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A generalized approach for sperm cryopreservation in the genus *Pomoxis*: Sperm cryopreservation and fertilization efficiency of black-stripe black crappie, *Pomoxis nigromaculatus*

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Abstract

Approaches for white crappie, *Pomoxis annularis* sperm cryopreservation have led to interest in applying similar methods to black-stripe black crappie, *Pomoxis nigromaculatus*. Their rarity in wild populations makes them a preferred phenotype for hatchery use. Sperm cryopreservation procedures were compared between black-stripe black crappie and white crappie for sperm motility and egg fertilization rate. There was no difference in black-stripe black crappie sperm motility after thawing between 5% dimethyl sulfoxide (DMSO, 45% motility) and 10% methanol (50% motility). However, fertilization rates were higher ($p < .001$) for sperm cryoprotected with 5% DMSO (38 ± 8%) than 10% methanol (22 ± 7%). Hatchery use requires sperm-to-egg ratios and fertilizing potential of single doses (i.e., 0.5 ml straw). Using black-stripe black crappie sperm (2.5×10^8 sperm/ml; 5% DMSO), the highest fertilization (27%) was found using single straws with 785 eggs (0.25 ml); total sperm:egg ratio: 159,000:1; motile sperm:egg ratio: 71,700:1. Therefore, sperm of two *Pomoxis* species could be cryopreserved using 350 mOsmol/kg Hanks' balanced salt solution as an extender, 5% DMSO as a cryoprotectant, cooling at 40°C/min, and thawing for 8 s at 40°C to maintain sperm motility and fertility. Basic protocols can be generalized within a genus if variables such as sperm concentration, process timing, and sample volumes are controlled.

Keywords

black-nose; Centrarchidae; crappie; cryopreservation; fertilization; Pomoxis; sperm

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1 | INTRODUCTION

Black crappie, *Pomoxis nigromaculatus* and white crappie, *Pomoxis annularis* are highly sought-after game fish throughout their ranges in North America and present unique management challenges including variable recruitment and the ability to overpopulate small impoundments (Busack & Baldwin, 1988; Mitzner, 1991; Swingle & Swingle, 1967). Recent advances in crappie aquaculture techniques, such as the ability to produce monosex, and sterile or triploid crappie (Al-ablani & Phelps, 1997; Arslan & Phelps, 2004; Cuevas-Uribe, Gomelsky, Mims, & Pomper, 2009; Culpepper & Allen, 2016a; Gomelsky, Mims, Onders, & Bean, 2002; Parsons, 1999), are helping managers address the issue of overpopulation in smaller impoundments.

Hybrid-sterile or hybrid-triploid crappie are produced to address overpopulation in small impoundments. Hybrids are produced by crossing a female white crappie with a male black crappie, and triploids can be induced by subjecting fertilized eggs to a high-pressure treatment (Parsons, 1992, 1996, 1999). For example, application of 6,000 psi (422 kg/cm^2) for 2 min to fertilized crappie eggs resulted in an 87% hatching rate and 100% triploidy (Parsons, 1999). The black-stripe black crappie is a naturally occurring phenotype expressing a unique predorsal stripe thought to be controlled by a mutant, dominant gene (Gomelsky, Mims, Onders, & Novelo, 2005). Phenotypic identifiers serve as tags that are beneficial because they cannot be lost (Henderson-Arzapalo et al., 1999) and do not expose fish to additional physical constraints that may increase predation (Catalano, Chipps, Bouchard, & Wahl, 2001), which is important for hatcheries and fisheries management (McFarlane, Wydoski, & Prince, 1990). Hatcheries have produced black-stripe black crappie as diploids and often triploid or hybrids (Parsons, 1996). The black-stripe black crappie phenotype is useful to biologists as a natural tag because of the rarity of the phenotype in the wild, facilitating identification of stocked fish (Isermann, Bettoli, Sammons, & Churchill, 2002). However, this rarity makes it difficult to reliably find enough broodstock for hatchery production during the spawning season. One way to address the need for more broodstock with the desired trait would be to cryopreserve the sperm for long-term storage and easy future access.

The ability to cryopreserve sperm has many benefits for aquaculture including storage of valuable genetic resources, reducing broodstock needs, ease of storage and transport, and the ability to have sperm available when female fish are ready for spawning (Cabrita et al., 2010). Methods for cryopreserving sperm have been documented in dozens of freshwater fishes including salmonids, sturgeons, carps, and catfishes (Cabrita et al., 2010), as well as temperate fishes of the same family as crappie (e.g., coppernose bluegill, *Lepomis macrochirus purpurescens*), or occupying similar habitats (e.g., white bass, *Morone chrysops*, and striped bass, *Morone saxatilis* (Brown & Brown, 2011). Recently, white crappie sperm was cryopreserved using 350 mOsmol/kg Hanks' balanced salt solution (HBSS350) as an extender, either 5% dimethyl sulfoxide (DMSO) or 10% methanol as cryoprotectant, and cooling at 40°C/min (Culpepper, Guitreau, Allred, Tiersch, & Allen, 2018). The capacity to cryopreserve sperm of the black-stripe black crappie following similar procedures could simplify strip-spawning and help maintain genetic diversity through quick access to sperm from multiple males.

Along with procedures for cryopreservation, to maximize the effectiveness of strip-spawning, techniques and procedures for fertilizing eggs using cryopreserved sperm need to be developed. For centrarchids, fertilization rates using cryopreserved bluegill sperm ranged from 15 to 50%, depending on cryoprotectant (Bates, Williams, Lang, & Tiersch, 2005). Fertilization rates using cryopreserved white crappie sperm yielded relatively low fertilization (13%) (Culpepper et al., 2018), indicating a need for refinement of fertilization techniques using cryopreserved sperm. Cryopreservation techniques are not commonly applied to produce hybrids in fishes, but have been used successfully (Gharrett, Smoker, Reisenbichler, & Taylor, 1999), sometimes with similar or higher fertilization rates to fresh sperm (Chao, Chao, Liu, & Liao, 1987). The ability to fertilize crappie eggs using cryopreserved black-stripe black crappie sperm could allow for increased efficiency and production of fish possessing the desired trait.

Developing sperm cryopreservation and egg fertilization methods would be beneficial to aquaculture and recreational hatcheries by allowing for black-stripe black crappie sperm to be reliably stored and easily accessible for strip-spawning. This would reduce the need for collecting and maintaining difficult-to-locate broodstock and increase fertilization percentages when using cryopreserved sperm. Therefore, the objectives of this study were to investigate whether (a) black-stripe black crappie sperm could be cryopreserved using methods established for white crappie (Culpepper et al., 2018), (b) compare the use of two cryoprotectants (i.e., 5% DMSO and 10% methanol) for black-stripe black crappie sperm, and (c) determine optimal egg volume (i.e., sperm:egg ratios) for fertilization using cryopreserved black-stripe black crappie sperm. These studies also offer insight on the generalization of cryopreservation across species, and practical application to support germplasm repository development.

2 | MATERIALS AND METHODS

2.1 | Fish collection and transportation

Male black-stripe black crappie (mean ± *SE*: weight: 511.12 ± 15.24 g; total length: 328 ± 7 mm) were obtained from William H. Donham State Fish Hatchery, in Corning, Arkansas, and were transported to the Mississippi Department of Wildlife, Fisheries, and Parks (MDWFP) North Mississippi Fish Hatchery (NMFH) in Enid, Mississippi. The fish were transported in 445-L (203 × 51 × 43 cm) rectangular tanks at 15°C and 3 ppt salinity (NaCl), conditions found to reduce disease and mortality in crappie (Culpepper & Allen, 2016b). Male white crappie (mean ± *SE*: weight: 454.47 ± 34.26 g; total length: 313 ± 4 mm) were collected from Enid Lake, Enid, MS in March 2017 in tributary arms branching off the main reservoir, via boat electrofishing using a 7.5-hp, generator-powered electrofisher shocking unit set at 60 Hz direct current (Smith-Root Inc, Vancouver, WA). Fish were placed in an onboard tank (61 × 115 × 54 cm, 380 L), during collection and supplied with circulating lake water until sampling concluded (~2 hr). Upon arrival, all fish were placed in a large recirculating aquaculture system (RAS) consisting of two rectangular tanks (630 × 89 × 61 cm, 3,420 L). Salinity was maintained at 3 ppt using artificial marine salt (Instant Ocean Sea Salt, Instant Ocean, Blacksburg, VA) and temperature maintained at 15°C, to prevent disease and reduce mortality, until the beginning of the experiment. Temperature was monitored

and maintained using a temperature controller (Single Stage Controller, 1/4–1/2 hp, 115 V, Aqua Logic Aquatic Inc., San Diego, CA) and a water chiller (1/2 hp, Cyclone Water Chiller, Aqua Logic Aquatic Inc., San Diego, CA). Water quality was maintained using 120-W high-output ultraviolent sterilizer (Emperor Aquatics, Pottstown, PA) and a bead filter (DF3, Aquaculture System Technologies, New Orleans, LA). Two weeks before transfer or spawning experiments, the RAS temperature was increased by 1°C daily for 6 days, until reaching spawning temperatures of 21°C (Culpepper & Allen, 2016b) and maintained at spawning temperatures for another week.

For sperm cryopreservation, fish were transported at 21°C to the Aquatic Germplasm and Genetic Resources Center of the Louisiana State University Agricultural Center in NMFH hatchery water with 3 ppt NaCl (Solar Salt). Oxygen was supplied throughout the trip using a compressed oxygen cylinder and airstones placed in the hauling tanks. Upon arrival, fish were acclimated to water temperatures of outdoor recirculating tanks (~21°C), where they were held separately by species until the experiment (~15 hr).

2.2 | Sperm cryopreservation

The experiment was designed to compare the motility of white crappie and black-stripe black crappie cryopreserved sperm, using two cryoprotectants (i.e., 5% DMSO and 10% methanol). A fish was selected from the recirculating system and placed in an anesthetic bath (100 mg/L tricaine methanesulfonate, 9 g/L NaCl and 400 mg/L $NaHCO_3$), until opercular and fin movement ceased. The fish was rinsed with HBSS350 to ensure water would not activate sperm after testes were removed (Testes gonadosomatic index: white crappie: 0.71 ± 0.06%; black-stripe black crappie: 0.59 ± 0.03%). The testes were removed via dissection and placed in a weigh boat containing enough HBSS350 to cover the entire organ. The testes were removed from the HBSS, minced, and filtered through a 0.8-mm screen to remove excess tissue. A 100-ml sperm suspension was made for each fish using HBSS350 to dilute neat sperm (1:19; sperm: HBSS350). The solution was vortexed to mix, and 5 μl was used to assess initial concentration of sperm for each fish by placing the sample on a Makler® counting chamber (SEFI Medical Instruments LTD, Irvine Scientific, Santa Ana, CA) and viewing with a dark-field microscope (Olympus CX41RF, Japan) at ×200 magnification. Each sample was counted three times and counts were averaged to obtain the initial sperm concentration per ml for each fish.

To estimate sperm motility before cryopreservation, a 4-μl sperm sample was placed on a Makler® counting chamber with 20 μl of water. Motility was estimated within 10 s of sperm activation using computer-assisted sperm analysis (CASA) (HTM-CEROS, version 14 Build 013, Hamilton Thorne Biosciences, Beverly, MA). System settings for assessing progressive motility were minimum contrast, 60; minimum cell size, 2 μm; number of frames for recording, 100; average-path velocity cut-off, 25 μ/s; straight-line velocity cut-off, 1 μ/s (Culpepper et al., 2018). Three measurements, each from a different field of view, were collected for each sample and the average initial motility calculated.

The cryoprotectants (5% DMSO and 10% methanol) were chosen for this study based on cryopreservation of white crappie sperm (Culpepper et al., 2018). For each male ($N = 5$ white crappie; $N = 6$ black-stripe black crappie), each cryoprotectant was prepared at double

the target concentration in HBSS350 and mixed 1:1 with sperm samples, to yield final sperm concentrations of 2.5×10^8 sperm/ml. After mixing, the solutions were placed on the Quattro Minitube system (Verona, WI), where 0.5 ml straws were filled, sealed, and labeled. The straws were arranged on a freezing rack and placed in a programmable freezer (Micro Digitcool, IMV, France). After 15 min total of equilibration, the straws were cooled at 40°C/min to −80°C, based on methods of Culpepper et al. (2018).

After cryopreservation, the straws were removed from the freezer, sorted by treatment, and placed in liquid nitrogen for storage. Post-thaw motility was assessed by randomly selecting three straws from each fish and treatment. Straws were thawed in a 40°C water bath for 8 s (Culpepper et al., 2018). Motility was estimated using the CASA system as described earlier. A motility estimate was assessed 3 times per straw, for 9 observations per cryoprotectant and fish and used to determine average post-thaw motility for each fish.

2.3 | Fertilization

White crappie were collected before the natural spawning season (i.e., March–April) from Enid Lake, Enid, MS, as described previously. Female white crappie were used for fertilization experiments as a protocol had already been developed for cryopreservation of white crappie sperm and use in fertilization of white crappie eggs (Culpepper et al., 2018). This allowed for a direct comparison with cryopreserved black-stripe black crappie sperm. Fish were held at the NMFH and sorted by sex following Culpepper and Allen (2016b), placed in a RAS, and held in conditions as described earlier.

After being held at spawning temperatures for 1 week, females were anesthetized and weighed. Previous experiments have shown gonadotropin-releasing hormone analog (GnRHa; Ovaprim, Syndel, Ferndale, WA) to be an effective ovulation-inducing hormone for white crappie (Culpepper, 2015), and therefore, it was selected for this experiment. The hormone was injected intramuscularly, about 2 cm below the dorsal fin using a 25-gauge, 2.5-cm hypodermic needle, in a priming dose (0.05 ml/kg; 10% of manufacturer's recommended dose [0.5 ml/kg recommended dose]), followed with a resolving dose (0.45 ml/kg; 90% of manufacturer's recommended dose) administered 24 hr later (Culpepper, 2015). After a latency period (i.e., time of ovulation post priming dose) identified in previous white crappie strip-spawning experiments (Shirley & Allen, 2020), females were observed every hour for ovulation starting 38 hr post priming dose.

If a female was deemed to be ovulating (i.e., presence of eggs in tank or release of eggs after light abdominal palpation), the fish was placed in an anesthetic bath as described earlier. Once lethargic, external moisture was removed from the female using a small hand towel to prevent eggs from water hardening before fertilization. Eggs were stripped using a strong downward motion on the lower abdomen, releasing eggs from the ovary, and were collected in a small glass bowl (~300 ml).

For each female ($n = 9$), 0.25 ml (~785 eggs) (Culpepper et al., 2018) were pipetted into nine, 250-ml plastic containers (VWR, Radnor, PA), three per treatment (black-stripe black crappie DMSO, black-stripe black crappie methanol, and white crappie methanol), for 72 containers. To allow adequate water flow while keeping each replicate separate, each

container had a 2.5 cm × 2.5 cm square cut out of both sides and covered with 300-μm nylon mesh (Pentair, Apopka, FL). After eggs were pipetted into their respective containers, they were fertilized with two straws of the desired treatment from one male randomly chosen and thawed for 8 s in a 40°C water bath, and dried externally with a kimwipe tissue. Sperm was used from all six black-stripe black crappie males. Ten milliliters of fresh water was added (10 ml water: 1 ml sperm; 11 ml volume including sperm), and the entire solution was gently mixed with a small brush and left stationary for 10 min to allow for fertilization. Afterwards, containers were placed in small race-ways with flowing water and aeration.

The remaining eggs from each female were fertilized using fresh sperm from a randomly selected white crappie male to confirm eggs were viable. Eggs from two female crappie were omitted from analyses because fresh sperm had a low fertilization percentage (<20%) indicating eggs were of low viability (n = 7 used for analyses). The male white crappie were euthanized in a portable electro-anesthesia system (Smith-Root, Vancouver, WA) and dissected to remove the testes. Testes were minced with a scalpel to obtain sperm, at which time a small sample (~100 μL) was placed on a slide and activated by a drop of water. Sperm were observed at ×100 magnification to confirm motility. After sperm motility was confirmed, the testes were strained through a small aquarium net to pass sperm and retain larger pieces of gonadal tissue. The mixtures of eggs and sperm were activated with 50 ml of fresh water. The solution was gently stirred using a turkey feather for 2 min, after which, 50 ml of fresh water was added, and the solution was stirred for another 5 min. The entire solution was carefully rinsed until debris was removed, excess water was decanted, and the egg solution was added to a McDonald hatching jar containing 3 L of water and 3 g of tannic acid (1 g/L), to remove the adhesive layer. The solution was aerated for 2 min and the jar was placed on a hatching table and supplied with a continuous flow of fresh water.

Fertilization percentages were calculated at 24 hr after fertilization by randomly selecting three 30-egg samples from each container, obtaining the number of fertilized eggs and dividing by 90. Fertilization was also determined from each hatching jar to confirm the female had produced viable eggs. Eggs were observed using a compound microscope at ×25 magnification. Fertilization was characterized by the presence of an intact chorion, vitelline membrane (Culpepper, 2015), and identifiable embryo near gastrula to neurula stages.

2.4 | Optimal egg volume

To determine the optimal volume of eggs for one straw containing 0.5 ml of cryopreserved sperm, an additional experiment was carried out using the sperm from males with the highest fertilization rate from the previous experiment. Female white crappie (mean *SE*: weight: 512 ± 80 g; total length: 323 ± 18 mm) were collected and induced to spawn as described earlier. Although saline extender solutions have been used to maintain unfertilized eggs for extended durations in other species (Dietrich, Dabrowski, Arslan, Ware, & Van Tassell, 2012; Goetz & Coffman, 2000), a preliminary experiment showed that white crappie eggs lose their viability rapidly when stored in HBSS350. Therefore, immediately after strip spawning, eggs from two females were combined and mixed using a small paint-brush. Eggs were pipetted into small (235 ml capacity) plastic cups using a 1,000-μL pipette with modified pipet tips. The small plastic cups were modified with rectangular cutouts on

opposite sides and 300-μm mesh adhered with aquarium silicone. These allowed for water movement through the cup, while preventing loss of eggs.

Five different volumes of eggs were tested: 0.125 ml (390 eggs), 0.25 ml (785 eggs), 0.5 ml (1,570 eggs), 1 ml (3,135 eggs), and 2 ml (6,270 eggs), with four replicates of each except for 1 and 2 ml, which had three replicates (based on an egg:volume ratio of 627 eggs: 0.2 ml determined from an average of 9 aliquots conducted previously). Sperm from four different males were used; a straw from each male was used for a separate replicate at each volume of eggs. Egg quality was also checked using a separate aliquot of eggs and fresh sperm from a male sampled at the same time females were strip spawned; both females were found to have viable eggs (>20% fertilization rate).

Straws were removed from the liquid nitrogen dewar, placed in a precooled Styrofoam cooler containing liquid nitrogen, sorted (within 10 s), and transferred to a 40°C water bath and thawed for 8 s. Within 10 s after thawing, the sperm was poured over the eggs in a replicate small plastic container. Water (5 ml) was added immediately (using a 5-ml pipette) after sperm was added to eggs (5 ml water: 0.5 ml sperm; 5.5 ml volume including sperm), and containers were swirled to mix the gametes. After swirling, cups were not moved for 10 min to allow for fertilization. Containers were placed in a water bath with flow-through well water at 21°C. Fertilization was checked 20 hr after addition of sperm as previously described.

2.5 | Statistical analysis

Data were analyzed using the statistical package SAS 9.4 (SAS Analytics Software & Solutions, Cary, NC), and significance was determined at $\alpha = 0.05$. Data were tested for normality using the Shapiro–Wilk test, and Levene's test was used to assess homogeneity of variance. Data were transformed using arcsine transformations as needed or logit for all percentage data to meet assumptions of normality and homogeneity of variance.

The initial concentration of fresh sperm from each species was compared using a Student's *t*-test to identify the most effective sperm cryoprotectant for black-stripe Black Crappie (i.e., 5% DMSO or 10% methanol). A 2 × 3 factorial arrangement of treatments was used to test for differences in average sperm motility between species (i.e., white crappie [$N = 5$] and black-stripe black crappie [$N = 6$]), cryoprotectant (i.e., 5% DMSO and 10% methanol), and time (i.e., pre-cryopreservation or post-cryopreservation) using analysis of variance (ANOVA). Least squares analysis was used to identify significant differences among treatments.

A randomized complete block design and ANOVA was used to compare fertilization rates among the three different cryoprotectant treatments (black-stripe black crappie DMSO, black-stripe black crappie methanol, and white crappie methanol), with individual females from which eggs were obtained as the block, and each egg container (i.e., containing a separate batch of fertilized eggs) representing the experimental unit. For the effective sperm volume experiment (i.e., optimal sperm:egg ratio), a one-way ANOVA followed by a Tukey's *post hoc* test was used.

3 | RESULTS

3.1 | Sperm motility

There were no significant differences in initial concentrations of fresh sperm ($p = .432$) obtained from either black or white crappie (Table 1). Regarding sperm motility, species, cryoprotectant, and time did not significantly interact to affect the mean motility of cryopreserved sperm ($p = .944$). Species and cryoprotectant ($p = .220$), species and time ($p = .515$), and cryoprotectant and time ($p = .207$) did not significantly interact to affect mean sperm motility. Similarly, neither cryoprotectant ($p = .235$) nor time ($p = .951$) affected mean motility. However, species did significantly affect the average motility ($p = .017$), as black-stripe black crappie sperm was significantly more motile than white crappie sperm (Table 1).

3.2 | Fertilization experiment

The cryoprotectant agent (i.e., 5% DMSO or 10% methanol) significantly affected fertilization percentage ($p < .001$) (Figure 1). Eggs fertilized using black-stripe black crappie sperm cryopreserved with 5% DMSO had a significantly higher fertilization rate than did eggs fertilized with black-stripe black crappie and white crappie sperm with 10% methanol (Figure 1). A significant block effect (block = one female) was detected for fertilization percent, indicating egg quality variation among females. However, this was not problematic because all females had the same treatments applied. A significant interaction was observed between treatment and female ($p = .003$), indicating there was some treatment variability in fertilization rates among females, however black-stripe black crappie sperm cryopreserved in 5% DMSO (38 ± 8%) generally outperformed sperm cryopreserved in 10% methanol (black-stripe black crappie: 25 ± 8%; white crappie sperm 22 ± 7%).

3.3 | Optimal egg volume

Fertilization using black-stripe black crappie sperm cryoprotected using 5% DMSO ranged from 0% to 27%, with egg volumes of 0.125 ml (390 eggs), 0.25 ml (785 eggs), and 0.5 ml (1,570 eggs) having greater fertilization than 2.0 ml (6,270 eggs) (Figure 2A). The highest fertilization percent for a single straw (0.5 ml) was for 785 eggs (0.25 ml). At this ratio, with a sperm concentration of 2.5×10^8 sperm/ml, a volume of 0.5 ml, and a post-thaw motility of 45%, the ratio of sperm:egg was 159,235:1 and the ratio of motile sperm:egg was 71,656:1 (Figure 2B).

4 | DISCUSSION

Black crappie possessing the black-stripe phenotype are often difficult to collect in sufficient numbers for hatchery production. Current procedures for crappie spawning require a male crappie to be euthanized and the testes excised for every female strip-spawned (Shirley & Allen, 2020; Culpepper & Allen, 2016a, 2016b). Recent protocols for cryopreserving white crappie sperm (Culpepper et al., 2018) have provided opportunity for sperm cryopreservation of black-stripe black crappie. Results from the present study indicate that sperm of black-stripe black crappie can be cryopreserved using the same approach identified for white crappie. The ability to transfer approaches across species would greatly facilitate

the application of cryopreservation and development of repositories to protect genetic resources of aquatic species (Liu, Taylor, Blackburn, & Tiersch, 2019; Tiersch, 2011). It is often assumed that cryopreservation protocols must be developed on a species-by-species basis, but much of the observed variability in results can be procedural rather than biological (e.g., male-to-male) (Dong, Huang, & Tiersch, 2007; Torres & Tiersch, 2018).

Evaluating sperm motility before cryopreservation and after thawing is crucial to optimize protocols for sperm cryopreservation due to the importance of sperm motility in egg fertilization (Cabrita et al., 2010). In this study, sperm of black-stripe black crappie and white crappie were each cryopreserved, allowing direct comparison between the species. Comparisons between pre-freeze and post-thaw motility resulted in small (3–7%) observed changes in motility for 5% DMSO and 10% methanol. Overall, black-stripe black crappie sperm had a higher mean motility (48%) than white crappie (39%). This post-thaw motility is comparable to another centrarchid, bluegill (26–56%) (Bates et al., 2005), and striped bass (40%) (He & Woods, 2003).

Methanol and DMSO have been commonly used as cryoprotectants for fish sperm, and their effectiveness has been evaluated through fertilization experiments. For several salmonid species, sperm cryopreserved with methanol was more effective at fertilizing eggs than DMSO (Lahnsteiner, Weismann, & Patzner, 1997). In centrarchids, bluegill sperm motility and fertilization were higher using 10% methanol than 10% DMSO, with fertilization rates as high as 75% using methanol and 50% using DMSO (Bates et al., 2005). In striped bass, motility and fertilization were higher in DMSO than methanol (He & Woods, 2003) with fertilization of 26–54% in DMSO (He & Woods, 2004). In contrast, channel catfish post-thaw motility was higher with methanol compared to DMSO, with fertilization rates averaging 64% in methanol (Tiersch, Goudie, & Carmichael, 1994).

A previous study using white crappie sperm cryopreserved with 5% DMSO or 10% methanol found that there was no difference in fertilization rates between the two cryoprotectants, although fertilization rates were low (mean: 13%) (Culpepper et al., 2018). In comparison, the present study found black-stripe black crappie sperm with 5% DMSO was more effective at fertilizing white crappie eggs (38%) than sperm cryopreserved in 10% methanol (22%). Although not directly comparable due to higher sperm:egg ratios used by Culpepper et al. (2018) (3.19×10^5 vs. 1.59×10^5 in the present study), this study had higher fertilization rates in white crappie eggs than those of Culpepper et al.'s study. Interestingly, black-stripe black crappie sperm was more effective at fertilizing white crappie eggs than white crappie sperm. It is possible that white crappie sperm could have had a higher fertilization rate if preserved in 5% DMSO, although results of a previous study were equivocal (Culpepper et al., 2018). Although sperm concentration was similar among treatments in the present study, black-stripe black crappie sperm had higher overall motility than white crappie sperm, which may explain higher fertilization rates. A more optimal sperm:egg ratio based on the findings of this study, as well as improvements in water quality (e.g., parasites) and a better understanding of latency period duration for hormone treatments (Shirley & Allen, 2020), may have also contributed to the increase in fertilization in the present study. Further research improving egg quality and fertilization would be beneficial, as well as evaluating hatching rate.

Because fertilization efficiency is at the heart of hatchery operation, the ratio of cryopreserved sperm to eggs was studied. Fertilization was the highest (27%) at a ratio of 785 eggs (0.25 ml) per straw (0.5 ml) of sperm cryopreserved with 5% DMSO. Observed optimal ratios of sperm to eggs for cryopreservation could vary widely. In this study for black-stripe black crappie sperm (with white crappie eggs), the best results were obtained with a ratio of 159,000 total sperm per egg and 71,700 motile sperm per egg. For comparison to other freshwater species, the thawed sperm:egg ratio documented for blue catfish (*Ictalurus furcatus*) was 135,000 total and 60,800 motile sperm:egg, with catfish species ranging from 11,300 to 69,000,000 total sperm:egg (Hu, Bosworth, Baxter, & Tiersch, 2014). Studies in other temperate fishes have found optimal ratios of 350,000 sperm:egg for Whitefish (*Coregonus muksun*; Piironen, 1987), 300,000 sperm:egg for Brook Trout (*Salvelinus fontinalis*; Nynca, Dietrich, Dobosz, Zalewski, & Ciereszko, 2015), and 900,000–2,400,000 sperm:egg for other salmonids (Lahnsteiner et al., 1997). In fishes, sperm:egg ratios can vary widely depending on species and protocol (Beirão et al., 2019).

In the present study, fertilization rate of cryopreserved sperm proved sensitive to increasing volume of eggs. A similar relationship has been found previously for carp (Kurokura, Hirano, Tomita, & Iwahashi, 1984). This is presumably a concentration-dependent phenomenon: higher concentrations would provide additional sperm to fertilize more eggs, and lowering of the curve would occur at higher egg numbers. Ensuring that appropriate numbers of sperm are available for reliable fertilization is fundamentally a function of the sperm concentration selected for cryopreservation, and the number of eggs that generally constitute a batch in hatchery production. Such concepts have been studied quantitatively for cryopreserved blue catfish sperm (for hatchery production of hybrids) at commercial scale (Hu et al., 2014) using industrial engineering and simulation modeling approaches (Hu, Liao, & Tiersch, 2015). Those studies were based on use of high-throughput processing (Hu, Yang, & Tiersch, 2011) developed using commercial dairy industry approaches (Lang, Riley, Chandler, & Tiersch, 2003).

Indeed, the work in the present study with crappie can be scaled up for use with hundreds of fish and multiple hatcheries by direct use of the results from the previous studies in blue catfish, because the approaches are the same, including the use of 0.5-ml French straws that can be filled, sealed, and labeled using automated equipment (e.g., the Minitube Quattro system used in the present study can process 15,000 straws per hour). In addition, cryopreservation in crappie can be directly transferred from a central facility (such as the AGGRC) to on-site work within an existing fish hatchery by use of high-throughput mobile cryopreservation capabilities (Childress, Caffey, & Tiersch, 2018) or establishment of in-house cryopreservation based on previous economic analysis (Caffey & Tiersch, 2000). Developing in-house cryopreservation capabilities at existing hatcheries will be greatly strengthened by recent developments in three-dimensional printing (e.g., Hu, Childress, & Tiersch, 2017) including fabrication of temperature probes (Shamkhalichenar, Choi, & Tiersch, 2019), and the potential for sharing of open-source design files for production of inexpensive, reproducible freezing devices that can be integrated with strong quality control programs (e.g., Hu, Liao, & Tiersch, 2013; Torres, Hu, & Tiersch, 2016). Thus, substantial commercial-level benefits can be realized by generalizing cryopreservation at the

application level, rather than trying to optimize new protocols on a species-by-species basis and restricting this work to the research level (Torres & Tiersch, 2018).

Because the temporary storage (0.5–5 hr) of white crappie eggs in HBSS350 prior to fertilization in a pilot study associated with this study produced no fertilized eggs, the batch size could be limited for now to the number of eggs produced per single female. Based on prior experience with other white crappie, egg quality appeared good for this batch of eggs ($N = 6$ females). Given that this study was performed near isotonic osmolality, loss of fertilization capability may have been due to ionic disturbances during storage. Future studies should address short-term storage of unfertilized crappie eggs (e.g., time, temperature, and various extender solutions) including quality assessments at collection and after short-term storage to avoid wasting of hatchery resources on poor-quality eggs (e.g., Glenn & Tiersch, 2002).

The ability to cryopreserve crappie sperm can assure reliable and economic meeting of production demands, and greater flexibility and efficiency in spawning at hatcheries. Furthermore, benefits of cryopreservation extend to germplasm repositories, which can facilitate conservation efforts or directions for new industry, such as the development of domesticated lines. The success in the present study of using a generalized approach for both species in the genus *Pomoxis* provides further evidence that cryopreservation protocols need not be assumed to be species-specific. For example, a single generalized protocol has been applied to more than 20 species within the genus *Xiphophorus* and two other species in the genus *Poecilia* to facilitate repository development to safeguard genetic resources of biomedical model species (Liu et al., 2019). Overall, more research is needed to quantitatively assess factors important to practical hatchery operation with cryopreserved sperm of aquatic species in general (e.g., Hu et al., 2014), and standardization of procedures and reporting is necessary to enable meaningful comparisons across studies (Torres & Tiersch, 2018).

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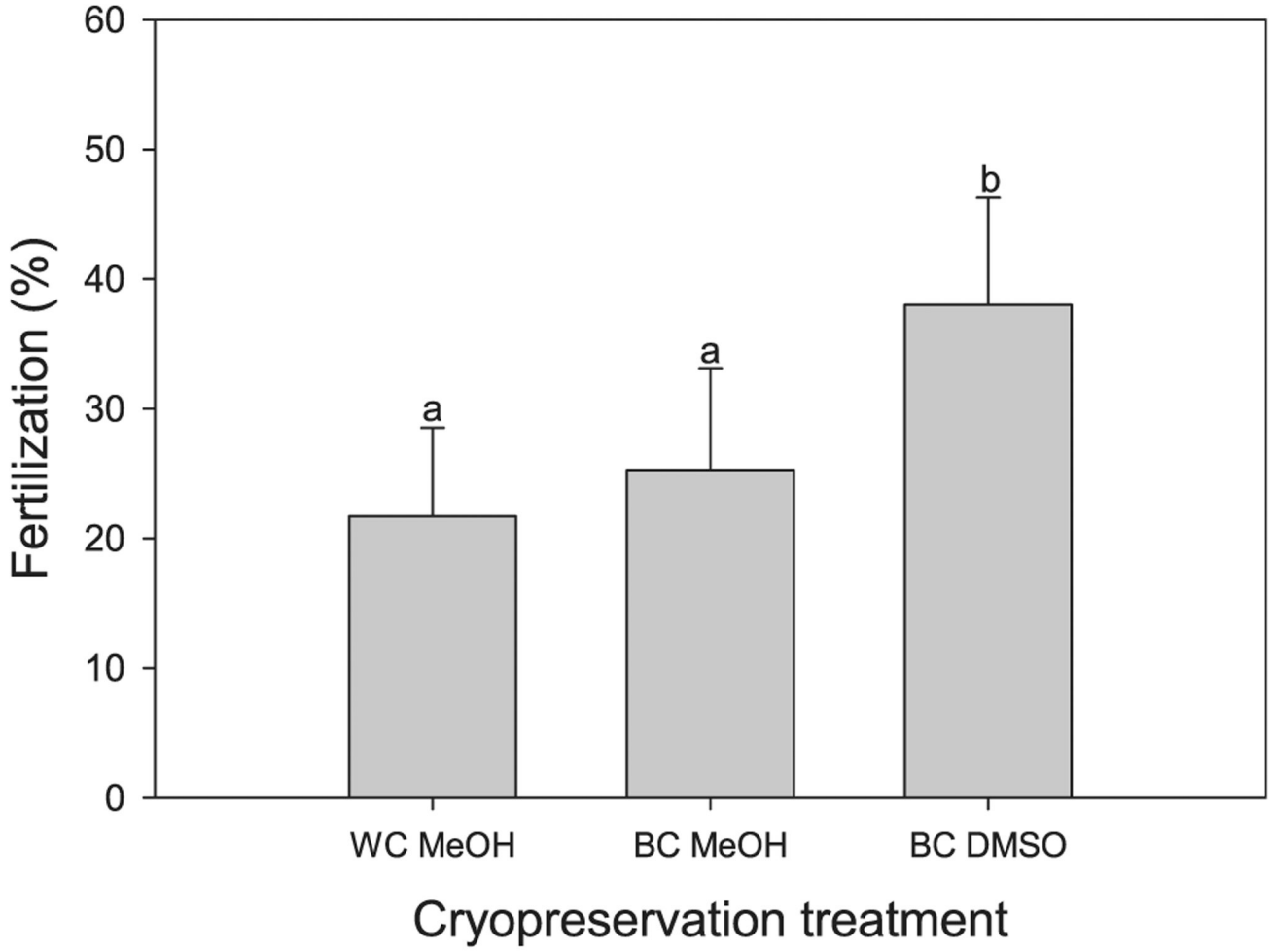

FIGURE 1.

Mean (±*SE*) percent fertilization of white crappie, *Pomoxis annularis* eggs fertilized using three different treatments (WC MeOH, white crappie sperm cryopreserved using 10% methanol; BC MeOH, black-stripe black crappie, *Pomoxis nigromaculatus* sperm cryopreserved using 10% methanol; BC DMSO, black-stripe black crappie sperm cryopreserved using 5% dimethyl sulfoxide) of cryopreserved sperm. Different lowercase letters indicate significant differences ($p < .05$) among treatments

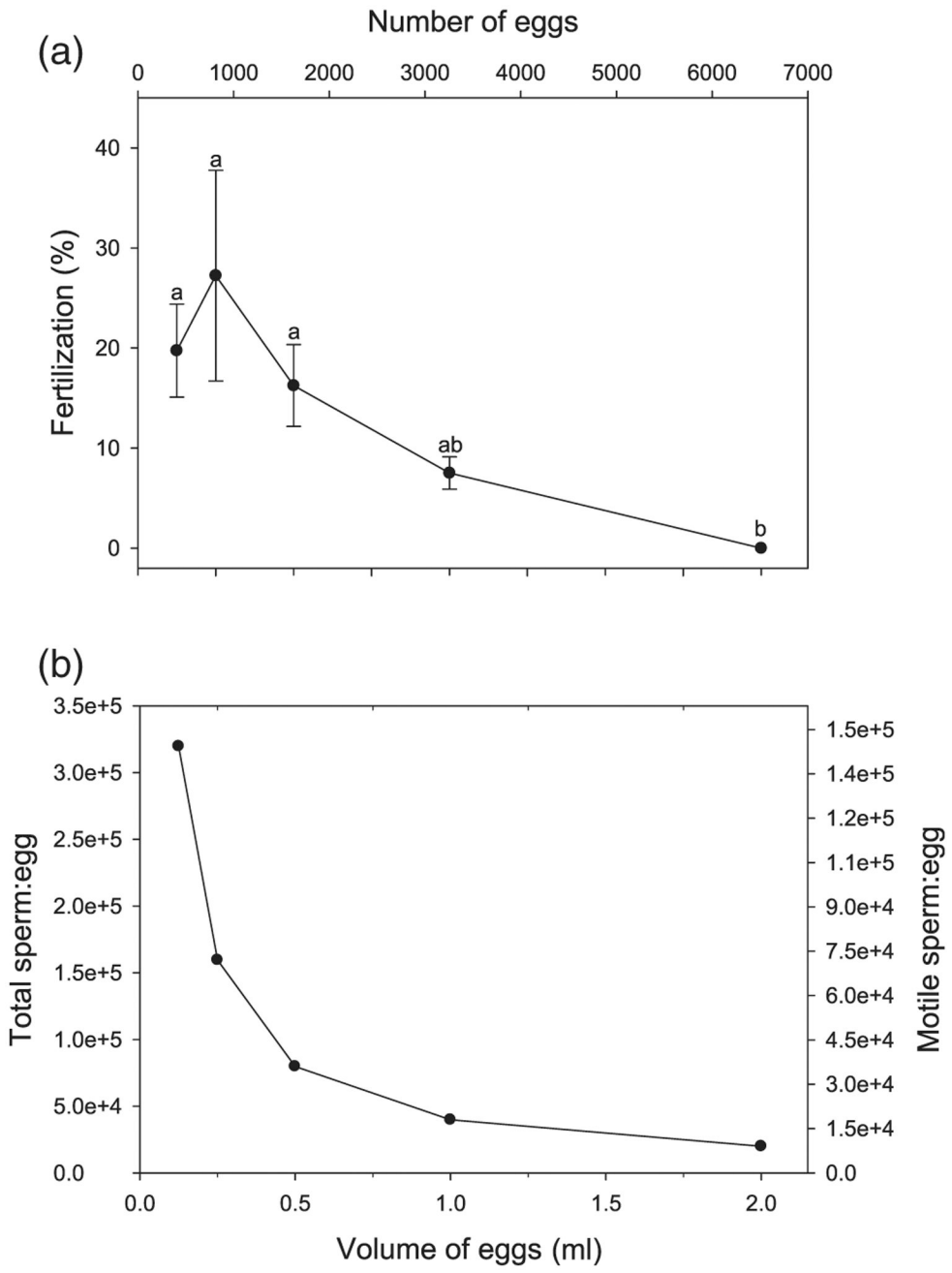

FIGURE 2.

(a) Mean (±*SE*) percent fertilization of white crappie, *Pomoxis annularis* eggs fertilized using black-stripe black crappie, *Pomoxis nigromaculatus* sperm cryopreserved using 5% dimethyl sulfoxide (DMSO) and associated (b) total and motile sperm to egg ratios. Different lowercase letters indicate significant differences ($p < .05$) among treatments

TABLE 1

Initial concentration and motility of fresh sperm and motility of black-stripe black crappie, *Pomoxis nigromaculatus* and white crappie, *Pomoxis annularis* sperm before freezing and after thawing

	Black-stripe black crappie	**White crappie**
Variable	**Mean ± *SE***	**Mean ± *SE***
Initial concentration (cells/ml)	$(5.77 \times 10^9) \pm (3.84 \times 10^8)$	$(6.31 \times 10^9) \pm (5.56 \times 10^8)$
Initial motility	68% ± 3.7	65% ± 2.0
5% DMSO pre-freeze motility	52% ± 2.9	36% ± 8.3
5% DMSO post-thaw motility	45% ±3.1	33% ± 8.8
10% methanol pre-freeze motility	47% ± 2.2	40% ± 3.8
10% methanol post-thaw motility	50% ± 4.1	47% ± 6.3
Mean sperm motility (5% DMSO and 10% methanol)	48%* ± 3.5	39% ± 4.5

Note: An asterisk indicates a significant difference among treatments (Student's *t*-test; $p < .05$; $n = 5$ white crappie, $n = 6$ black-stripe black crappie).

Abbreviation: DMSO, dimethyl sulfoxide.