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Refining Spawning Protocols for Crappie

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By

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White Crappie (*Pomoxis annularis*) and Black Crappie (*P. nigromaculatus*) are popular North American gamefish; however, frequent fluctuations in year class strength present a management challenge for recreational fisheries. Intensive aquaculture production has the potential to address this challenge through controlled hatchery reproduction for supplemental stocking, but further study is needed to refine and optimize techniques. Therefore, this study evaluated the effects of hormone injection timing on latency period and spawning success, examined effective cryopreservation techniques for black-stripe Black Crappie sperm (a preferred hatchery phenotype), and compared simulated spring duration on out-of-season spawning success. Latency period for White Crappie did not depend on the diel time of gonadotrophin-releasing hormone injection. Cryopreservation of black-stripe Black Crappie sperm and subsequent fertilization of White Crappie eggs was more effective using 5% dimethyl-sulfoxide than 10% methanol. A longer duration at final spring spawning conditions (3 vs. 2 weeks) increased egg fertilization in out-of-season spawning experiments.

DEDICATION

I would like to dedicate this research to my family for their continuous encouragement and support throughout this project.

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CHAPTER I

LITERATURE REVIEW

Introduction

White Crappie (*Pomoxis annularis*) and Black Crappie (*P. nigromaculatus*) are members of the Centrarchidae family of fishes (Ross 2001) and are a highly sought-after game fish (McDonough and Buchanan 1991; Boxrucker and Irwin 2002). Crappie fisheries in the southeastern USA may provide up to 80% of fish harvest in some fresh water lakes (Miranda et al. 2013) and can provide a substantial economic impact in the state of Mississippi (Dorr et al. 2002). Despite popularity among anglers, crappie present many unique management challenges to fisheries biologists including: overpopulation of smaller impoundments (< 20 ha), variable recruitment from year to year, fluctuating strength of year classes with a stronger year class produced every 3-5 years, and fluctuating populations (Swingle and Swingle 1967; Busack and Baldwin 1988; Mitzner 1991). Crappie have been studied as aquaculture species to support supplemental stockings to address these management issues. Despite popularity and research on the culture of other members of Centrarchidae, (i.e., Largemouth Bass (*Micropterus salmoides*) and Bluegill (*Lepomis macrochirus*)), crappie aquaculture has not been well studied (Morris and Clayton 2009). However, the success of tank spawning (Bryan et al. 1994), indoor culture (Mischke and Morris 1997), and out-of-season spawning (Mischke

and Morris 1997; Matthews and Stout 2013) of these Centrarchidae species has led to an interest in developing crappie aquaculture techniques.

Captive spawning

Recent advances in crappie aquaculture have allowed for more intensive culture techniques and production methods to be further explored. Crappie are primarily produced extensively in ponds by allowing fish to spawn naturally (Smeltzer and Flickenger 1991; Myers and Rowe, 2001; Bunnell et al. 2007). Studies that have produced crappie in tanks have mainly focused on producing monosex, sterile, or sex-reversed fish (Al-ablani and Phelps 1997; Gomelsky et al. 2002; Arslan and Phelps 2004; Cuevas-Uribe 2009). However, recent advances in tank acclimation and disease prevention have led to a greater examination of intensive culture techniques. Culpepper (2015) found crappie could be acclimated to indoor recirculating aquaculture systems (RAS) by lowering water temperatures to < 15°C and maintaining a salinity of 3-5 ppt. Recent studies have focused on producing fish for stocking programs by manipulating water temperatures, photoperiod, and the use of ovulation inducing hormones (Culpepper 2015; Culpepper and Allen 2016a; Culpepper and Allen 2016b; Allred et al. in review). Gonadotropin-releasing hormone analog (GnRHa) has been identified as the desired hormone for inducing ovulation in crappie when compared to human chorionic gonadotropin (HCG) and luteinizing hormone-releasing hormone analog (LHRHa), because of a high ovulation success rate, moderate egg fertilization, and high survival of broodstock after hormone injection (Culpepper 2015). Another study using GnRHa to induce ovulation in crappie was able to identify a latency period (i.e., time from initial

injection to time of ovulation) of 40 – 50 hours post initial injection in White Crappie that were held at 21° C water temperature and a natural spring (16 hours light) photoperiod (Allred et al. in review). However, ovulation occurred outside of normal hatchery operating hours, either due to the time of injection or a preference for spawning at night. Further studies need to evaluate whether hormone injection time effects latency period and spawning success of White Crappie.

Sperm cryopreservation

Sperm cryopreservation benefits aquaculture operations by providing large quantities of readily accessible sperm with desirable traits that can be easily stored or transported, and reducing requirements for male broodstock. In an intensive aquaculture setting, sperm needed for spawning crappie are obtained by euthanizing male fish and removing the testes (Culpepper and Allen 2016b). This method requires capturing wild fish before each spawning season or maintaining broodstock year-round. One way to address this issue is through the use of cryopreserved sperm. Other species closely related to crappie, such as Bluegill, have been successfully spawned using cryopreserved sperm, with up to 50% fertilization rates (Bates et al. 2005), leading to interest in developing techniques for fertilization of crappie eggs using cryopreserved sperm. Methods for the cryopreservation of White Crappie sperm have recently been documented (Culpepper et al. 2017), but hatchery application would more likely use black-stripe Black Crappie sperm due to easy identification of stocked fish with this rare phenotype, as is done in Mississippi with the Magnolia Crappie (Cole Cochran, personal communication). Black-stripe Black Crappie are Black Crappie possessing a dominant mutant genotype, causing

a pre-dorsal black stripe to appear on the fish (Gomelsky et al. 2005). The stripe can be used in hatcheries as a genetic marker for population and spawning studies.

The use of cryopreserved sperm in the intensive spawning of White Crappie needs further refinement to improve fertilization rates. Although Culpepper et al. (2017) were successful in cryopreserving White Crappie sperm, fertilization rates were low (~13%). Methods also need to evaluate whether buffer, cryoprotectants, freezing rate and thawing regime used for White Crappie are equally effective for the cryopreservation of black-stripe Black Crappie sperm.

Out-of-season spawning

Recent studies by Culpepper and Allen (2016a) and Allred et al (in review) have been important in establishing basic techniques for out-of-season spawning of crappie. However, improvements are still needed before broader implementation in hatchery settings. Culpepper and Allen (2016a) and Allred et al. (in review) established methods for out-of-season spawning by manipulating photoperiod and water temperature and inducing ovulation with GnRHa. Although these studies demonstrated fertilization, success was variable (fertilization rate 10.5-82.7%). Further improvements may come from a change in focus on length of the spring transitional period (i.e., 3, 6, or 9 weeks) to the duration of time fish are held at final spring conditions (22° C and 16 hours light) before hormones are administered. Culpepper and Allen (2016a) compared 3-week and 6-week transition periods, and while spawning success was limited, the 6-week transition period produced more ovulating females and a higher egg fertilization rate (55%). No difference was found in spawning success of White Crappie when 6-week and 9-week

spring transitions were compared (Allred et al. in review). However, Allred et al. (in review) held fish for 2 weeks at final spring spawning conditions, while Culpepper and Allen (2016a) only held fish at final spring spawning conditions for 1 week. Allred et al. (in review) had higher average fertilization percent, number of females ovulating, and ovulated egg volume than Culpepper and Allen (2016a), presumably due to the increased duration at final spring spawning conditions. The effects of the duration of time at final spawning conditions in out-of-season spawning of crappie merits further investigation.

Objectives

Based on this literature review, the following objectives will be addressed:

1. Determine whether time of hormone injection (GnRHa) affects latency period, egg fertilization rate, ovulated egg volume and the percent of females ovulated.
2. Investigate whether black-stripe Black Crappie sperm can be cryopreserved using similar methods to those established for White Crappie and compare two cryoprotectants (i.e., 5% DMSO or 10% methanol) for fertilization efficacy, and
3. Compare out-of-season spawning success of White Crappie held for 2 and 3 weeks at spawning conditions.

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CHAPTER II

EFFECTS OF GONADOTROPHIN-RELEASING HORMONE ANALOG (GNRHA) INJECTION TIME ON WHITE CRAPPIE (*POMOXIS ANNULARIS*) LATENCY PERIOD

Abstract

Relatively little is known of the effects of time of day of hormone injection on latency period in most fishes. Interest in improved culture techniques for White Crappie (*Pomoxis annularis*), a popular recreational gamefish, has led to advances in tank holding and induced spawning methods. However, an understanding of latency period is needed for synchronizing and optimizing White Crappie hatchery production. Therefore, the effects of hormone injection timing on latency period and egg viability were examined. Crappie were injected with gonadotropin-releasing hormone analog (GnRHa) at 1600 (evening treatment) and 0800 (morning treatment), monitored for latency period duration, and spawning success was determined by percent of females ovulated, ovulated egg volume, and egg fertilization percent. In the evening treatment group, 75% of the females ovulated, with eggs ovulated (mean ± SE) 47.11 ± 1.95 hours post initial injection, and mean egg fertilization of 63%; while in the morning injection treatment, 91% of females ovulated with eggs ovulated (mean ± SE) 44.80 ± 1.53 hours post initial injection, and mean fertilization of 56%. No significant difference in mean latency period or spawning

success was observed between treatments. Therefore, diel timing of hormone injection did not affect latency period or spawning success in female White Crappie, allowing for flexibility in hatchery production.

Introduction

Although crappie, *Pomoxis* spp., are popular game fishes throughout the Southeastern and Midwestern US (Boxrucker and Irwin, 2002; McDonough and Buchanan, 1991), they present unique management challenges to biologists. Crappie often overpopulate smaller impoundments (<20 ha), and have variable annual recruitment, producing a strong year class every 3-5 years (Busack and Baldwin, 1988; Mitzner 1991; Swingle and Swingle, 1967). To address population fluctuations, hatchery-reared crappie are often stocked into public waters by regulatory agencies. However, intensive culture of crappie, which is used to supply stocking programs, is relatively new with little information regarding intensive production, spawning, and rearing techniques.

Successful spawning of captive fish is essential for producing the fish numbers needed for stocking programs. Currently, crappie production is primarily accomplished by allowing fish to spawn naturally in hatchery ponds (Bunnell et al. 2007; Smeltzer and Flickenger, 1991; Myers and Rowe, 2001). Production is also conducted by strip-spawning wild females, but requires the collection of ovulating females and immediate spawning, requiring intensive field sampling (Culpepper and Allen, 2016b). Hormone-induced tank spawning techniques have been documented, although studies primarily focused on producing monosex, sterile, or sex-reversed fish (Al-ablani and Phelps, 1997; Arslan and Phelps, 2004; Cuevas-Uribe, 2009; Gomelsky et al. 2002).

While previous studies show the potential for intensive crappie production using hormone-induced spawning, comparison of techniques and hormone efficiency were not made. Recently, Culpepper (2015) compared commonly available hormones, including human chorionic gonadotropin (HCG), luteinizing hormone-releasing hormone analog (LHRHa), and gonadotropin-releasing hormone analog (GnRHa), for ovulation induction efficacy and egg fertilization percentages in White Crappie. In that study, GnRHa was one of the top performing hormones, with a high ovulation induction efficacy, high fertilization rate, and low post-injection mortality rate in broodstock (Culpepper, 2015).

Water temperature and photoperiod are known to naturally cue fish to spawn (Fortuny et al. 1988; Lam 1982; Matthews and Stout, 2013; Morris and Clayton, 2009), and using these environmental cues can benefit synchronization of induced spawning. Studies on the Common Sole, *Solea solea* (Ramos, 1986), Indian Major Carp, *Cirrhina mrigala* (Kaul and Rishi, 1986), Milkfish, *Chanos chanos* (Marte et al. 1988), Goldfish, *Carassius auratus* (Sokolowska et al. 1984), and Spotted Seatrout, *Cynoscion nebulosus* (Thomas and Boyd, 1988) report fish held in natural spawning photoperiods and water temperatures between 17° C and 32° C ovulate eggs 24-48 hours after GnRHa injection. Similarly, White Crappie, which spawn naturally at water temperatures from 14 to 24°C (Tin, 1982), have been induced to spawn by using a simulated natural photoperiod, a water temperature of 22°C and GnRHa injection (Allred et al. in review; Culpepper, 2015). Under these conditions, White Crappie ovulate between 38 and 53 hours after initial hormone injection (Allred et al. in review), indicating a predictable latency period.

Although hormone-induced spawning is widely used in aquaculture, relatively few studies have examined whether the time of day of hormone injection affects latency period and measures of spawning success. In the few fish species examined, the time of day at which ovulation-inducing hormones (i.e., GnRHa and LHRHa) are injected can influence latency period and egg quality (Rasines et al. 2013), although results appear to be species specific. A study on Barramundi (*Lates calcarifer*) found that latency period post-injection of LHRHa differed depending on the time of hormone injection (Garcia, 1990). Other studies found ovulation was independent of the time of day hormone was injected (Arabaci et al. 2001; Drori et al. 1994), but fertilization and hatching rates were affected. Egg fertilization and hatching rates of the Japanese Eel (*Anguilla japonica*) were found to be dependent on hormone injection timing, with the best fertilization and hatching rates occurring when eels were injected in the afternoon. In the Common Carp (*Cyprinus carpio*), fertilization and hatching rates were higher when hormone was injected in the morning rather than the afternoon (Rasines et al. 2013). However, for most aquaculture fishes, including centrarchids such as crappie, there is no literature documenting how time of day of hormone injection effects latency period and spawning success.

Understanding the effects of hormone injection time on latency period would be beneficial for synchronizing and optimizing induced-spawning procedures for White Crappie, as well as for many other cultured fish species. Therefore, this study was conducted to determine whether time of hormone injection affects latency period, egg fertilization rate, ovulated egg volume and the percent of females ovulated.

Methods

Fish collection and tank acclimation

All experiments were approved by an institutional animal care and use committee at Mississippi State University; protocol # 17-056. White Crappie were collected from Enid Lake, Enid, MS in March 2017 from tributary arms branching off of 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) with 3 m poles on the front of the boat. During collection, fish were placed in an onboard tank (61 x 115 x 54 cm, 379 L), which was supplied with circulating lake water until sampling was completed (~ 2 hours).

A total of 60 White Crappie (23 females and 37 males) were transported to the Mississippi Department of Wildlife, Fisheries, and Parks North Mississippi Fish Hatchery (NMFH), Enid, MS. At the hatchery, fish were sorted by sex using visual cues including color, size and urogenital opening characteristics (i.e., female urogenital opening generally larger than the anus while male openings are similar in size) (Culpepper and Allen 2016b) and placed in 4 sections of a large recirculating aquaculture system (RAS). The RAS consisted of 2 rectangular tanks (630 x 89 x 61 cm, 3,420 L) separated into 4 sections each using prefabricated metal dividers. Salinity was maintained at 3 ppt using artificial marine salt (Instant Ocean Sea Salt, Instant Ocean, Blacksburg, VA), and temperature was maintained at 15° C to prevent disease and reduce mortality following tank acclimation protocol described by Culpepper and Allen (2016b). Temperature was monitored and maintained using a temperature controller (Single Stage Controller, Aqua

Logic Inc., San Diego, CA) and a water chiller (1/2 HP, Cyclone Water Chiller). Water quality was maintained using 120 watt high-output ultraviolent sterilizer (Emperor Aquatics, Pottstown, PA) and a bead filter (DF3, Aquaculture System Technologies, New Orleans, LA). Fish were held in these conditions for 3 weeks before water temperature manipulation began.

Hormone injection

Two weeks before hormone injection, temperature in the RAS was manipulated to stimulate natural spring spawning conditions. Water temperature was increased 1° C a day for six days until reaching spawning temperatures at 21° C (Culpepper, 2015). Spawning temperatures were then maintained for 1 week, at which time hormone injections took place.

Crappie were divided into two treatment groups based on the time of hormone injection. One treatment group (evening injection group) was designed to place the ovulation window during normal, daily hatchery operating hours (i.e. 0800-1800). Fish in the evening injection group began receiving injections at 1600. The second treatment group (morning injection group) was designed to place the ovulation window during the night (i.e. 2400-1000), with fish in this group received injections starting at 0800.

Before hormone injection, fish were anesthetized in a MS-222 bath (100mg/L tricaine methanesulfonate; 9g/L NaCl; and 400mg/L $NaHCO_3$), and weight was recorded (nearest 0.01 kg). Each fish was assigned a unique fin clip (i.e., left or right pectoral fin, caudal fin, anal fin, or no fin clip) to aid in recognizing individual fish. GnRHa was injected via a priming dose (0.05 mL/kg; 10% of the manufacturer's recommended dose)

followed by a resolving dose (0.45 mL/kg; 90% of the manufacturer's recommended dose) 24 hours later. All injections were given intramuscularly, approximately 2 cm below the dorsal fin using a 25 gauge, 2.5cm hypodermic needle, following Culpepper and Allen (2016a). After the priming dose, fish were randomly placed into raceway sections.

This experiment used 6 separate flow-through raceways (3 per treatment) equally divided into 4 sections using fabricated metal screens. The first two sections of each raceway (i.e. closest to inflow) were assigned 2 or 3 females, and the third section was left empty to prevent fish from volitionally spawning. The last section of each raceway (i.e., closest to outflow) was assigned 5 males. Each raceway received aerated surface water from an onsite settling pond, and airstones were used to provide additional aeration. Water temperature was monitored 3 times daily using a multi-parameter probe (YSI Pro2030, Yellow Springs, Ohio).

Spawning protocol

After the 90% resolving dose was injected, females were monitored every 3 hours for ovulation. Turbidity in the water from the settling pond limited visibility in tanks, so to check for ovulation, females were gently captured using dipnets and lightly palpated along the abdomen. If light palpation of the abdomen caused egg release, the female was considered ovulated and ready to spawn; the time was recorded, the fish was identified by its fin clip, and the strip-spawning procedure was conducted.

Once a female was ready to spawn, a male was selected from the same raceway and identified via a unique fin clip. The female was sedated in an anesthetic bath (similar

to that previously described), and the male was euthanized in a portable electroanesthesia system (Smith-Root, Vancouver, WA). The male crappie was measured to total length (nearest mm) and the testes were removed, and placed in a small petri dish and weighed (nearest 0.01 g). A small sample of milt (~ 100 µL) was then collected using a pipette, and activated by adding 1-2 mL of water and observing movement at 10x magnification. The testes were then minced with a scalpel to obtain sperm.

When lethargic, the ovulating female was collected from the anesthesia bath and measured to total length (nearest mm). Before expressing eggs via a stripping procedure, the fish were blotted dry using small towels to prevent eggs from water hardening. Eggs were then stripped by applying a strong downward motion on the lower abdomen, and eggs were collected in a small glass bowl (~300 mL). Eggs were then poured into a graduated cylinder to obtain a pre-fertilization volumetric measure of eggs obtained from each female. Next, eggs were transferred to a larger glass bowl (~ 1 L), and the minced testes were squeezed through a small aquarium net to obtain sperm and retain larger pieces of tissue, and the fertilization time was recorded.

The resulting egg and milt mixture was then rinsed with 50 mL of a salt-urea solution (3 g urea/ L H_2O, 4 g NaCl/ L H_2O). This solution was gently stirred for two minutes using a turkey feather followed by an additional 50 mL of salt-urea solution and a 10-minute stirring period. The solution was carefully decanted to remove debris and rinsed 3 times using fresh well water. Excess water was then poured off, and the remaining egg solution was poured into a large graduated cylinder to obtain a post-fertilization volume of eggs (mL). Eggs were added to a McDonald hatching jar

containing 1 g/L tannic acid and aerated using small air stones for 2 minutes to remove the adhesive layer on the eggs. The hatching jar was then placed on an incubation table and supplied with a steady flow of surface water until hatching was completed. After eggs were stripped from the female, the fish was euthanized in the portable electroanesthesia system and ovaries removed and weighed to the nearest 0.01 gram. Gonadosomatic index (GSI) was later calculated using the following formula: GSI= (Gonad weight/ total body weight) *100.

Additionally, a female pre-spawn GSI was calculated. Gonad weight pre-ovulation was estimated using the equation (Gonad weight pre-ovulation = Gonad weight + weight of ovulated eggs), and pre-ovulation GSI was conducted using the GSI equation above. Gonad weight was obtained by weighing the ovaries after strip-spawn and dissection. Weight of ovulated eggs was obtained by weighing eggs before fertilization and calculating a correction factor (1.02) for volume of eggs to mass of eggs. The correction factor was calculated using the following equation: [Correction factor = (mass of eggs before fertilization/volume of eggs before fertilization)].

Fertilization percentages

To determine egg fertilization percentages, 3 samples of eggs (30 eggs/sample) were collected from each hatching jar 24 hours post-fertilization. Each sample was observed for fertilization under a compound microscope at 100x magnification. Fertilization percentages were calculated using the following equation:

$$Fertilization\ percentage = \left(\frac{mean\ number\ of\ fertilized\ eggs}{30}\right) * 100 \qquad \text{Equation 2.1}$$

Egg fertilization was determined by the presence of an intact chorion and vitelline membrane following Culpepper and Allen (2016a).

Egg counts

To determine the number of eggs released per female, a 200 µL sample of eggs was collected pre-fertilization from 9 females. Samples were placed in 1 mL of 10% neutrally buffered formalin and counted using a microscope (VWR Stereo Zoom Universal Stand Binocular Microscope, VWR, Radnor, PA). An average number of eggs per 200 µL (627eggs per 200 µL) was calculated and then multiplied by 5 to estimate an average number of eggs per mL. Females ovulated an average of 3,135 eggs.

Statistical analysis

The experiment was set up as a completely random design with each raceway section defined as the experimental unit, fish as subsamples within experimental units, and the time of hormone injection defined as the fixed effect factor. Latency period, egg fertilization percentage, percent of females ovulated, number of eggs ovulated, and GSI were analyzed using Student's T-Tests since only two treatments were compared. Data were assessed for normality using the Shapiro-Wilk's test, and homogeneity of variance was assessed using a Levene's test. Data were transformed using $\log_{10}$ as needed or logit for percentage data. Data were analyzed using the statistical packages SAS 9.4 (SAS Analytics Software & Solutions, Cary, NC), and in all cases significance was determined at $\alpha = 0.05$.

Results

Latency period

Water temperature in raceways ranged from 20.3°C to 22.4°C with a mean ± SE of 21.6 ± 0.10. A total of 19 females ovulated during the experiment (evening injection group= 9 females ovulated; morning injection group= 10 females ovulated). Latency period for fish in the evening injection treatment group was 47.11 hours post initial injection (mean ± SE; 47.11 ± 1.95; range 39 -59 hrs; n=9), and latency period for fish in the morning injection treatment group was 44.80 hours post initial injection (mean ± SE; 44.80 ± 1.53; range 33 -51 hrs; n=10). Seven of nine females in the evening treatment group (78%) ovulated eggs between 42 and 52 hours, and 9 of 10 females in the morning injection group (90%) ovulated eggs within this ovulation window. Latency period did not significantly differ between treatment groups (P= 0.3593), indicating latency period is not dependent on time of day when injection occurs in White Crappie. The majority (84%) of females in both treatment groups ovulated eggs 42 and 52 hours post initial injection, indicating White Crappie will spawn within a reliable and predictable timeframe regardless of time of day when the hormone is injected.

Spawning success

A total of 23 females were injected with GnRHa (evening injection group n= 12; morning injection group n=11), 19 of which ovulated during the experiment (83%). Of the 12 females placed in the evening injection group, 9 ovulated (75%; 2 fish did not ovulate and one mortality), and of the 11 females in the morning injection group, 10 ovulated (91%; 1 fish did not ovulate).

Egg fertilization percentage was 59% from all fish spawned in the experiment. There was no difference between the mean fertilization percent of the evening injection group (63% ± 4.8) and the morning injection group (56 % ± 5.6) (P =0.3343) (Table 1.1). Ovulated egg volume (mL) did not differ between treatments (P =0.3713), with fish in the evening injection group ovulating a mean (± SE) of 21.1 ± 3.0 mL and fish in the morning injection group ovulating a mean (± SE) of 17.9 ± 1.9 mL (Table 1.1).

Egg counts and gonadosomatic index

Overall, there was a mean of 627 eggs per 200 µL sample for a 1 mL average of 3,135 eggs. This average was then multiplied by egg volume pre-fertilization of each fish to obtain the total number of eggs produced per fish. Evening injection treatment fish ovulated 66,009 ± 9,537 eggs (mean ± SE), while fish in the morning injection treatment fish ovulated 55,970 ± 5,865 eggs (Table 1.1) and which was not significantly different (P =0.3718). A mean (±SE) of 102 ± 8.90 eggs per gram of fish body mass was ovulated by females in this study.

Gonadosomatic index for female White Crappie post ovulation in the evening injection was 4.77% ± 0.89 (mean ± SE), and for females in the morning treatment group, the mean GSI post ovulation was 4.63% ± 0.54 (Table 1.1). GSI did not significantly differ between treatments (P =0.8933). Fish in the evening injection treatment had a mean pre-ovulation GSI of 8.62% ± 1.23 (mean ± SE), and fish in the morning injection treatment had a mean pre-ovulation GSI of 7.46%± 0.76 (mean ± SE) (Table 1.1). No significant difference was observed in pre-ovulation GSI among the two treatments (P

=0.2026). There was no correlation between female body size and volume of eggs ovulated ($r^2 = 0.07$).

Discussion

Hatchery production of crappie in closed systems is limited by a lack of knowledge of some important steps in the reproduction and rearing of these species. Recent advances in crappie aquaculture techniques have shown that White Crappie can be successfully spawned in a controlled environment using GnRHa to induce egg ovulation (Allred et al. in review; Culpepper, 2015; Culpepper and Allen, 2016a). In this study, White Crappie were injected with GnRHa at two different times to examine whether the latency period and spawning success varies based on diel time of injection. The results of this study indicate latency period is not affected by the time of day when hormones are injected, as females ovulated eggs both diurnally and nocturnally within a similar period. The majority of females (84%) ovulated eggs between 42 and 52 hours post initial injection, indicating time of day when a female White Crappie will ovulate eggs is dependent on time of the initial hormone injection. Results also indicate the timing of the initial injection does not affect egg fertilization, percent of females ovulated, or ovulated egg volume.

Latency period, the time between hormone injection and egg ovulation, is known to be affected by water temperature. Fish held in warmer water have shorter latency periods, compared to fish held in cooler water (Fortuny et al. 1988; Lam, 1982). White Crappie induced to ovulate using GnRHa have been reported to release eggs within a predictable time frame (38-53 hours post initial injection) when held in a water

temperature of 22°C (Allred et al. in review). In the current study, the majority of White Crappie ovulated eggs within this time window, indicating latency period of White Crappie is similar between year classes.

While hormone induced spawning is widely used in fishes, in contrast to studies on temperature effects, few studies have examined whether time of day of hormone injection affects latency period. Previous studies have found the latency period of some fishes (i.e., Japanese Eel and Common Carp) was independent of the time of hormone injection (Arabaci et al. 2001; Drori et al. 1994; Kagawa et al. 1997), while in other fishes (i.e., Barramundi and European Seabass) the latency period differed depending on the time of day at which the hormone was injected (Alvarino et al. 1992; Garcia, 1990). In the present study, White Crappie spawned within the predicted ovulation window following hormone injection, regardless of the time of day of hormone injection, with fish spawning both diurnally and nocturnally, as predicted. In this study, the majority of fish (84%) in both treatment groups ovulated eggs between 42 and 52 hours after hormone injection. This indicates latency period of White Crappie injected with GnRHa is not dependent on when hormones are injected, but rather the time of day when White Crappie ovulate eggs is dependent upon the timing of initial hormone injection.

Egg quality and ovulated quantity are also reported to be dependent on time of hormone injection in some fish species. The quality of eggs via hormone-induced ovulation has been reported to be variable in different fish species (i.e., Japanese Eel and Senegalese sole) depending on time of day at which ovulation occurred (Kagawa et al. 1997; Rasines et al. 2013); possibly due to the daily cycles of sensitivity of the pituitary

gland to GnRHa (Rasines et al. 2013). Regarding egg quantity, Barramundi produced a larger volume of eggs when GnRHa was injected during daytime compared to volumes produced by fish injected at nighttime (Garcia, 1990). In this study, there was no difference in egg quantity or fertilization rate of eggs ovulated at different times of day, indicating the time of day White Crappie are injected with GnRHa does not affect the quality or quantity of eggs ovulated.

Centrarchid species such as Largemouth Bass (*Micropterus salmoides*) and Bluegill (*Lepomis* m*acrochirus*) have been studied more thoroughly than White Crappie, and protocols for tank culture, artificial spawning, and out-of-season spawning have been established (Morris and Clayton, 2009). The development of protocols for these species led to an interest in the development of protocols for crappie, although most crappie production has been done extensively in ponds (Morris and Clayton, 2009) with variable techniques and success (Culpepper and Allen, 2016b). However, the recent development of techniques for hormone induced spawning (Al-ablani and Phelps, 1997; Arslan and Phelps, 2004; Cuevas-Uribe, 2009; Culpepper and Allen, 2016b; Gomelsky et al. 2002), tank holding procedures, out-of-season spawning techniques (Allred et al. in review; Culpepper and Allen 2016a) and sperm cryopreservation (Culpepper et al. 2017) have allowed for more intensive crappie spawning techniques to be studied.

Variables often used to describe the success of fishes intensively spawned include: percent of fish (females) spawned, egg fertilization percentage, and volume of eggs ovulated. While previous studies have induced crappie to spawn using various hormones (HCG and LHRHa) (Al-ablani and Phelps, 1997; Arslan and Phelps, 2004;

Cuevas-Uribe, 2009; Gomelsky et al. 2002), spawning success was not the subject of these studies and was not reported, with the exception of Al-ablani and Phelps (1997) who reported a spawning success of 86% when using HCG to induce spawning. Culpepper (2015) demonstrated GnRHa was an effective hormone for inducing spawning in White Crappie, with 50% of females spawned, egg fertilization percentages from 34% to 68%, and a mean post-fertilization egg volume of 46.4 mL ovulated per female. The present study was able to achieve a spawning success of 83% percent of females spawned, egg fertilization of 59%, and a mean post-fertilization egg volume of 36.4 mL ovulated (0.03 mL of ovulated eggs/gram of fish weight) per female, indicating successful spawning induction.

Additionally, GSI was used to assess the development of gonads in this study. Two different GSI measurements were taken, GSI pre-ovulation and GSI post-ovulation. A study conducted on naturally spawning White Crappie found that during the peak spawn, the GSI of wild female fish that had not yet ovulated was approximately 8% (Thomas and Kilambi, 1981). The present study found similar GSI pre-ovulation values in White Crappie (8.04%), suggesting temporary (~5 weeks) holding of White Crappie in recirculating systems does not negatively affect GSI. Female GSI post-ovulation, measured after fish were strip-spawned, did not differ between evening and morning injection treatments (4.77% and 4.63% respectively). This indicates similar proportions of ovaries were induced to ovulate, and hormone efficacy was not affected by the time of day of injection.

The identification of the latency period of White Crappie following hormone injection, and determination that latency period, fertilization percentage, percent of females ovulated, and ovulated egg volume is not dependent on injection time, has many benefits for crappie production practices. Understanding and using latency period will allow hatchery managers to schedule strip-spawning procedures when hatchery ponds are ready for larval rearing and available for use. The synchronous spawning of fish may have benefits for reducing size and age variability when fish are stocked in hatchery ponds, potentially reducing cannibalism, and in turn, increasing the survivability of larval fish. The ability to predict when ovulation will occur will allow hatchery operations to limit handling of fish to check for ovulation to only the hours when females are most likely ovulating eggs, potentially reducing fish stress. Reducing stress during the later stages of gonad development may have benefits for increasing the quantity, quality, and survival of eggs ovulated (Campbell et al. 1994). Using RAS to keep adult fish alive and healthy, in combination with the ability to accurately predict when females will ovulate, could also eliminate the need for continuous sampling for ovulating females during the spawning season. Wild, White Crappie could be collected before the spawning season begins and held in recirculating systems, allowing for large numbers of fish to be collected for production of larval fish when conditions, hatchery schedules, and stocking schedules dictate, leading to more synchronous development of larval fish.

Tables

Table 2.1 Spawning success variables in induced-spawning of White Crappie (*Pomoxis annularis*).

Injection	Evening				Morning Injection			
Variable	**Mean**	**±**	**SE**	**n**	**Mean**	**±**	**SE**	**N**
Pre-fertilization volume of eggs ovulated per fish (mL)	21.1	±	3.0	9	17.9	±	1.9	10
Post-fertilization volume of eggs ovulated per fish (mL)	40.2	±	5.1	9	33.0	±	4.2	10
Total eggs ovulated per fish	66,009	±	9,537	9	55,970	±	5,865	10
Gonadosomatic index pre-ovulation	8.62%	±	1.23	9	7.46%	±	0.76	9
Gonadosomatic index post-ovulation	4.77%	±	0.89	9	4.63%	±	0.54	9
Egg fertilization percent	63%	±	4.8	9	56%	±	5.6	10
Percent of females ovulated	75%			12	91%			11

No statistical difference was observed for any of the above measurements of spawning success between the treatment groups (i.e., evening injection and morning injection treatment) (P>0.05).

Figures

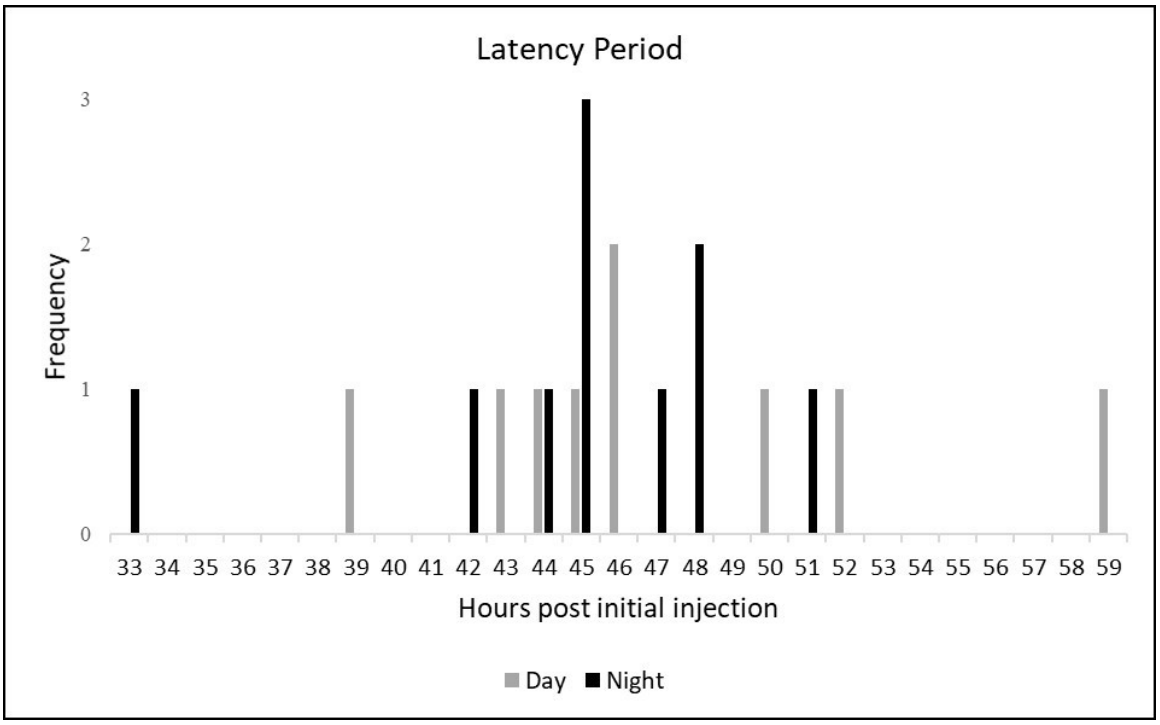

Figure 2.1 White Crappie (*Pomoxis annularis*) time of ovulation (hours) (i.e., latency period) following the initial, priming injection of gonadotropin-releasing hormone analog (GnRHa).

The majority (84%) of fish spawned 42-52 hours post-injection of GnRHa. There was no statistical difference in latency period between the two treatment groups, with 78% of females in the evening injection group and 90% of females in the morning injection group ovulating eggs between 42 and 52 hours post initial injection ($P > 0.05$).

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CHAPTER III

EVALUATING TECHNIQUES FOR CRYOPRESERVING BLACK-STRIPE BLACK CRAPPIE (POMOXIS NIGROMACULATUS) SPERM AND USE IN EGG FERTILIZATION

Abstract

Recent techniques for White Crappie (*Pomoxis annularis*) sperm cryopreservation have led to an interest in applying similar methods to a phenotypic variant of Black Crappie (*P. nigromaculatus*), known as black-stripe Black Crappie. Black-stripe Black Crappie have a conspicuous black stripe extending from the anterior base of the dorsal fin to the upper maxilla and are uncommon in wild populations. Their rarity makes them a preferred phenotype for hatchery use, particularly for ease of post-stocking identification. Therefore, procedures established for cryopreserving White Crappie sperm (i.e., using Hanks Balanced Salt Solution (HBSS) as an extender and two cryoprotectants, 5% dimethyl-sulfoxide (DMSO) and 10% methanol) were compared between black-stripe Black Crappie and White Crappie for pre-cryopreservation and post-thaw sperm motility and fertilization rate of White Crappie eggs. There was no significant difference in black-stripe Black Crappie sperm motility between sperm cryopreserved with 5% DMSO or 10% methanol (45% and 50% sperm motility, respectively). However, fertilization rates

were higher for black-stripe Black Crappie sperm cryoprotected with 5% DMSO than black-stripe Black Crappie sperm cryoprotected with 10% methanol (38% and 22% fertilization, respectively). This study indicated black-stripe Black Crappie sperm could be cryopreserved using HBSS as an extender, 5% DMSO as a cryoprotectant, frozen at 40° C/minute, and can be thawed for 8 seconds at 40°C to maintain sperm motility and fertility.

Introduction

Black Crappie (*Pomoxis nigromaculatus*) and White Crappie (*P. annularis*) are highly sought-after game fish throughout their range and present unique management challenges including variable recruitment and the ability to overpopulate small impoundments (Swingle and Swingle 1967; Busack and Baldwin 1988; Mitzner 1991). Recent advances in crappie aquaculture techniques, such as the ability to produce monosex, sterile or triploid crappie, (Al-ablani and Phelps 1997; Gomelsky et al. 2002; Arslan and Phelps 2004; Cuevas-Uribe 2009; 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 may be subsequently induced by subjecting fertilized eggs to a high-pressure treatment (Parsons 1992, 1996, 1999). Parsons (1999) found a pressure shock treatment on fertilized crappie eggs of 6,000 psi (422 kg/cm^2) for 2 minutes resulted in an 87% hatching rate and 100% triploidy. The black-stripe Black

Crappie is a naturally occurring Black Crappie phenotype expressing a unique pre-dorsal stripe thought to be controlled by a mutant, dominant gene, and has been used by hatcheries to produce easily identifiable crappie, which are often triploid or hybrid (Parsons 1996; Gomelsky et al. 2005). The black-stripe Black Crappie phenotype is useful to biologists as a natural tag, facilitating identification of stocked fish, because of the rarity of the phenotype in the wild (Gomelsky et al. 2005). However, this rarity makes it difficult to find enough broodstock for hatchery production use 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 storing sperm containing desired gametes, reducing broodstock needed, ease of storage and transport, and ability to have viable sperm on hand when female fish are available for spawning (Cabrita et al. 2010). Methods for cryopreserving fish sperm have been documented in freshwater fish including salmonids, sturgeon, carp and catfish (Cabrita et al. 2010), as well as Coppernose Bluegill (*Lepomis macrochirus purpurescens*), White Bass (*Morone chrysops*), and Striped Bass (*Morone saxatilis*) (Brown and Brown 2011). Recently, Culpepper et al. (2017) demonstrated White Crappie sperm can be cryopreserved using 350 mOsmol/kg Hanks Balanced Salt Solution (350 HBSS) as a buffer, either 5% dimethyl-sulfoxide (DMSO) or 10% methanol as the cryoprotectant, and cooling at 40°C/minute. The capacity to successfully 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 many males.

Along with developing optimal procedures for cryopreserving sperm, the developing techniques and procedures for fertilizing eggs using cryopreserved sperm is essential to maximizing the effectiveness of strip-spawning. For centrarchids, egg fertilization rates using cryopreserved Bluegill sperm have ranged from 15 to 50%, depending on cryoprotectant (Bates et al. 2005). Egg fertilization rates using cryopreserved White Crappie sperm yielded relatively low fertilization rates (13%) (Culpepper et al. 2017) indicating a need for refining egg fertilization techniques using cryopreserved sperm. Cryopreservation techniques are not commonly applied to produce hybrids in fishes, but have been done so successfully (Gharrett et al. 1999), sometimes with similar or higher fertilization rates (Chao et al. 1987). The ability to successfully fertilize crappie eggs using cryopreserved black-stripe Black Crappie sperm could allow for more 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 large amounts of black-stripe Black Crappie sperm to be stored and be easily accessible during strip-spawning. This could reduce the need for collecting and maintaining broodstock of the difficult to locate black-stripe Black Crappie and increase egg fertilization percentages when using cryopreserved sperm. Therefore, the objectives of this study were to investigate whether black-stripe Black Crappie sperm can be cryopreserved using similar

methods to those established for White Crappie (Culpepper et al. 2017), and compare two cryoprotectants (i.e., 5% DMSO and 10% methanol) for fertilization efficacy.

Methods

Fish collection and transportation

Male black-stripe Black Crappie were obtained from William H. Donham State Fish Hatchery, in Corning, Arkansas. Fish were transported to the Mississippi Department of Wildlife, Fisheries, and Parks North Mississippi Fish Hatchery (NMFH) in Enid, Mississippi. The fish were transported in 445 L (203 x 51 x 43 cm) rectangular tanks at 15°C and 3 ppt salinity (NaCl), conditions found to reduce disease and mortality in crappie (Culpepper and Allen 2016a). White Crappie 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 x 115 x 54 cm, 380 L) during collection and supplied with circulating lake water until sampling concluded (~2 hours). Upon arrival all fish were placed in a large recirculating aquaculture system consisting of two rectangular tanks (630 x 89 x 61 cm, 3,420L) separated by prefabricated metal dividers into four sections each, and fish were held in one section. 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, 115v**,** Aqua Logic Aquatic Inc., San Diego, California) and a water chiller (1/2 hp, Cyclone Water Chiller, Aqua Logic Aquatic Inc., San Diego, California). Water quality was maintained using 120-watt high output ultraviolent sterilizer (Emperor Aquatics, Pottstown, PA) and a beadfilter (DF3, Aquaculture System Technologies, New Orleans, LA).

Fish were transported to the Louisiana State University Agriculture Research Center from the NMFH in 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 hours).

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 (100mg/L tricaine methanesulfonate, 9g/L NaCl and 400mg/L $NaHCO_3$), until operculum and fin movement ceased. The fish was then rinsed with 350 mOsm/L Hanks Balanced Salt Solution (350 HBSS) to ensure water would not activate sperm once testes were removed. The testes were removed via dissection and placed in a weigh boat containing enough 350 HBSS to cover the entire organ. The testes were then 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 350 HBSS to dilute neat sperm (1:19; sperm:350 HBSS). The solution was vortexed to mix, and 5 µL was removed to determine an initial concentration of sperm for each fish. Initial sperm concentration was determined by placing the 5 mL sample on a Makler® counting chamber (SEFI Medical Instruments LTD, Irvine Scientific, Santa Ana, CA) and viewed under a microscope (Olympus CX41RF, Japan) at 200x magnification. Each sample was counted 3 times and counts were averaged to obtain the initial sperm concentration per mL for each fish.

A 4 µL sperm sample was placed on the Makler counting chamber and 20 µl water added to activate sperm to estimate and ensure sperm motility before cryopreservation. After activation, the counting chamber was placed under the microscope and motility was estimated using the computer-calculated motility characteristics. System settings followed those used by Culpepper et al. (2017) and 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. Three measurements, each from a different field of view, were taken for each sample and the average initial motility calculated.

The cryoprotectants 5% DMSO and 10% methanol were chosen for this study based on successful cryopreservation of White Crappie sperm (Culpepper et al. 2017). For each male (n= 5 White Crappie; n=6 black-stripe Black Crappie), both cryoprotectants were prepared at double the target concentration in 350 HBSS and mixed

1:1 with sperm samples, to yield final sperm concentrations of $2.5x10^8$ sperm/mL. After mixing the cryoprotectant and sperm, the solution was placed in the Quattro Minitube system (Verona, WI), where straws were filled, sealed, and labeled. The straws were then arranged on a freezing rack, and placed in a programmable freezer (Micro Digitcool, IMV, France). The straws were cryopreserved at 40°C/minute, based on methods of Culpepper et al. (2017).

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 3 straws from each fish and treatment. Straws were thawed in a 40° C water bath for 8 seconds (Culpepper et al. 2017). Motility was estimated using the computer analysis system described previously. Three straws from each male in each treatment were thawed and used in post-thaw sperm motility estimations. 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.

Egg fertilization

Female fish were collected from Enid Lake, Enid, Mississippi, via boat electrofishing, similar to previously described methods, before the natural spawning season (i.e., March-April). Fish were held in an onboard tank and supplied with circulating lake water during sampling. After collection, fish were transported to the NMFH in Enid, MS. Crappie were sorted by sex, using visual cues including color (females often lighter than males), size of abdomen (swollen ovaries), and urogenital

opening characteristics (female urogenital opening generally larger than the anus while male openings are similar in size) based on Culpepper and Allen (2016a), and placed in a recirculating aquaculture system (RAS) and held in conditions as described above. Two weeks before the scheduled spawning temperature, the RAS was increased by 1°C daily for 6 days, until reaching spawning temperatures of 21°C (Culpepper and Allen 2016a) and maintained at spawning temperatures for another week.

After being held at spawning temperatures for one week, females were anesthetized in an anesthetic bath (100 mg/L tricaine methanesulfonate; 9 g/L NaCl; and 400 mg/L $NaHCO_3$) and weighed to the nearest 0.01 kg. Previous experiments have shown the gonadotropin-releasing hormone analog (GnRHa) is 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, via a priming dose (0.05 mL/kg; 10% of manufacturer's recommended dose [0.5mL/kg recommended dose]), with a resolving dose (0.45 mL/kg; 90% of manufacturer's recommended dose) administered 24 hours later (Culpepper 2015). After a latency period (i.e. time of ovulation post priming dose) identified in previous White Crappie strip-spawning experiments (Shirley and Allen, in review), females were observed every hour for ovulation starting 38 hours 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 above. 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 collected in a small glass bowl (~300 mL).

Eight female White Crappie were strip spawned following the procedures described above. For each female, 0.25 mL (~750 eggs) (Culpepper et al. 2017) 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 x 2.5 cm square cut out of both sides and covered with 300 µm nylon mesh (Pentair, Apopka, FL). Once eggs were pipetted into their respective containers, for each container two straws of the desired cryoprotectant from one male were randomly chosen and thawed for 8 seconds in a 40° C water bath, dried externally with a kimwipe, and used to fertilize the eggs in each container. Sperm was used from all six black-stripe Black Crappie males. Ten mL of fresh water was added and the entire solution was gently mixed with a small brush and left stationary for 10 minutes to allow for fertilization. After 10 minutes, containers were placed in small raceways 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. The male White Crappie were euthanized in a portable electroanesthesia system (Smith-Root, Vancouver,

WA) and dissected to remove the testes. Testes were then minced with a scalpel to obtain sperm, at which time a small sample (~100 μL) was scraped onto a slide and activated by a drop of water. Sperm were observed at 100X 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 mixture of egg and milt was then activated with 50 mL of fresh water. The solution was gently stirred using a turkey feather for 2 minutes. After 2 minutes, 50 mL of fresh water was added, and the solution was stirred for another 5 minutes. The entire solution was then carefully rinsed until debris was removed, excess water was decanted, and the egg solution was added to a McDonald hatching jar containing 3Lwater and 3 g tannic acid (1 g/L), to remove the adhesive layer. The solution was aerated for 2 minutes and then the jar was placed on a hatching table and supplied with a continuous flow of fresh water.

Fertilization percentages were calculated 24 hours post fertilization by randomly selecting three, 30 egg samples from each container, obtaining the number of fertilized eggs and dividing by 90. Egg fertilization was also determined from each hatching jar to confirm the female had produced viable eggs. Eggs were observed using a compound microscope at 25X magnification. Egg fertilization was characterized by the presence of an intact chorion and vitelline membrane (Culpepper and Allen 2016b).

Statistical analysis

Data were analyzed using the statistical package SAS 9.4 (SAS Analytics Software & Solutions, Cary, NC), and significance was determined at $\alpha = 0.05$. This

experiment was designed to directly compare the effects of sperm cryopreservation techniques on the motility of sperm from White Crappie and black-stripe Black Crappie. Data were tested for normality using the Shapiro-Wilk test, and Levenes'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 both 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 2x3 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-significant differences were used to identify significant differences among treatments.

A randomized complete block design and ANOVA was used to compare fertilization rates between the 3 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.

Results

Sperm motility

There were no significant differences in initial concentrations of fresh sperm (P=0.4322) obtained from either Black or White Crappie (Table 1). Regarding sperm motility, species, cryoprotectant, and time did not significantly interact to affect the mean sperm motility of cryopreserved sperm (P=0.9442). Species and cryoprotectant (P= 0.2196), species and time (P= 0.5150), and cryoprotectant and time (P= 0.2074) did not significantly interact to affect the mean sperm motility. Similarly, neither cryoprotectant (P=0.2346) nor time (P= 0.9507) affected the mean sperm motility. However, species did significantly affect the average sperm motility (P=0.0167), as black-stripe Black Crappie sperm was significantly more motile than White Crappie sperm (Table 1).

Fertilization experiment

The cryoprotectant agent (i.e. 5% DMSO or 10% methanol) significantly affected egg fertilization percentage (P <0.0001) (Figure 1). Eggs fertilized using black-stripe Black Crappie sperm cryopreserved using 5% DMSO had a significantly higher fertilization rate than eggs fertilized with black-stripe Black Crappie sperm cryopreserved with 10% methanol and White Crappie sperm cryopreserved with 10% methanol (Figure 1). A significant block effect (block = one female) was detected for egg fertilization percent, indicating egg quality varied 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=0.0032), indicating there was some variability in treatment fertilization rates of eggs among females, however black-stripe

Black Crappie sperm cryopreserved in 5% DMSO (38%) generally outperformed black-stripe Black Crappie sperm cryopreserved in 10% methanol (25%) and White Crappie sperm cryopreserved in 10% methanol (22%) by greater than 10%.

Discussion

Recently documented protocols for cryopreserving White Crappie sperm (Culpepper et al. 2017) have led to an interest in cryopreserving the sperm of black-stripe Black Crappie, an uncommon phenotype preferentially used by hatcheries. Results from this study indicate the sperm of black-stripe Black Crappie can be cryopreserved using the same extender (350 HBSS), cryoprotectants (5% DMSO and 10% methanol), freezing rate (40° C/minute), and thawing regime identified for White Crappie. Notably, black-stripe Black Crappie sperm cryopreserved using 5% DMSO yielded higher egg fertilization than 10% methanol cryopreserved sperm from either White or black-stripe Black Crappie, indicating its preferential future use.

Evaluating sperm motility after cryopreservation and thawing is crucial to optimizing protocols for sperm cryopreservation due to the importance of sperm motility in egg fertilization (Cabrita et al. 2010). In this study, both black-stripe Black Crappie and White Crappie sperm were cryopreserved following methods established by Culpepper et al. (2017), allowing direct comparison of sperm motility between the two species. Regardless of cryoprotectant used, black-stripe Black Crappie sperm, had a higher mean sperm motility (48%) than White Crappie cryopreserved sperm (39%). Cryopreserved sperm motility is comparable to the centrarchid, Bluegill (26-56%) (Bates

et al. 2005), and striped bass (40%) (He and Woods 2003), and higher than Channel Catfish (*Ictalurus punctatus)* (5-10%) (Tiersch et al. 1994).

Methanol and DMSO have been commonly used as cryoprotectants of fish sperm, and their sperm cryopreservation effectiveness has been evaluated through egg fertilization experiments. Studies suggest the optimal cryoprotectant used to cryopreserve sperm may differ among fish. Studies of fertilization rates using cryopreserved sperm of several salmonid species found sperm cryopreserved with methanol was more effective at fertilizing eggs than DMSO (Lahnsteiner et al. 1997). In centrarchids, cryopreserved sperm experiments with Bluegill found sperm motility and egg fertilization were higher using 10% methanol than 10% DMSO, with fertilization rates up to 75% using methanol and 50% using DMSO (Bates et al. 2005). In Striped Bass, sperm motility and egg fertilization rates were higher in DMSO than methanol (He and Woods 2003) with fertilization rates of 26-54% in DMSO treatments (He and Woods 2004). In contrast, Channel Catfish, sperm motility and egg fertilization rates (24%-97%) were higher in sperm cryopreserved with methanol compared to DMSO (Tiersch et al. 1994).

A previous study using White Crappie sperm cryopreserved with 5% DMSO or 10% methanol found there was no difference in fertilization rates between the two cryoprotectants, although fertilization rates were low (overall mean: 13%) (Culpepper et al. 2017). In comparison, this study found black-stripe Black Crappie sperm cryopreserved with 5% DMSO was more effective at fertilizing White Crappie eggs (38%) than sperm cryopreserved in 10% methanol (22%). Although not directly

comparable, this study had higher fertilization rates in White Crappie eggs than Culpepper et al. (2017). Improvements in water quality (parasites observed during fertilization checks by Culpepper et al. (2017)) and a better understanding of latency period duration may have contributed to the increase in egg fertilization in the present study. Further research improving egg fertilization percentage or establishing optimal ratios of cryopreserved sperm: eggs for fertilization may be beneficial.

This study used White Crappie eggs for fertilization and used both White Crappie and black-stripe Black Crappie sperm. Interestingly, black-stripe Black Crappie sperm was more effective at fertilizing White Crappie eggs than White Crappie sperm. However, this study only used one White Crappie cryopreserved sperm treatment (10% methanol), while black-stripe Black Crappie sperm cryopreserved in both 5% DMSO and 10% methanol were used. It is possible 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. 2017). Although sperm concentration was similar in the present study, black-stripe Black Crappie sperm had higher motility than White Crappie sperm, which may explain higher fertilization rates.

The black-stripe Black Crappie is of particular interest in crappie fisheries management, because of its unique pre-dorsal black stripe. This characteristic is used by some hatcheries to aid in identifying hatchery produced fish stocked into a fishery (Cole Cochran, MDWFP; personal communication), and is also easily recognized by the general public. Marking or tagging fish is a vital tool for management and study of fish

and the fisheries they inhabit facilitating identification for analyses of survival, recruitment, movement, and stocking success (McFarlane et al. 1990). The ability to tag fish, the ability of fish to retain a tag, and the ability for fisheries managers to accurately identify tagged fish, are important components of fisheries management (McFarlane et al. 1990). Beyond easy identification, an important benefit of producing black-stripe Black Crappie for hatcheries, is tags will never be lost or have maladaptive impacts on fish. It is imperative to fisheries management that fish do not lose implanted or external tags (Henderson-Arzapalo et al. 1999), die from unforeseen effects of having a tag, or be more susceptible to predation (Catalano et al. 2001).

Black Crappie possessing the black-stripe phenotype are often difficult to locate in enough numbers needed for hatchery production. Current crappie strip-spawning requires a male crappie to be euthanized and the testes excised for every female strip-spawned (Culpepper and Allen 2016a; Shirley and Allen, in review). This study identifies protocols for cryopreservation of black-stripe Black Crappie sperm and demonstrates the efficacy of cryopreserved sperm in fertilization of White Crappie eggs. Interestingly, black-stripe Black Crappie sperm had higher motility than White Crappie sperm and protocols established for White Crappie sperm were more effective for cryopreserving black-stripe Black Crappie sperm. Notably, for black-stripe Black Crappie sperm, a 5% DMSO cryoprotectant yielded better egg fertilization than 10% methanol. The ability to cryopreserve black-stripe Black Crappie sperm may assure meeting demands and greater flexibility in spawning at hatcheries.

Tables

Table 3.1 Initial concentration and motility of fresh sperm and motility of black-stripe Black Crappie (*Pomoxis nigromaculatus*) and White Crappie (*P. annularis*) sperm before freezing and after cryopreservation and thawing.

	Black-stripe Black Crappie			White Crappie		
Variable	**Mean**	**±**	**SE**	**Mean**	**±**	**SE**
Initial Concentration (cells/mL)	5.77×10^9	±	3.84×10^8	6.31×10^9	±	5.56×10^8
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 & 10% Methanol)	48%*	±	3.5	39%	±	4.5

An asterisk indicates a significant difference among treatments (Student's t-test; $P<0.05$; n=5 White Crappie, n=6 black-stripe Black Crappie).

Figures

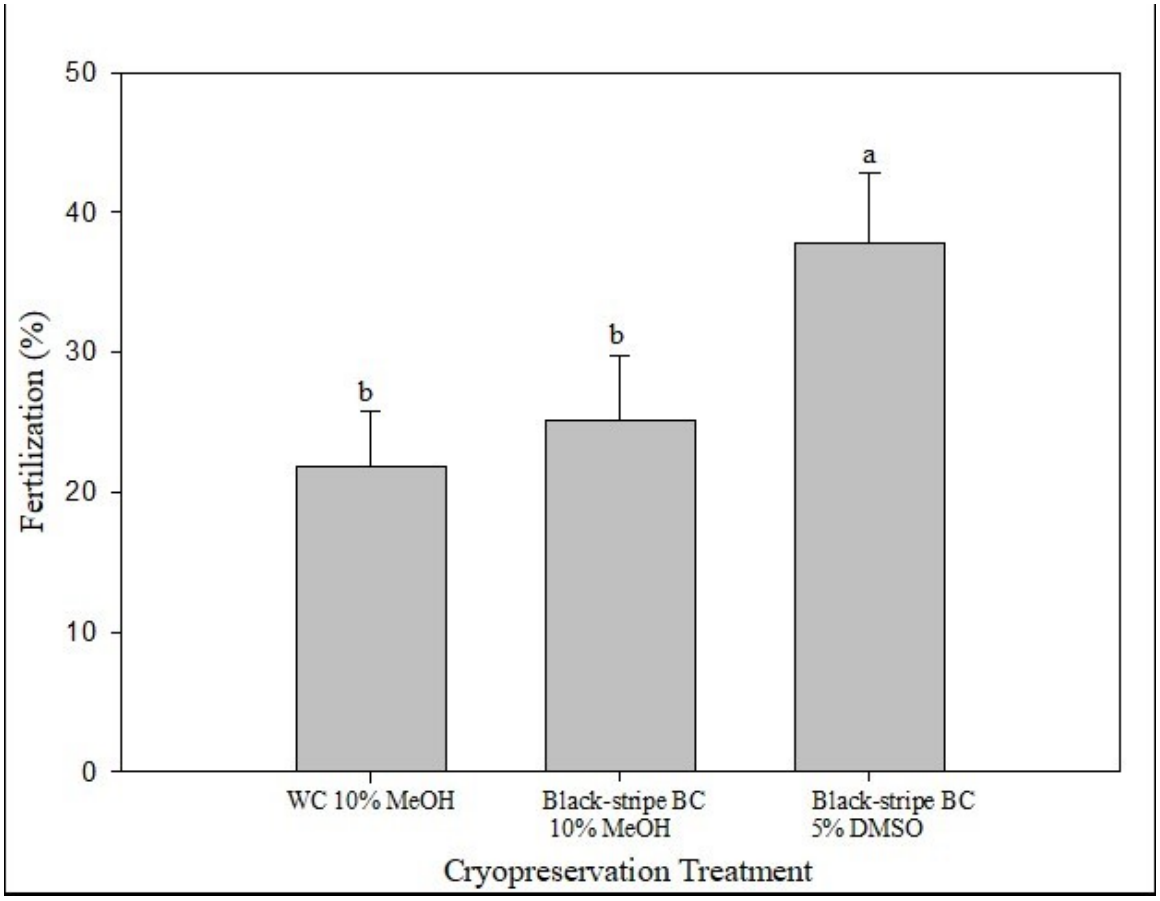

Figure 3.1. Mean (± standard error) egg fertilization percent of White Crappie (WC; *Pomoxis annularis*) eggs fertilized using 3 different treatments (WC 10% MeOH, White Crappie sperm cryopreserved using 10% methanol; black-stripe BC 10% MeOH, black-stripe Black Crappie sperm cryopreserved using 10% methanol; black-stripe BC 5% DMSO, black-stripe Black Crappie sperm cryopreserved using 5% dimethyl-sulfoxide) of cryopreserved sperm. Different lowercase letters indicate significant differences ($P < 0.05$) among treatments.

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CHAPTER IV

EVALUATING THE EFFECT OF SIMULATED SPRING DURATION ON WHITE CRAPPIE (*POMOXIS ANNULARIS*) SPAWNING SUCCESS

Abstract

Interest in increasing crappie (*Pomoxis* spp.) production has led to research on developing out-of-season spawning protocols. Previous studies have established out-of-season spawning White Crappie (*P. annularis*) is possible after simulated winter and gradual adjustment to spring conditions, with duration at final spring conditions suggested to improve spawning success. Therefore, the effects of 2 or 3 weeks at spring conditions (22°C, 16-hour light) were examined for White Crappie spawning success (i.e., percent females spawned, egg fertilization percent, ovulated egg volume, gonadosomatic index (GSI), and mean egg diameter). Fish were exposed to a simulated 2-week winter (10°C, 10-hour light), a 6-week spring transition phase, and held for either 2 or 3 weeks at final spring conditions. Fish were induced to spawn with intramuscular injections of gonadotropin-releasing hormone analog (GnRHa) and strip-spawned after ovulation. In the 2-week spring treatment, all females (n=9) ovulated, with a mean (± SE) egg fertilization of 34 ± 8%, ovulated egg volume of 17.5 ± 3.1 mL and egg diameter of 0.82 ± 0.01 mm. All females in the 3-week treatment ovulated (n=10) with a mean (± SE) egg fertilization of 65 ± 10%, ovulated egg volume of 16.9 ± 4.5 mL, and egg diameter of

0.81 ± 0.02 mm. Egg fertilization percent was greater in the 3-week spring treatment, indicating White Crappie out-of-season spawning success benefits from increased duration of spring conditions.

Introduction

While popular among recreational and competitive anglers throughout the Southeastern and Midwestern US, crappie (*Pomoxis* spp.) often present management challenges including: overpopulation of smaller impoundments (<20 ha), population fluctuations, and variable recruitment leading to a strong year class every 3-5 years (Busack and Baldwin, 1988; McDonough and Buchanan, 1991; Mitzner, 1991; Boxrucker and Irwin, 2002). These management challenges have led to increasing interest in the intensive production of hatchery-reared crappie for supplemental stocking into public waters. While extensive crappie production has been practiced for some time (Culpepper and Allen 2016a), intensive crappie culture is not well studied, and knowledge is limited regarding techniques and protocols for spawning, rearing and producing crappie indoors.

Until recently, intensive crappie culture has primarily focused on developing techniques for producing mono-sex or sterile crappie (Al-ablani and Phelps 1997; Gomelsky et al. 2002; Arslan and Phelps 2004; Cuevas-Uribe et al. 2009), whereas methods for producing diploid larval fish for stocking have not been well developed. Recent studies on crappie intensive production have focused on understanding induced spawning through identifying factors affecting percent of females spawned, egg fertilization percentage, gonadosomatic index (GSI) and volume of eggs ovulated

(Culpepper 2015; Culpepper and Allen 2016a; Culpepper and Allen 2016b). Several commonly used ovulation-inducing hormones have been evaluated for spawning efficacy, including human chorionic gonadotropin (hCG), luteinizing hormone-releasing hormone analog (LHRHa), and gonadotrophin-releasing hormone analog (GnRHa), with GnRHa consistently effective for inducing ovulation in crappie (Culpepper, 2015). Other recent advances in intensive crappie culture include demonstrating induced spawning (Al-ablani and Phelps 1997; Gomelsky et al. 2002; Arslan and Phelps 2004; Cuevas-Uribe et al. 2009), successfully cryopreserving White Crappie sperm (Culpepper et al. 2017), and identifying the latency period after hormone-induced spawning (Shirley and Allen in review).

Most studies intensively spawning crappie have taken place during the natural spawning season (Al-ablani and Phelps, 1997; Arslan and Phelps, 2004; Cuevas-Uribe et al. 2009; Gomelsky et al. 2002; Culpepper, 2015; Shirley and Allen, in review). However, fish of the same family as crappie (Centrarchidae), including Largemouth Bass (*Micropterus salmoides*) and Bluegill (*Lepomis macrochirus*), have been successfully spawned out-of-season through manipulating water temperature and photoperiod (Mischke and Morris., 1997; Matthews and Stout, 2013), leading to interest in spawning White Crappie (*Pomoxis annularis*) out-of-season.

Recent studies have demonstrated out-of-season spawning is possible for White Crappie (Culpepper and Allen, 2016b; Allred et al. in review). These studies established a procedure for simulating winter and spring necessary for tank acclimation and inducing gonad maturation in White Crappie and examined effects of three different seasonal

adjustments (i.e., winter-spring) periods (i.e., 3, 6, and 9 weeks) on spawning success. Culpepper and Allen (2016b) used seasonal adjustments of 3 and 6 weeks and determined out-of-season spawning was possible, although spawning success (i.e., egg fertilization and the number of females spawned) was limited, with only one 3-week female and two 6-week females ovulating viable eggs. Egg fertilization was low in the 3-week period (10%), but higher (75%) in the 6-week adjustment period (Culpepper and Allen 2016b). When seasonal adjustments of 6 and 9 weeks were used, spawning success was comparatively high (60-89%). However, no difference between treatments in egg fertilization, GSI, or ovulated egg volume was observed (Allred et al. in review). This increase in the percent of females spawning may be attributed to the greater duration fish are held at final spring conditions, from < 1 week (5 days)(Culpepper and Allen 2016b), to 2 weeks (15 days) (Allred et al. in review).

Studies have suggested fish spending additional time at spring temperature and photoperiod show higher spawning success (Banner and Hyatt 1975; Mischke and Morris 1997; Cargnelli and Neff, 2006). Therefore, the objective of this study was to compare spawning success of White Crappie held for 2 and 3 weeks at spring conditions by evaluating latency period, percent of females ovulated, fertilization percentage, egg volumes, gonadosomatic index (GSI), and egg diameter to improve out-of-season spawning protocols.

Methods

Fish collection and tank acclimation

White Crappie were collected from anglers at Lake Washington, MS on December 9, 2017. Fish were transported in two, 445 L (203x51x43 cm) rectangular tanks supplied with compressed oxygen via diffuser stones to the South Farm Aquaculture Facility, Starkville, MS. The tanks were filled with well water at the South Farm Aquaculture Facility and chilled to <15°C and salinity was maintained at 3 ppt with solar salt (NaCl).

Upon arrival at the South Farm Aquaculture Facility, fish were placed in 20, 430-L tanks on two recirculating aquaculture systems (RAS). Salinity was maintained from 3-5 ppt with artificial marine salt (Instant Ocean Sea Salt, Instant Ocean, Blacksburg, VA) to reduce stress and prevent disease (Culpepper and Allen 2016a). Each RAS (~9,000-L) consisted of an 85-L bead filter with a mixing motor (PBF-3, Aquaculture Systems Technologies, New Orleans, LA), an 80-watt high output ultraviolet sterilizer (Emperor Aquatics, Pottstown, PA), a heat exchanger (Titan HP-7 in-line heat, Aqua Logic, San Diego, CA), two in-line heaters (EHE25T, Elecro Engineering, Stevenage Hertfordshire, UK), a moving bed bioreactor (1,020-L) and a sump tank (268-L). Temperature, dissolved oxygen, salinity, and pH were measured daily throughout the experiment, with a multiparameter probe (YSI model 85; YSI Inc., Yellow Springs, Ohio) and a pH meter (Ecosense pH100A; YSI Inc.). Nitrite, total ammonia nitrogen, and alkalinity were monitored weekly with a colorimeter (Hach DR-890; Hach Co., Loveland, Colorado) and commercial test kits (Model AQ-3; LaMotte Company, Chester, Maryland) (Table 1). Crappie were supplied with > 3 live Silver Shiners (*Notropis photogenis*) per crappie;

consumed shiners were replaced every morning to keep crappie fed to satiation throughout the experiment.

Temperature and photoperiod manipulation

Tanks were in an enclosed room, facilitating temperature and photoperiod control. Fish were first placed in 430-L tanks (91 cm diameter) partly covered with foam boards to provide shading. Water temperature and photoperiod were adjusted to simulate winter (10°C and 8 hours light). These conditions were maintained for 2 weeks before increasing temperature 1°C every 4 days and increasing photoperiod 1 hour of light every 4 days until spring conditions were reached (22°C and 16 hours light) following Culpepper and Allen, (2016b) and Allred et al. (in review). When spring conditions were reached, fish were anesthetized in a water bath (100 mg/L tricaine methanesulfonate; 9 g/L NaCl; and 400 mg/L $NaHCO_3$). Fish were then sexed by either obtaining eggs via cannulation, presence of milt, or based on visual cues (i.e., urogenital opening size and shape: males have similar-sized urogenital opening and anus while female urogenital opening is often larger when compared to the anus (Culpepper and Allen, 2016b)). Two or three fish of the same sex were then placed in a tank. Each treatment (i.e., 2 or 3 weeks at spring conditions) was assigned to 10 tanks (5 female tanks and 5 male tanks). Forty-two fish were used during the experiment (21 fish per treatment).

Induced spawning protocol

Before hormone injection, fish were anesthetized (similar methods to previous), and weight was recorded (nearest 5 g). GnRHa (Pentair Aquatic Eco-Systems, Apopka,

FL) was administered via a priming dose (0.05 mL/kg; 10% of the manufacturer's recommended dose) followed by a resolving dose (0.45 mL/kg; 90% of manufacture's recommended dose) 24 hours later. The hormone was injected intramuscularly about 2 cm below the dorsal fin with a 25-gauge, 2.5 cm hypodermic needle, following Culpepper (2015). After injection, fish were returned to original tanks and revived.

Based on a previous study on latency period in White Crappie (Shirley and Allen; in review), starting at 34 hours post initial injection, tanks were monitored every hour for ovulating females. This was done by checking tank bottoms and individual females via gentle abdominal palpation for ovulated eggs. If eggs were easily released from the urogenital opening, the female was considered to be ovulating.

Once a female was determined to be ovulating, the time was recorded, and a male was selected from the same treatment. The female was sedated in an anesthetic bath (previously described), and the male was euthanized by longer exposure to the anesthetic bath. Testes were dissected from male crappie, placed in a small petri dish and weighed to the nearest 0.01 g. A milt sample (~ 100µL) was collected with a pipette and activated by adding a drop of water, and a subsample was assessed for motility with a compound microscope at 100X magnification. Once motility was confirmed, the testes were minced with a scalpel to obtain sperm.

When lethargic, the ovulating female was removed from the anesthetic bath and blotted dry with cloth towels to prevent eggs from water hardening. Eggs were stripped by applying a strong downward motion on the lower abdomen and collected in a small glass bowl (~300 mL). Next, eggs were transferred to a larger glass bowl (~ 1 L), and the

minced testes were squeezed through a small aquarium net to pass sperm but retain larger pieces of tissue, and the fertilization time was recorded.

The resulting egg and milt mixture was rinsed with 50 mL salt-urea solution (3 g urea/ L H_2O, 4 g NaCl/ L H_2O). This solution was gently stirred for two minutes with a turkey feather, followed by an additional 50 mL salt-urea solution and 5-minutes stirring. The solution was decanted to remove debris and rinsed 3 times with fresh well water. Excess water was then poured off, and the remaining egg solution was poured into a large graduated cylinder to obtain a post-fertilization volume of eggs. Eggs were added to a McDonald hatching jar containing 1g/L tannic acid, aerated with small air stones for 2 minutes to remove the adhesive layer on the eggs, and then rinsed with fresh well water. The hatching jar was then placed on a RAS until hatching was complete. The hatching RAS contained a UV sterilizer (80-watt; Smart High Output UV Sterilizer, Pentair Aquatic Eco-systems) and bag filters (FV1, Pentair Aquatic Eco-systems). Water temperature in the hatching system was maintained at 23 ± 1°C with a 1700-Watt heater (S1EBT1.7118A, Process Technology).

After eggs were stripped from the female, the fish was euthanized by a longer exposure time in an anesthetic bath. The ovaries were removed via dissection and weighed to calculate a gonadosomatic index (GSI). GSI was later calculated:

$$GSI = \frac{Gonad\ weight\ (g)}{Fish\ body\ weight\ (g)} * 100 \qquad \text{Equation 1}$$

A GSI for female fish before strip-spawning was also calculated. Gonad weight pre-ovulation of eggs was estimated with the equation:

Gonad weight pre-ovulation = gonad weight (g) + weight of ovulated eggs Equation 2

This pre-ovulation gonad weight was then used in Equation 1 to obtain a pre-ovulation GSI. The weight: volume ratio of ovulated eggs (1.02) was obtained from a previous study (Shirley and Allen, in review).

Fertilization percentages

To determine egg fertilization percentages, 3 samples of eggs (30 eggs/sample) were taken from each hatching jar 24 hours post-fertilization. Each sample of eggs was observed under a compound microscope at 25x magnification for fertilization. Egg fertilization was determined by the presence of an intact chorion and vitelline membrane following Culpepper and Allen (2016a). Fertilization percentages were calculated with the following equation:

$$Fertilization\ percentage = \left(\frac{mean\ number\ of\ fertilized\ eggs}{30}\right) * 100 \qquad \text{Equation 3}$$

Egg diameter

Whole ovaries were preserved in 10% neutrally buffered formalin and later used to obtain an average egg diameter. Ten eggs were collected from the left and right lobes and the base of the ovary, for 30 eggs from each ovary. Eggs were placed in a small petri dish, and egg diameters were measured with a stereo microscope at 20X magnification, with computer software (Motic Imaging 2.0; Motic, British Columbia, Canada). Only complete eggs with intact nuclei and vitelline membranes were measured.

Statistical analysis

The experiment was a completely randomized design, testing the effects of two different durations of time at which fish were held at spring conditions (i.e., 2 or 3 weeks). Latency period, fertilization percentage of eggs, percent of females ovulated, GSI, ovulated egg volume, and egg diameters were compared between treatment groups using Student's t-tests. Normality was assessed with the Shapiro-Wilk test, and homogeneity of variance was assessed using the Levene's test. Percentage data were logit transformed before analysis. All data were analyzed with SAS 9.4 (SAS Analytics Software & Solutions, Cary, NC). Significance was determined at $\alpha = 0.05$.

Results

Latency period

The mean latency period of female White Crappie was not different between 2- and 3-week spring treatments ($P = 0.79$; Table 2), indicating latency period is not affected by time female White Crappie are held at spring conditions.

Spawning success and fertilization percent

Twenty females were injected with GnRHa (2-week group n= 9; 3-week group n=11). The difference in females per treatment was because of difficulty in accurately assigning sex when visual cues had to be relied upon. All females strip-spawned in both the 2-week and the 3-week spring treatments ovulated viable eggs, as indicated by successful fertilization, indicating simulated spring condition time did not affect egg viability. However, two fish in the 3-week treatment released eggs into the tank; viability could not be confirmed due to water hardening.

Egg fertilization was lower in the 2-week spring treatment compared to the 3-week spring treatment (Table 2) (P=0.0306). This difference indicates holding White Crappie at spring conditions for 3-weeks may yield an increased egg fertilization percentage (65%) when compared to White Crappie held at the same conditions for 2-weeks (34%); an approximately 2-fold increase.

Ovulated egg volume

Ovulated egg volume was not significantly different between the two treatment groups (P=0.9103) (Table 2), indicating, ovulated egg volume was not affected by the time fish were held at spring conditions.

Gonadosomatic index

Females in the 2-week spring treatment had a similar mean GSI as females in the 3-week spring treatment (Table 2), and GSI did not significantly differ between the two treatments (P= 0.7631). A pre-ovulation GSI was also calculated for females, with the equation previously described, pre-ovulation GSI was not significantly different between the two treatments (P=0.5203) (Table 2).

Egg diameter

Mean egg diameter from ovaries of strip-spawned fish did not significantly differ between the 2 and 3-week spring conditions treatments (P= 0.9130) (Table 2), indicating the diameter of White Crappie eggs was not affected by time at final spring conditions.

Discussion

Previously, White Crappie intensive spawning has been restricted seasonally to the natural spring spawning season. However, recent advances including identifying an

effective ovulation inducing hormone (GnRHa) (Culpepper, 2015) and documenting a seasonal adjustment schedule for out-of-season spawning (Culpepper and Allen, 2016b; Allred et al, in review) allow for further exploring out-of-season spawning White Crappie. This study identifies the effects of holding White Crappie at two different final spring condition durations on measures of spawning success, to potentially increase out-of-season production of White Crappie. Importantly, results of this study indicate while the percent of females spawned, ovulated egg volume, GSI, and egg diameters are not different between fish held at spring conditions for 2 or 3 weeks, egg fertilization percent increases.

Out-of-season spawning protocols have been previously identified for other Centrarchidae species (i.e., Largemouth Bass and Bluegill) (Mischke et al. 1997; Matthews and Stout, 2013). The ability to successfully spawn these species out-of-season led to an interest in expanding intensive production of crappie throughout the year. Previous experiments have shown White Crappie can be intensively strip-spawned outside the normal spring, spawning season by manipulating water temperature and photoperiod and using ovulation inducing hormones (Culpepper and Allen, 2016b; Allred et al. in review). These experiments focused on the spring transition from winter conditions to final spawning conditions, not the time fish were held at spring conditions (i.e., 22° C and 16 hours light) (Culpepper and Allen 2016b; Allred et al. in review). However, Allred et al. (in review) held fish at spring conditions for about two weeks (15 days) while Culpepper and Allen (2016b) held fish at spring conditions for about one week (5 days). Allred et al. (in review) had 74% of females ovulate, with an average of 11.5 mL eggs ovulated per female, with an average fertilization rate of 65%, whereas

Culpepper and Allen (2016b) had 13% of females ovulate, with an average of 2.4 mL eggs ovulated per female, with an average fertilization rate of 41%. The increase in these spawning success variables may be from extending spring conditions (22°C and 16 hours light) by Allred et al. (in review). A study on a closely related fish, Bluegill, has shown a reproductive success was highest during the middle of the spawning season, or when fish had been at spring conditions for a longer time (Cargnelli and Neff 2006). Also, other out-of-season spawning experiments have successfully spawned Bluegill by using a final spring 2 weeks or longer (Mischke and Morris 1997). In the present study, while most measures of White Crappie spawning success were not different, egg fertilization was higher for fish held at spring conditions for 3 weeks (65%) when compared to the egg fertilization of fish held at spring conditions for 2 weeks (34%) (Table 2). This increase in fertilization percentage suggests holding White Crappie at final spring conditions can increase egg fertilization, possibly from increased egg quality.

Egg quality can be defined by the ability of the egg to be fertilized and then develop into an embryo (Bobe and Labbé 2010). Factors including temperature, photoperiod, and broodstock nutrition are known to affect gamete quality of both males and females (Bobe and Labbe, 2010); however, fish in the present experiment were all fed to satiation and temperature and photoperiod were adjusted at the same rates for all fish. The only difference was the duration (2-weeks or 3-weeks) fish spent at the final spring conditions before being strip-spawned. The consumption of limited amounts of prey before spawning has been shown to limit the reproduction success of female White Crappie (Bunnell et al. 2007). Thus, the additional week of feeding (3 weeks at spring

conditions) may have led to the increased fertilization rate, from improved gamete development (Izquierdo et al. 2001).

To examine whether egg diameter and possibly nutrient deposition may be affected by duration at spring conditions, egg diameters were examined but did not differ between treatments. In comparison with diameters of mature White Crappie eggs from wild populations, eggs were of similar size. Previous studies define crappie eggs as mature when egg diameters were > 0.82 mm (Morgan 1954; Whiteside 1964), > 0.75 mm (Mathur et al. 1979), and > 0.58 (Thomas and Kilambi 1981). Egg diameters obtained from White Crappie ovaries in this study were on average 0.81 mm. The gonadosomatic index was also assessed as an additional measure of reproductive readiness of fish to spawn. Female pre-ovulation GSI (GSI calculated for ovaries before being strip-spawned) was lower (5.7%, 2-week treatment and 5.0%, 3-week treatment) (Table 2) than the reported pre-ovulation GSI of wild White Crappie (i.e., pre-ovulation GSI) (~8.0 %) (Thomas and Kilami, 1981; Shirley and Allen, in review) indicating while White Crappie can be induced to spawn by simulating natural water temperature changes, complete gonad maturation may not be achieved.

This study indicates out-of-season spawning of White Crappie is benefited by holding broodstock at final spring conditions for greater times (i.e., 3 weeks vs. 2 weeks), which increases egg fertilization. Manipulating natural cues (photoperiod and temperature) and using ovulation inducing hormones (GnRHa), can be used to induce ovulation, which may increase the number of fish available for stocking. Future studies would benefit from examining strategies for feeding larval fish in indoor tanks, as

transferring larval fish to fertilized ponds for grow-out is not a viable option during the winter.

Tables

Table 4.1. Mean (± standard error) daily water quality in experimental tanks.

Variable	Mean	±	SE
Dissolved Oxygen (mg/L)	9.33	±	0.01
Salinity (ppt)	3.5	±	0.1
pH	7.92	±	0.04
Total Ammonia Nitrogen	0.08	±	0.03
Unionized Ammonia	0.002	±	0.001
Nitrite	0.03	±	0.01
Alkalinity	91.11	±	2.28

All variables were maintained at acceptable concentrations throughout experiment (Piper et al. 1982**).**

Table 4.2 Mean (± standard error) variables of spawning success for White Crappie (*Pomoxis annularis*) induced to spawn after 2- or 3- week simulated spring conditions.

	2-week spring conditions				3-week spring conditions			
Variable	**Mean**	**±**	**SE**	**n**	**Mean**	**±**	**SE**	**N**
Latency period (hours)	39.90	±	0.77	9	40.02	±	0.84	10
Post-fertilization egg volume (mL)	17.5	±	3.2	9	16.9	±	4.5	10
Gonadosomatic index pre-ovulation	5.69	±	0.70	9	5.04	±	0.66	10
Gonadosomatic index post-ovulation	3.93	±	0.42	9	3.72	±	0.51	10
Egg fertilization percent	34	±	8	9	65*	±	10	10
Egg diameter (mm)	0.82	±	0.01	9	0.81	±	0.02	9

An asterisk (*) indicates a significant difference between treatments (P<0.05).

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APPENDIX A

STRIP-SPAWNING PROTOCOLS FOR WHITE CRAPPIE

(*POMOXIS ANNULARIS*)

Strip-spawning protocols

1. Collect fish via electrofishing or trap nets before natural spawning occurring
2. Sex fish by cannulation using PE 50 tubing or by visual cues
    a. Urogenital opening size and shape: males have a similar-sized urogenital opening, and the female urogenital opening is often larger than the anus
3. Tank acclimate fish in RAS system at 15°C and 3-5 ppt salinity (Instant Ocean Sea Salt)
4. Two weeks before when a manager wants females to ovulate, adjust temperature 1°C/d for 6 days until reach final spawning water temperature (21°C), and hold fish at final spawning water temperature for 1 week
5. After 6 days at spring conditions, anesthetize fish in MS-222 bath
    a. 100mg/L tricaine methanesulfonate; 9g/L NaCl; and 400mg/L $NaHCO_3$
6. Inject fish (males and females) with initial priming ovaprim (GnRHa) dose (10% of total dose) at 4 p.m. 2 days before the day when a manager wants females to ovulate eggs
    a. Total hormone = $0.5\frac{ml}{kg}$
7. Inject fish (males and females) with resolving dose (90% of total dose) 24 hours after initial injection.
    ex: If ovulation desired on a Wednesday, give the initial injection at 4 p.m. on Monday, followed by resolving dose at 4 p.m. on Tuesday. Females should begin ovulating eggs around 6-8 a.m. Wednesday morning (38-52 hrs after priming dose)
8. Ovulation is determined if eggs flow readily by applying light pressure to the abdomen or if presence observed on the bottom of the tank. Do not force eggs; they will not fertilize if forced before ovulation.
9. Do not force eggs; best quality is when they are easily released.
10. Sedate female in MS-222 bath
    a. 100 mg/L tricaine methanesulfonate; 9 g/L NaCl; and 400 mg/L $NaHCO_3$
11. Euthanize a male by overdose in MS-222 bath
12. Remove testes and mince using a scalpel
    a. Obtain a small sample of sperm and confirm sperm motility under the microscope
        i. Activate sperm sample with ~ 1-2 mL water and observe for movement
13. Dry female thoroughly using cloth hand towels. Strip eggs into a bowl by applying strong, downward pressure on the lower abdomen
14. Squeeze fresh sperm through standard aquarium net onto eggs to remove large tissue pieces
15. Add 50 ml of salt-urea mix onto eggs and sperm and gently stir solution for 2 minutes with a turkey feather
    a. 11.34g Urea and 15.12g NaCl/ 1 liter well water
16. Add another 50 ml salt-urea to the solution and stir for additional 5 minutes

17. After 5 minutes, rinse eggs 2-3 times by slowing filling bowl with fresh water and decanting off water and floating debris until solution is free of floating debris. Eggs will be settled on the bottom under a clear solution
18. Pour solution into 50-100 ml graduated cylinder, allow to settle, and take a volumetric measurement of eggs
19. Place eggs in an aerated McDonald hatching jar contained 3 liters well water and 1 gram/l tannic acid (3 grams total) for 2 minutes to remove an adhesive layer from eggs
20. Rinse hatching jar with fresh water until the solution is clear in color (2-3 times)
21. Place hatching jar on an incubation table and supply with a slow, continuous flow of aerated water at the desired temperature (22-24°C) until hatching is complete
22. Check for fertilization percent 24 hours after eggs were fertilized
    a. Egg fertilization is determined by the presence of an intact chorion and vitelline membrane

Table A.1 Materials needed for spawning

Towels	Petri dishes	Scalpel	Forceps	Balance	Spatula	Gallon container	Graduated Cylinders	PE 50 tubing
Pour container	Timers	Turkey baster	Flashlights	Small funnels	Turkey feathers	Bowls	Syringes 1 cc	Fillet knife
Needles 25 ga	Ms-222	NaCl	Ovaprim	NaHCO3	Tannic acid	Slides & cover slips lined & regular	Eye droppers	Nets

APPENDIX B

FERTILIZING WHITE CRAPPIE (*POMOXIS ANNULARIS*) EGGS WITH BLACK-STRIPE BLACK CRAPPIE (*POMOXIS NIGROMACULATUS*) CRYOPRESERVED SPERM

Fertilizing eggs with cryopreserved sperm protocols

1. Collect female fish via electrofishing or trap nets before natural spawning occurring
2. Tank acclimate fish in RAS system at 15°C and 3-5 ppt salinity
3. (Instant Ocean Sea Salt)
4. Two weeks before when a manager wants females to ovulate adjust temperature 1°C/d for 6 days until the system reaches final spawning water temperature (21°C), and hold fish at final spawning water temperature for 1 week
5. Obtain cryopreserved sperm and store in liquid nitrogen
    a. 5% DMSO is the desired cryoprotectant
6. After 6 days at spring conditions, anesthetize fish in the MS-222 bath
    a. 100mg/L tricaine methanesulfonate; 9g/L NaCl; and 400mg/L $NaHCO_3$
7. Inject fish (females) with initial ovaprim (GnRHa) priming dose (10% of the total dose) at 4 p.m. 2 days before the day when a manager wants females to ovulate eggs
    a. Total hormone = $0.5\frac{ml}{kg}$
8. Inject fish (females) with resolving dose (90% of total dose) 24 hours after initial injection.
    ex: If ovulation desired on a Wednesday, give the initial injection at 4 p.m. on Monday, followed by resolving dose at 4 p.m. on Tuesday. Females should begin ovulating eggs around 6-8 a.m. Wednesday morning (38-52 hours after priming dose)
9. Ovulation is determined if eggs flow readily by applying light pressure to the abdomen or if presence observed on the bottom of the tank. Do not force eggs; they will not fertilize if forced before ovulation.
10. Sedate female in the MS-222 bath
    a. 100 mg/L tricaine methanesulfonate; 9 g/L NaCl; and 400 mg/L $NaHCO_3$
11. Dry female thoroughly using dry hand towels. Strip eggs into a bowl by applying strong, downward pressure on the lower abdomen
12. Thaw 2 straws of cryopreserved sperm in a 40°C water bath for 8 seconds
    a. Further experiments needed to determine optimal sperm to egg ratio
13. Wipe straws dry, cut ends off using scissors and drain sperm directly onto eggs
14. Add <50 ml of fresh water (only add enough just to cover eggs) to the solution, stir, and allow to sit for 10 minutes
15. After 5 minutes, rinse eggs 2-3 times by slowing filling bowl with fresh water and decanting off water and floating debris until solution is free of floating debris. Eggs will be settled on the bottom under a clear solution
16. Pour solution into 50-100 ml graduated cylinder, allow to settle, and take a volumetric measurement of eggs
17. Place eggs in an aerated McDonald hatching jar contained 3 liters well water and 1 gram/l tannic acid (3 grams total) for 2 minutes to remove adhesive layer from eggs
18. Rinse hatching jar with fresh water until the solution is clear in color (2-3 times)

19. Place hatching jar on an incubation table and supply with a slow, continuous flow of aerated water at the desired temperature (22-24°C) until hatching is complete
20. Check for fertilization percent 24 hours after eggs were fertilized
    a. Egg fertilization is determined by the presence of an intact chorion and vitelline membrane

Table B.1 Materials needed for spawning

Towels	Petri dishes	Cryopreserved sperm (5% DMSO)	Forceps	Balance	Spatula	Gallon container	Graduated Cylinders	PE 50 tubing
Pour container	Timers	Turkey baster	Flashlights	Small funnels	Turkey feathers	Bowls	Syringes 1 cc	Fillet knife
Needles 25 ga	Ms-222	NaCl	Ovaprim	NaHCO3	Liquid nitrogen	Slides Lined & regular	Tannic acid	Dewar

APPENDIX C

OUT-OF-SEASON WHITE CRAPPIE (*POMOXIS ANNULARIS*) COLLECTION AND STRIP-SPAWNING PROTOCOL

Out-of-season strip spawning protocol

1. Collect fish via boat electrofishing or trap nets when water temperatures cool down (i.e. < 15° C) November and December
2. Tank acclimate fish in RAS system at <15°C and 3-5 ppt salinity (Instant Ocean Sea Salt) for 1-2 weeks
3. Simulate winter temperature (10°C) and photoperiod (8 hours light) for 2 weeks
4. Transition to final spring conditions (22°C and 16 hours light) by increasing temperature by 1°C and photoperiod 1 hour every 4 days
5. Sex fish by cannulation using PE 50 tubing or by visual cues
    a. Urogentital opening size and shape: males have similar-sized urogenital opening and anus while female urogenital opening is larger when compared to the anus
6. Hold fish at spring conditions (22°C and 16 hours light) for 3 weeks
7. After 3 weeks at spring conditions, anesthetize fish in the MS-222 bath
    a. 100mg/L tricaine methanesulfonate; 9g/L NaCl; and 400mg/L $NaHCO_3$
8. Inject fish (males and females) with initial priming ovaprim (GnRHa) dose (10% of total dose) at 4 p.m. 2 days before the day when a manager wants females to ovulate eggs
    a. Total hormone = $0.5\frac{ml}{kg}$
9. Anesthetize and inject fish (males and females) with resolving dose (90% of total dose) 24 hours after initial injection.
    ex: If ovulation desired on a Wednesday, give the initial injection at 4 p.m. on Monday, followed by resolving dose at 4 p.m. on Tuesday. Females should begin ovulating eggs around 6-8 a.m. Wednesday morning (38 – 52 hrs after priming dose)
10. Ovulation is determined if eggs flow readily by applying light pressure to the abdomen or if presence observed on the bottom of the tank. Do not force eggs; they will not fertilize if forced before ovulation.
11. Sedate female in the MS-222 bath
12. Euthanize a male by overdose in the MS-222 bath
13. Remove testes and mince using a scalpel
    a. Obtain a small sample of sperm and confirm sperm motility under the microscope
        i. Activate with ~1-2 mL water and observe for movement
14. Dry female thoroughly using dry hand towels. Strip eggs into a bowl by applying strong, downward pressure on the lower abdomen
15. Squeeze fresh sperm through standard aquarium net onto eggs
16. Put 50 ml of salt-urea mix onto eggs and sperm and gently stir solution for 2 minutes with a turkey feather
    a. 11.34g Urea and 15.12g NaCl/ 1 liter well water
17. Add another 50 ml salt-urea to the solution and stir for additional 5 minutes
18. After 5 minutes, rinse eggs 2-3 times by slowing filling bowl with fresh water and decanting off water and floating debris until solution is free of floating debris. Eggs will be settled on the bottom under a clear solution

19. Pour solution into 50-100 ml graduated cylinder, allow to settle, and take a volumetric measurement of eggs
20. Place eggs in an aerated McDonald hatching jar contained 3 liter well water and 1 gram/l tannic acid (3 grams total or 3 liters) for 2 minutes to remove adhesive layer from eggs
21. Rinse hatching jar with fresh water until the solution is clear in color (2-3 times)
22. Place hatching jar on an incubation table and supply with a slow, continuous flow of aerated water at the desired temperature (22-24°C) until hatching is complete
23. Check for fertilization percent 24 hours after eggs were fertilized
    a. Egg fertilization is determined by the presence of an intact chorion and vitelline membrane

Table C.1 Materials needed for spawning

Towels	Petri dishes	Scalpel	Forceps	Balance	Spatula	Gallon container	Graduated Cylinders	PE 50 tubing
Pour container	Timers	Turkey baster	Flashlights	Small funnels	Turkey feathers	Bowls	Syringes 1 cc	Fillet knife
Needles 25 ga	Ms-222	NaCl	Ovaprim	NaHCO3	Tannic acid	Slides & cover slips lined & regular	Eye droppers	Nets