used in previous sperm cryopreservation studies of freshwater fish. Coppernose bluegill sperm have been cryopreserved using 10% DMSO or 10% methanol (Bates et al. 2005). White bass and striped bass sperm were cryopreserved with a 4% DMSO cryoprotectant concentration, with the addition of 10% trehalose at 100 mg/mL which increased motility (Brown and Brown 2011). A concentration of 7.5% DMSO has also been recommended for striped bass (Woods 2011).

Because sperm are not adapted to extreme cold temperatures, cold shock caused by the cryopreservation process can damage sperm cellular membranes and reduce post-thaw motility (Medeiros et al. 2002). The rate of cooling during the cryopreservation process can help to minimize damage to the sperm cellular membranes and maintain post-thaw motility. In this study, a 40 C/min cooling rate, equal to the recommended rates for rainbow trout (Lahnsteiner 2011) and striped bass (Woods 2011), produced the highest sperm motility following thawing.

Post-thaw fertilization assessments indicated the cryoprotectant agents (5% DMSO and 10% methanol), and osmotic activators (water and salt/urea), all yielded similar fertilization rates (overall mean: 13.3%) after sperm were stored for 1 yr in liquid nitrogen. Freshly excised, undiluted sperm yielded higher fertilization rates (overall mean: 49.7%) but was not directly comparable to cryopreserved sperm due to much higher concentrations of sperm. Similarly, fertilization experiments conducted with coppernose bluegill using a 10% DMSO cryoprotectant found that fertilization was 15 vs. 95% for freshly excised, undiluted sperm, while a 10% methanol cryoprotectant resulted in higher post-thaw fertilization (50%) (Bates et al. 2005). In rainbow trout, the highest fertilization (82%) after thawing (25 C for 30 sec) was in a 5% methanol cryoprotectant (Lahnsteiner 2011).

Dilution in extender and refrigerated storage of sperm samples are useful tools for gamete collection in the field, where cryopreservation is difficult to accomplish (Tiersch et al. 2011). Results from short-term storage and transport indicate overnight shipping of white crappie sperm, using a 1:10 dilution with 350HBSS, is a useful technique for transporting sperm samples to a facility where cryopreservation can be accomplished. Further experiments could be conducted to compare transportation methods (e.g., plastic tubes versus petri dishes) and examine the effect of oxygen supplementation. Because large variation in post-thaw sperm motility exists among published studies (ca. 15–70%), direct comparisons of cryopreservation quality among any species are problematic.

This study developed practical cryopreservation protocols for white crappie sperm based on using either HBSS350 or Ca-free HBSS350 as an extender. The highest post-thaw motilities were achieved with a cooling rate of 40 C/min, where both 5% DMSO and 10% methanol cryoprotectants yielded similar motility (24–28%) and fertilization rates (8–16%) following thawing. Short-term (< 24 h) storage of sperm samples, with a 1:10 ratio of HBSS350 at 4 C, maintained sperm survival during transportation. These storage and transport methods provide a viable alternative to immediate use after harvesting of testes from wild-caught male crappie, which can be difficult and costly to acquire in adequate numbers during the short natural spawning season. Long-term storage of crappie sperm can have potential utility for out-of-season spawning and production of phenotypically marked triploid hybrid crappie.

Further studies on improvements in cryopreserved sperm fertilization rate, determining optimal refrigerated storage conditions, and development of cryopreservation methods for black-stripe black crappie would be beneficial.

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