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Development of White Crappie Pomoxis Annularis Reproduction Methods in Closed Aquaculture Systems

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Development of white crappie *Pomoxis annularis* reproduction methods in closed aquaculture systems

By

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Aquaculture methods are limited for white crappie (*Pomoxis annularis*), reducing production potential. Therefore, reproduction methods, including induced spawning, sperm cryopreservation and out-of-season spawning, were developed in tank systems. A two week acclimation period (15°C; 3-5 ppt salinity) was necessary to reduce disease-related mortality. Afterwards, four spawning induction hormones and a control were examined to induce spawning. Luteinizing hormone releasing hormone analogue and salmonid gonadotropin releasing hormone analogue performed the best in terms of spawning success and %-fertilization. Sperm cryopreservation was effective using Hanks or Ca^{2+}-free Hanks balanced salt solutions with 10%-methanol or 5%-dimethyl-sulfoxide as a cryoprotectant, frozen at 40°C/min. Out-of-season spawning experiments manipulated photoperiod and temperature over 3-wk (9% spawning success; 11% fertilization) and 6-wk (16% spawning success; 55% fertilization) seasonal shifts. Post-experiment maturation data indicate that females were in an intermediate development stage. These experiments demonstrate the potential of advanced spawning techniques to improve annual production of white crappie.

DEDICATION

I would like to dedicate this research to my wife, Anna Culpepper, for her continuous support and encouragement throughout this project.

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CHAPTER I

AQUACULTURE TECHNIQUES FOR THE CULTURE OF CRAPPIE POMOXIS SPP

Abstract

Extensive crappie *Pomoxis* spp. culture has been practiced for decades, however, knowledge of crappie aquaculture methods is limited. The following review synthesizes existing research on crappie aquaculture and identifies knowledge gaps where further research is needed. Topics such as: life history, tank culture, feeding, reproduction and spawning, larval rearing, transport and harvest, triploidy, hybridization, out-of-season spawning, and sperm cryopreservation were reviewed. The outcome is a better understanding of hindrances preventing crappie aquaculture development in the past, particularly tank culture and induced spawning techniques, and specific research objectives with potential to enhance recreational and commercial production.

Introduction

Crappie *Pomoxis* spp. are preferred game species throughout North America, and as a result their sport-fisheries are highly utilized (Mitzner 1984; Busack and Baldwin 1988; Colvin 1991; McDonough and Buchanan 1991). Within some regions of the southeastern United States, crappie fisheries may provide up to 80% of total annual fish harvest (Miranda et al. 2013). Predominantly located in reservoirs, crappie fisheries provide a substantial source of income to local, regional and state economies throughout the southeastern US (Dorr, Munn and Meals 2002). The US Fish and Wildlife Service

reports in 2011, 6.1 million US residents fished for crappie, representing 23% of all freshwater recreational fishing in the United States (USFWS 2011). Despite crappie fisheries popularity and socioeconomic value, crappie population management has provided a difficult challenge to fisheries biologists for decades. Challenges identified as hindering crappie fisheries management include small impoundment overpopulation (< 20 ha)(Busack and Baldwin 1988; Mitzner 1991) and quasi-cyclic population fluctuations, with a dominating single year-class produced every 2-5 yr and numerous stunted year-classes occurring in between (reviewed by Boxrucker and Irwin 2002).

Crappie aquaculture has been practiced for decades by state hatcheries and commercial farms, primarily to address problems with fisheries management of cyclic populations through supplemental stocking and private pond stocking. Annual production potential has been limited by a lack of research on aquaculture techniques for crappie. A majority of current crappie production is restricted to extensive pond culture, where broodstock spawn in ponds and larvae are reared using algae and zooplankton blooms. Recent research has focused on larval and juvenile culture methods, although methods for adult tank culture and feeding have not been investigated. Research has demonstrated juvenile crappie can be cultured in high-density tank systems (Willis and Flickinger 1981; Parsons 1999; Dudenhoeffer et al. 2014) and feed trained in both tanks and raceways (Smeltzer and Flickinger 1991; Thomas 1995; Hayward and Arnold 1996; Parsons 1999; Dudenhoeffer et al. 2014). Reproductive research has focused primarily on the production of infertile, monosex or triploid crappie for stocking in small water bodies (Al-ablani and Phelps 1997; Parsons 1999; Arslan and Phelps 2004 and Cuevas-Uribe et al. 2009). Several publications indicate crappie can be induced to spawn using luteinizing

hormone releasing hormone analogue (LHRHa) and human chorionic gonadotropin (HCG) either volitionally in aquaria (Al-ablani and Phelps 1997; Arslan and Phelps 2004; Gomelsky et al. 2005; Cuevas-Uribe et al. 2009) or by strip spawning (Gomelsky et al. 2000 and 2002), although these studies did not focus on spawning methods nor perform comparative experiments to determine the preferred hormone for ovulation induction.

Advanced spawning and culture methods, such as adult tank culture, artificial spawning and out-of-season spawning for several other popular centrarchid species (largemouth bass, *Micropterus salmoides*; bluegill, *Lepomis macrochirus*) have been more intensively researched (Morris and Clayton 2009). Research advances in these species have facilitated culturing adults indoors in recirculating systems allowing for year-round production (Willis and Flickinger 1981; Mischke and Morris 1997), and in-tank spawning method development (Banner and Hyatt 1975; Bryan et al. 1994). Reproductive timing has been manipulated to spawn fish out-of-season (Mischke and Morris 1997; Matthews and Stout 2013) providing greater flexibility in culture. These advances demonstrate the production potential of centrarchids through aquaculture, yet research in these same production methods with crappie is limited (Smeltzer and Flickinger 1991; Morris and Clayton 2009).

Investigations into the early life history requirements and intensive culture methods of crappie are necessary for crappie aquaculture advancement. Therefore, this literature review was conducted to synthesize the extent of existing research in crappie aquaculture, determine current crappie production rates and methods used throughout the U.S. by private and public facilities, present related research on aquaculture of other centrarchid species, and provide a necessary foundation for future research objectives.

Topics, such as: life history, tank culture, reproduction and spawning, larval rearing, transport and harvest, triploidy, hybridization, out-of-season spawning, feeding and sperm cryopreservation were reviewed. The outcome is a better understanding of the hindrances preventing crappie aquaculture development in the past, and identifying specific research objectives with potential to enhance current production.

Life History

White (*P. annularis*) and black crappie (*P. nigromaculatus*) (other common names: papermouth; speckled perch; bachelor perch; sac-a-lait) are in the Centrarchidae family of fishes. Centrarchids are ubiquitous throughout North American waters, lakes and river systems to marine environments (Kerns et al. 2005). The adaptability of these fishes to a broad range of environmental conditions and habitat niches make them unique among fish families around the globe (Kieffer and Cooke 2009). The natural geographic range of crappie extends from southern Texas and Alabama, north to the southern Great Lakes and west into Nebraska (Kerns et al 2005). Their average life span is 7-9 yr, with sexual maturation occurring from 1-3 yr , depending on varying geographic regions, temperature regimes, nutritional condition and habitat suitability (Edwards et al. 1982a; Wallace and Simmon 2008).

Average total length (TL) of a mature crappie is 19-22 cm, with little sexual dimorphism outside of breeding season conditions (Edwards et al. 1982b). Physical characteristics of white crappie include: a laterally compressed body; silver-green coloration with 5-10 vertical-black bars, often faint; 6 dorsal rays; anal, caudal and dorsal fins with black spots; and a large upper-jaw, which extends back towards the middle of the eye (Edwards et al. 1982b). The black crappie, which commonly co-inhabits

environments with white crappie and shares very similar physiology, morphology and ecological traits, can be distinguished by an absence of vertical bars, 7-8 dorsal rays, and darker silver sides with black speckles or blotches (Edwards et. al 1982b; Kerns et al. 2005).

While the physiological tolerances of several centrarchid species have been documented (e.g. largemouth bass and bluegill), there is relatively little information available on crappie (Kieffer and Cooke 2009). Beitinger et al. (2000) found the critical thermal maxima of white crappie to be 32.5° C, while the critical thermal maxima of black crappie was 35° C. Optimum performance and growth occurs at 23.5° C for white crappie, while black crappie optimal temperature is from 28-30° C (Keiffer and Cooke 2009). Although little information exists, the physiological tolerances of crappie can be inferred to be generally similar to that of other centrarchids. Crappie are endemic to nearly the same geographic regions of North America as largemouth bass and share similar physical characteristics, ecology, nutritional requirements and habitat use (Edwards et al 1982a,b; Struber et. al 1982).

As predominantly open-water species, the habitat preference of white and black crappie are similar. Both crappie species are most abundant in lakes and reservoirs > 2 ha, as well as the low-velocity backwater habitats in many southeastern U.S. rivers (Edwards et al 1982a,b; Pflieger 1997). Adults and juveniles generally maintain a depth of 5 m, utilizing submerged woody debris, boulders and aquatic vegetation as protective cover from predation and for foraging (Edwards et al 1982a,b). The only documented environmental preference differing between white and black crappie is turbidity. White crappie prefer a more turbid environment with total-dissolved-solids from 100-350 ppm,

such as tributaries of reservoirs and lakes and backwater river sections (Edwards et al. 1982b). Black crappie prefer clear water, and are found in shallow areas within large rivers, lakes and reservoirs (Edwards et al. 1982a).

Wallace and Simon (2008) reported fry remain in covered littoral habitat until reaching a minimum size of 8-10 mm TL, with most juveniles moving into open water at 50-60 mm TL. Crappie fry subsist as planktivore/insectivores until reaching a large enough size to consume small fish. Gut content analysis by Dubuc and DeVries (2002) found larval crappie from several Alabama reservoirs (< 10 mm) exhibited positive selection for small zooplankton (< 0.25 mm), with crustacean zooplankton dominating their diets by weight and rotifers by numerical counts. Juvenile and adult white crappie are nearly exclusively piscivorous, with fathead minnows (*Pimephales promelas*) and various shad species (*Alosa* spp.) being their primary food source in large lakes and reservoirs (Edwards et al. 1982 a, b; O'Brian et al. 1984; Kerns et al. 2005).

Spawning begins in March in the southern U.S. when water temperature reaches 13-14° C, with peak spawning occurring at 16-20° C (Siefert 1968). According to Sammons et al. (2001), during the spawning season, white crappie and black crappie utilize ephemeral, littoral-habitats containing abundant inundated vegetation, sand, clay or gravel substrates, low turbidity (< 50 nephelometric turbidity units) and low water velocity. As a nest-brooding species, males construct and guard nests, 12-30 cm in diameter and at an average depth of 0.42 m, within colony sites generally containing dozens of nests. Females lay 5,000-30,000 eggs during a single spawn. Mean incubation is 4-6 d, generally taking place from March through May in the southeast with peak hatching occurring when water temperatures reach 20-22° C. In an indoor hatchery

setting at 18-21° C, hatching has been observed approximately 72hrs post-spawning (Parsons 1999).

Fisheries Management Challenges

Seasonal recruitment is controlled by numerous environmental and biological variables, which are assumed responsible for inconsistent crappie recruitment dynamics and population fluctuations (Allen and Miranda 1998; 2001). Variables such as the extent of shore-line development, fluctuating winter pool levels, spring zooplankton biomass, and spring water temperatures have an influence on spawn timing and subsequent larval crappie growth and survival (Guy and Willis 1995; Maceina and Stimpert 1998; Pine and Allen 2001; Dubuc and DeVries 2002). However, the interaction among these variables and their influence on crappie year-class recruitment remains unclear, making appropriate and accurate management decisions difficult.

Fisheries management strategies to control recruitment variability have included: creel limit manipulation, changing angling regulations (e.g., permitting night fishing; lowering length limits), prescribed poisoning, increasing forage fish populations and control via increasing largemouth bass predation pressure (Busack and Baldwin 1988; Allen and Miranda 1998 & 2001; Boxrucker and Irwin 2002). Inconclusive results have been reported from these management strategies, with research being too limited in scale and duration to draw conclusions that can be applied at the reservoir-level spatial scale (> 80.9 ha)(Dubuc and DeVries 2002). It is also important to note these management strategies are not applicable to water bodies < 2 ha, as crappie quickly overpopulate smaller aquatic systems (Busack and Baldwin 1988; Guy and Willis 1995). Alternative strategies may be beneficial for maintaining crappie populations.

Crappie Aquaculture

There is a relative paucity of information on the culture of crappie species (Myers and Rowe III 2001; Morris and Clayton 2009). While limited published research on specific culture methods exists, crappie have been a documented culture species since at least 1908 (Leary 1909) and are currently being propagated in the U.S. by public and private aquaculture facilities. As a part of this review, to determine current production practices and mean annual production rates, a telephone survey of U.S. state hatcheries was conducted. Crappie are produced annually by 10 of the 48 state fisheries agencies within the continental U.S., averaging 3,238,000 black crappie and 745,000 white crappie per year (Table 1). Mississippi is currently the only state actively producing and stocking triploid-hybrid crappie (100,000/year), although several states, including Tennessee and Alabama, have produced triploid-hybrid crappie in the past (Pers. Comm.: David Roddy, Tennessee Wildlife Resources Agency; Brian Rinehard, Alabama Dept. of Conservation and Natural Resources; 2015).

According to the United States Department of Agriculture (USDA) Census of Aquaculture (2013), 3,140,094 crappie were commercially produced in the U.S. ($558,725 total annual sales) (Table 2). Production has occurred in 25 states, with most operations located in mid-western and southern states (USDA 2005) (Table 3). Personnel communication with public and private industry professionals indicate two methods for spawning crappie are currently used by aquaculturists. For state hatcheries, mature wild broodstock are collected from local lakes or reservoirs via electroshocking during the spring spawning season (March-May in the southeastern U.S.), while commercial farms maintain adult crappie year-round in low density broodstock ponds (Malone and Son Fish

Farm, Lonoke, AR; Dunn's Fish Farm of Oklahoma, Fitstown, OK). The most common culture method uses natural spawning in ponds and fry collection by seining (Smeltzer and Flickinger 1991; Myers and Rowe III 2001; Bunnell et al. 2007). In the second method, adults are strip spawned, with the embryos incubated and hatched in flow-through systems (Busack and Baldwin 1988; Parsons 1992, 1996 and 1999), however using strip-spawning is limited in both public and private production to only a few producers (i.e., North Mississippi Fish Hatchery, Enid, MS; Malone and Son Fish Farm, Lonoke, AR). These methods will be described in greater detail below.

Pond Culture

Crappie culture primarily occurs in ponds, with efforts focused on the fry-fingerling grow-out production phase, (0-76 mm TL)(Busack and Baldwin 1988; Smeltzer and Flickinger 1991; Myers and Rowe III 2001). While crappie pond culture is practiced routinely, optimal variables for fish production (fry stocking density, size at harvest, broodstock density and feed types and rates) are not established and vary greatly depending on the source. Several experiments have been conducted on rearing post-larval stage crappie (> 76 mm TL) to a larger-size class (> 200 mm) in ponds before stocking into larger water bodies (Smeltzer and Flickinger 1991; Bunnell et al 2007). However, most hatchery practices do not attempt to grow-out crappie beyond the post-larval stage due to factors associated with cannibalism, refusal of formulated feed at sizes > 200 mm and problems associated with handling, harvest and transport (Smeltzer and Flickinger 1991; Parsons 1996; Gomelsky et al. 2000).

Crappie have a dietary shift between the fingerling to juvenile life stages, changing from planktivores to piscivores. This transition makes it impractical for

culturists to provide a live food source in a large-scale production situation. Black crappie have been reared to the juvenile stage in ponds (TL of 105 ± 41 mm) with an average pond yield of 52,681 fish/ha on a zooplankton diet, using a fertilization regime (ammonium nitrate (NH_4NO_3): 34N-0P-0K and triple super phosphate ($Ca(H_2PO_4)_2$); 0N-46P-0K) to provide a food source (Myers and Rowe III 2001). No studies for pond rearing juvenile to adult crappie have been documented.

Brood fish stocking densities and sex ratios in pond spawning experiments range greatly, depending on the study. The greatest spawning success and highest fingerling yield occurs at male-female sex ratios of 5:1 and 10:1 and a stocking density from 247-371 adults/ha (Myers and Rowe III 2001). For pond-reared female white crappie, Bunnell et al. (2007) found gonadal development, individual spawning success and egg size/development were influenced by environmental and physiological factors such as: nutrition, feeding regimes, condition, water quality, and physical stressors (i.e., harvest and transport). Private U.S. aquaculture farms reported holding and spawning adult black crappie in extensive pond systems at densities ranging from 14-125 adults/ha, but all experienced highly variable production success (Pers. Comm.: Owen and Williams Fish Farm, Hawkinsville, GA; Dunn's Fish Farm, Fittstown, OK; Schultz Fish Hatchery, Lake Ariel, PA, Shangaloo Fisheries, Inc., Hampton, FL).

Harvest and Transportation

Harvest and transport methods can be a significant source of mortality, although maintaining cool water temperatures (< 16° C) and using salt appears to improve survival during transport. Smeltzer and Flickinger (1991), for example, experienced juvenile (25-35 mm TL) crappie harvest mortality from 70-94% when water temperatures were > 15°

C. Myers and Rowe III (2001), however, experienced far lower mortality (< 5%) associated with handling when conducting nocturnal harvest (23:00-23:30 was ideal) of larger crappie (76-216 mm TL versus 25-35 mm) during January when water temperatures are lowest, however no specific temperature was documented. Similarly, Parsons (1999) found higher survival as body size increased. Besides temperature and body size, salt addition (i.e., 500 mg/L NaCl) to transport tanks has also been shown to reduce mortalities associated with harvest and transport (Parsons 1999; Bunnell et al. 2007).

Tank Rearing and Feed-Training

Hatchery research advances in crappie aquaculture over the past few decades indicate crappie can be cultured in high-density indoor systems (Willis and Flickinger 1981; Parsons 1999; Dudenhoeffer et al. 2014). However, much of the research has focused on production of infertile, monosex or triploid crappie for stocking in small water bodies (Al-ablani and Phelps 1997; Parsons 1999; Arslan and Phelps 2004 and Cuevas-Uribe et al. 2009) and feed training larval crappie in tanks and raceways (Smeltzer and Flickinger 1991; Thomas 1995; Hayward and Arnold 1996; Parsons 1999; Dudenhoeffer et al. 2014). Diet, tank size, flow rate, feed suspension time and fish size appear to be important factors for successful feed training.

Parsons (1999) conducted feed experiments on fry-sized crappie (4-53 mm TL) in flow-through systems (tank volume not documented) to determine the viability of rearing fry in tanks to obtain a larger size before pond stocking but reported only marginal success, with most feed regimes failing to be accepted and 100% mortality in most tanks. The following food sources were examined, singularly and in various combinations, but

rejected by crappie fry: *Artemia* nauplii, hatchfry encapsulon larval feed at 30, 50 and 100 µm (Argent Labs, Redmond, Washington), wild caught plankton, green water culture (bloom of phytoplankton and zooplankton in the tank), rotifers, and pond water culture (tank supplied with flow-through water from a nearby pond). The only feed regime that had marginal success used natural pond water in a flow-through design with a 300-L holding tank. The pond water was fertilized with cottonseed meal to create a dense zooplankton bloom and supplemented by finely ground, commercially prepared striped bass (*Morone saxatillis*) feed. Fish grew from 4.0 mm to 52.9 mm over 5 mo, but growth rates drastically declined after the first 2 mo of culture, suggesting a dietary shift occurring at this size/age of crappie. Gut content examination revealed otoliths in numerous fish, indicating cannibalism occurred. Parsons (1999) concluded although the feed experiment was successful in growing out crappie fry, mortality was unacceptably high, however, no exact number was reported.

Busack and Balwin (1988) also mentioned unsuccessful fry feeding trials on a combination of the following feeds following induced triploidy experiments: infusoria mixture (composed of protists, protozoa and various other unicellular algae), copepods *Cyclops* sp. and *Daphnia* spp. Arslan and Phelps (2004) had limited success weaning crappie fry to a commercial dry diet (Aquamax Trout Chow #0, 50% crude, Purina Mills, Minneapolis, MN) starting at 45 daysposthatch, after 5 d where fry were offered live brine shrimp several times per day. No survival rate was documented. While these experiments had marginal success feeding crappie in tanks, fry grow-out has not been achieved without high mortality rates.

Recently, Dudenhoeffer et al. (2014) successfully reared crappie on commercial feeds in recirculating aquaculture systems and provided information on juvenile crappie artificial feed training. The effects of tank density (0.81, 1.62, 3.25 and 6.50 g/L), feed type (type 1: 2 mm, slow-sinking rainbow trout *Oncorhynchus mykiss* feed with 45% minimum protein; type 2: 1.2 mm, sinking with 50% minimum protein), feeding interval (12- or 24-hr belt feeders), tank cover (with or without) and bottom color (lightblue or black) were experimentally tested on both white and black crappie. Results indicated no difference between species performance of larval crappie in final weight, feed conversion ratio (FCR) and final biomass. Type 2 feed, with a higher protein content and smaller size yielded the best final biomass, weight gain, specific growth rate (SGR) and final mean body weight. The difference in feed performance was attributed to a smaller feed size and higher protein content. No difference was found between lightblue tanks with or without a cover; however, black tank bottoms had the lowest weight gain and consumption levels. There was no difference in survival between tank densities. Also, no difference in growth performance relative to tank densities was observed until > 3.25 g/L, where final body weight and SGR decreased significantly, indicating a tank density from 1.62-3.25 g/L is optimal. FCR was higher using 24-hr belt feeders, however no other growth differences were observed between the two feed intervals tested. Dudenhoeffer et al. (2014) demonstrated both white and black crappie juveniles (< 35 g) can be trained to consume formulated diets in tank systems with comparable growth performance to bluegill. However, these experiments did not investigate the feeding and culture requirements of larger crappie.

For feed-training juvenile crappie, Smeltzer and Flickinger (1991) concluded a carp (*Cyprinus carpio*) egg to commercial diet (Biodiet, Bio-Oregon®, Oregon, U.S.) feeding regime had the best feed trainability success with 90.3% acceptance by 25-35 mm black crappie. Tank size appeared to be a factor as well, as they found 102-L rectangular tanks had a 20% increase in feed acceptance compared to 15-L buckets (69.5% acceptance). Trainability was greatly reduced in the larger crappie, as larger, 45-65 mm, black crappie had a 45% feed acceptance in 15-L buckets. The authors speculated extended feed suspension in the 102-L rectangular tanks could be the cause of increased feed acceptance. Long-term food acceptance of feed-trained 25-35 mm crappie as they grew to larger sizes was not reported. Therefore, it can be inferred a larger tank with extended feed suspension would have the greatest chance of success for training juvenile crappie to accept formulated diets, especially as they continue to grow.

Few attempts to feed-train adult crappie have been documented. Notably, adult black crappie have been successfully feed-trained in large recirculating aquaria (tank volume = 567,750 L) starting with fresh, chopped smelt (*Osmeridae* spp.) and eventually weaning the adults to a diet of mixed, frozen smelt, cod (*Gadus morhua*), whiting (*Merluccius bilinearis*) and haddock (*Melanogrammus aeglefinus*)(Rob Mottice, Manager, Tennessee Aquarium, personal communication). Adult black crappie have been held successfully at low density (~0.0015 fish/m^3) in 0.4-ha ponds until needed for spring strip spawning, by providing large numbers of live koi (*Cyprinus carpio*), approximately 5 cm TL (Charles Silkwood, Mississippi Dept. of Wildlife, Fisheries and Parks, personal communication).

Induced Spawning

Using hormones to control spawning in finfish has been an established practice in the aquaculture industry for decades (Stickney 2009). Reproduction in fish in aquaculture is controlled solely or by a combination of exogenous and endogenous factors. Exogenous factors are controlled by providing an environment mimicking natural spawning conditions, whereas endogenous factors are manipulated by artificial regulation of endocrine pathways controlling gonadal development and spawning. Temperature and photoperiod are the primary environmental cues used by centrarchids for reproductive development, while water quality, water velocity, moon phases, spawning substrate, behavioral cues and weather cycles are thought to play secondary roles (Morris and Clayton 2009; Mylonas et al. 2010). Because it is often infeasible to replicate spawning environments for finfish, direct control of the internal endocrine pathways mediating spawning has become the primary technique used by aquaculturists to produce consistent spawning results (Ram and Gupta 2011).

The brain-hypothalamus-pituitary-gonad axis is the internal endocrine pathway regulating reproduction in fish (Ram and Gupta 2011; Mylonas et al. 2010). The brain receives environmental stimuli and routes reproductively relevant information to the hypothalamus. Should the environmental stimuli be within the reproductive variables of the fish species, the hypothalamus produces gonadotropin-releasing hormone (GnRH). In non-reproductive conditions the hypothalamus produces gonadotropin-inhibiting hormones, primarily dopamine, to inhibit gonadal development and reproduction. GnRH stimulates the pituitary gland to release gonadotropin hormones (GtHs), which are prerequisites to ovulation. GtHs then act on the gonads to release steroids and

prostaglandins, which trigger two ovarian processes: egg maturation via the steroid progesterone and follicle rupture or ovulation, stimulated by prostaglandins. Spermiation in males is also controlled by the same GtH-steroid mechanism to induce the testes to produce sperm.

Aquaculturists have the ability to manipulate and control the brain-hypothalamus-pituitary-gonad axis through hormone promoting or inhibiting substances. Carp pituitary extract (CPE), the first commercially produced GtH created to induce ovulation in gravid female finfish, acts directly on the ovaries and testes to stimulate steroid/prostaglandin release and gonadal development, however no research was found on CPE induced spawning in crappie. An alternative to synthesized GtH, human chorionic gonadotropin (HCG), also mimics natural GtH and is commonly used as a gonadal stimulant in spawning white bass, striped bass, red drum and catfish (Porter 1997).

While HCG and CPE bypass the brain-pituitary link to act directly on the gonad, luteinizing hormone-release hormone (LHRH) acts directly on the hypothalamus to stimulate the pituitary to release the fish's own natural GtH. Synthetic LHRH analogs (LHRHa) are available commercially, such a Des-GLY (Bachem; Bubendorf, Switzerland). Recommended dosages of LHRHa range from 5-10 µg/kg, injected intramuscularly directly posterior to the dorsal fin. LHRH alone has been ineffective in numerous fish species, without using a dopamine blocker (Arabac et al. 2004; Arabac 2010; Silverstein et. al 1999). Dopamine prevents the hypothalamus from stimulating the pituitary gland, but through synthetic-dopamine blocker injections (e.g. haloperidol-fluorobutyrophenone) this process can be abated.

Both LHRHa and HCG have been used to artificially induce crappie to spawn in tanks. Gomelsky et al. (2000) induced male black crappie to spawn with a single dose at 50µg/kg, and induced female black crappie using 100µg/kg, with a 10% priming dose and 90% resolving dose 12hrs later. Studies by Al-ablani and Phelps (1997), Arslan and Phelps (2004) and Cuevas-Uribe et al. (2009) used HCG for spawning crappie, using the same 10% priming dose and 90% resolving dosage reported by Gomelsky et al. (2000), at a rate of 1000 IU/kg for females and 500 IU/kg for males. Spawning time following hormone inoculation from the aforementioned studies ranges from 24 - 48 hr. Al-ablani and Phelps (1997) reported a spawning success rate of 86% using HCG at a temperature of 24° C. However, because these studies focused on the production of monosex crappie, replicated experiments to determine the preferred artificial spawning methods for crappie have not been performed.

Spawning and Egg Incubation

If gravid broodstock are acquired from local reservoirs for artificial spawning, immediate transportation to the laboratory for stripping and fertilization is recommended for optimal hatch rates (Busack and Baldwin 1988; Parsons 1992, 1996 and 1999). Busack and Baldwin (1992) presented detailed information on spawning and fertilization of crappie eggs. Before stripping, the posterior-ventral surface of female crappie should be cleaned and dried with a towel. Stripping is achieved by applying gentle pressure to the abdomen, allowing the eggs to discharge into a sterile plastic dish. Sperm from males is obtained via maceration of excised testes. Post removal, the testes are immediately activated by adding 10-15 mL of water, and will remain motile and viable for approximately 2 min. Eggs are fertilized by adding 4-5 mL of dilute sperm/water

solution to the eggs, using a Pasteur pipet, and gently mixed for 30-40 sec using a turkey feather. Post fertilization, eggs are placed into Syracuse dishes, where they rapidly form a monolayer and adhere to the glass surface.

After adherence, a gentle water flow across the dish allows unfertilized eggs to be washed away, leaving only viable eggs to be placed into incubation chambers. Viable eggs (bright, yellow-orange in appearance) can be distinguished from under-ripe eggs (pale, white appearance) and over-ripe eggs (white, with a deteriorating membrane). Parsons (1992 and 1999) evaluated spawning methods and flow-through system crappie egg incubation, and reported ideal incubation conditions for crappie production (Table 1). The study found McDonald hatching jars were the best method for incubating crappie eggs (egg density: 10-12 eggs/cm^2), with higher hatch rates (37%) compared to Syracuse dishes (27.5%), and ease of use versus static aquaria incubation. Percent fertilization averaged 35-46% at temperatures from 15-21° C; the best %-hatch, fertilization and survival occurred at 21° C. Parsons (1999) also noted improved %-fertilization was observed when the concentration of calcium ions was > 10 ppm.

Triploidy and Hybridization

Using triploid-hybrid crappie (female *P. annularis x* male *P. nigromaculatus*) has potential as a powerful tool for managing crappie fisheries (Parsons 1992, 1996 and 1999). Inducing triploidy in fish causes reproductive sterility, preventing stocked fish from reproducing and allowing year-class structure to be controlled through stocking and exploitation alone. There are numerous advantages to producing and stocking hybrid-triploid crappie, which have the potential to greatly enhance crappie use as a sport-fish nationwide. Triploid crappie can be stocked into smaller bodies of water, even farm

ponds (~0.4-2.0-ha water bodies) and coexist in these environments in healthy, mixed-assemblages with other popular game fish, such as largemouth bass, bluegill and catfish (*Ictalurus* spp.)(Parsons 1996). Introducing triploid crappie could also break the cyclic year-class dominance and variable recruitment in crappie fisheries, though no research has investigated the long-term effectiveness of this technique (Busack and Baldwin 1988). Potentially beneficial morphological and phenotypic characteristics also exist in triploid crappie, but conclusive evidence of these characteristics is still lacking due to the difficulty of working with juvenile and adult crappie in controlled settings (Parsons 1996).

Triploidy in fish can be induced by preventing the second meiotic division of the egg (after the sperm enters the egg), thus creating an embryo with an addition set of chromosomes (2n female; 1n male)(Felip et al. 2001). Currently, thermal and pressure shock treatments (hydrostatic pressure shock being the industry standard) are used by aquaculturists to induce triploidy in numerous popular food fish and game fish, such as: rainbow trout, grass carp (*Ctenopharyngodon idella*) and tilapia (*Oreochromis* spp.)(Patino 1997; Felip et al. 2001; Stickney 2009). Parsons (1992, 1996 and 1999) found while cold temperature shock (5° C for 45 min) was effective at inducing triploidy, pressure shock treatment (6000 psi; 422 kg/cm^2 for 2 min) was the most successful method for inducing triploidy in crappie due to reduced physiological stress on the eggs, very high triploidy induction rates, low labor and time efficiency. Parsons (1999) reported a hatch rate of 87.3% with 100% triploidy using this method. The North Mississippi Fish Hatchery, which regularly produces triploid-hybrid crappie, uses a higher pressure (7000 psi; 492 kg/cm^2 for 2 min) for triploid induction than Parsons

(1999) found to be optimal. Hatch rates, however, are similar, indicating either pressure is effective in inducing triploidy (Charles Silkwood, Mississippi Dept. of Wildlife, Fisheries and Parks, personal communication).

Hybrid vigor has been documented in several other freshwater, teleost species (e.g. *Morone saxatilis* x *M. chrysops*; *Lepomis macrochirus* x *L. cyanellus*), and produces superior traits such as: enhanced growth rates, survival, disease resistance and general hardiness to culture (Hooe et al. 1994; Morris and Clayton 2009). Hooe and Buck (1991) established hybrid vigor also occurs in hybridized (black male x white female) crappie, and these beneficial traits may be further enhanced by induced triploidy. Using both hybridization and triploidy has many potential benefits to crappie production and management, such as: increased stress resistance, limited reproductive potential and accelerated growth. A morphological variant of black crappie, endemic to the Arkansas River and currently present in several large reservoirs in the southeastern U.S. (e.g., Grenada Lake, Grenada, MS), possess a black strip running along the dorsal surface of the head. This phenotypic marker has been used by hatcheries to produce triploid-hybrid crappie easily identified for future identification of hatchery produced crappie (Parsons 1996; Gomelsky et al. 2005). Triploid-hybrid crappie are currently used by several public and private hatcheries to stock small impoundments for these reasons (Charles Silkwood, Mississippi Dept. of Wildlife, Fisheries and Parks; Robert Glennon, Malone and Son Fish Farm, Lonoke, AR, personal communications).

Sperm Cryopreservation

The culture of most fishes uses freshly milted or excised semen (Brown and Brown 2011). Fish semen remains inactive until coming in contact with water, which

osmotically actives the spermatozoa. Sperm remain viable for only 1-5 min after activation, which constrains spawning and fertilization efforts to a short time (Brown and Brown 2011). Hagedorn and Kleinhans (2011) reported the aquaculture industry will see numerous benefits from using systematic germplasm cryopreservation in reproductive efforts in the coming years, such as: reducing interbreeding though the maintenance of large gene pools, increasing the availability of spermatozoa, decreasing impacts on endemic, wild populations, declining facility costs associated with maintaining broodstock, increasing reproductive and fertilization success by alleviating human errors and increasing consistency in gamete maturity.

Currently, no information is available regarding the extension of crappie sperm viability, however successful preservation, from a few days to more than 1 yr, using semen extender-buffer and cryopreservation has been documented in numerous fish species (sharp-tooth catfish, *Clarias gariepinus*; zebrafish, *Danio rerio*; rainbow trout; striped bass, *Morone saxatilis;* white bass, *Morone crysops*)(Perez-Cerezales et al. 2009; Brown and Brown 2011; Hagedorn and Kleinhans 2011; Urbanya et al. 2011; Woods III 2011). While sperm cryopreservation in these species has been successful, preservation methods (i.e., chemical composition, semen:preservant ratios, extender solution composition, and freezing and thawing regimes) are still variable among species (Hagedorn and Kleinhans 2011) and are lacking entirely for crappie. Sperm from other centrarchids, such as white and striped bass, have been preserved using a 4% dimethyl-sulfoxide + trehalose solution (Brown and Brown 2011). Kieffer and Cooke (2009) suggest a similar solution may be effective in preserving crappie sperm.

Out-of-Season Spawning

The few studies examining out-of-season spawning centrarchids indicate it is possible. However, out-of-season spawning has only been attempted once with crappie and was not the primary focus of the study (Parsons 1992). Bluegill have been spawned in the laboratory through photoperiod and temperature manipulation, without using reproductive hormones (Mischke and Morris 1997). However, bluegill are more easily cultured than crappie and have different feed and density requirements, even in pond aquaculture (Morris and Clayton 2009). Mischke and Morris (1997) present methods for tank spawning by photoperiod and temperature manipulation. Bluegill were stocked (2 males and 4 females/tank) in six 640-L fiberglass, circular tanks on a recirculating system, with 1% daily exchange rates and a 2.6 hour turnover. The experimental fish were initially held at 22° C with a 16 hr light/day photoperiod for 2 mo with two artificial nests placed in each spawning tank. Artificial nests were constructed from the bottom of 19-L buckets and filled with pea-gravel to a depth of 7-9 cm, with a bead of silicone placed around the top rim of the nest to aid in containing the substrate during nest building activities. The water temperature was gradually lowered to 15°C over 2 wk, while photoperiod was gradually decreased to 8 h light:16 h dark and held under these conditions for 1 mo. Temperatures were then gradually increased to 24° C and a 14 h light:10 h dark photoperiod and maintained until spawning occurred, approximately 2 wk after returning to summer environmental cues. The precise gradient of temperature increase in the experimental systems during the 2-wk acclimation was not documented. A second trial was conducted the following year in which the same photoperiod regime was followed, but water temperature was not controlled (Mischke and Morris 1997). In trial 1,

when temperature was controlled, 5 of the 6 culture-tanks had successful spawns, with a total of 46 spawns. In trial 2, when temperature was not controlled, 4 of the 6 tanks had successful spawns, with a total of 28 spawns. Thus, temperature manipulation does not appear to be required for successful out-of-season spawning of bluegill, although it did enhance the efficacy of spawning.

In addition to bluegill, Florida largemouth bass *Micropterus salmoides floridanus* can be spawned out-of-season, with studies using concrete raceways and photoperiod and thermal manipulation without hormone administration (Matthews and Stout 2013). Matthews and Stout (2013) placed wild-caught adult bass into 35,900-L concrete raceways (averaging 150 adults per raceway) and fed live koi (3% body weight/day) during an 8 wk acclimation. Following acclimation, raceway temperature and photoperiod was reduced (1° C/2-3 d; 2hr less light bi-weekly) to winter conditions (10-12° C; 8 hr light) and maintained for 3-4 wk. Temperature and photoperiod were then increased to spring conditions (23-24° C/14 hr light), using the same gradient of change, and maintained until spawning occurred within the raceways. Spawning was successful in all three years the out-of-season experiment was conducted, with data indicating no significant difference in spawning performance between natural spring spawning and out-of-season spawning. In contrast to crappie, bluegill and largemouth bass readily adapt to indoor culture conditions (Morris and Clayton 2009; Matthews and Stout 2013).

A limited out-of-season spawning study, conducted by Parsons (1992), examined the viability of holding adult crappie in tanks and artificially inducing spawning outside of the natural spawning season. Parsons (1992) acquired adult crappie, in pre-spawning condition, from a reservoir (Sardis Lake, MS, USA) during late winter. Crappie were

held in a single 1130-L tank at the same temperature as the reservoir for 1 wk acclimation. Then, the tank's photoperiod and temperature were gradually adjusted from winter conditions (16 h dark:8 h light, 10° C) to spring spawning conditions (8 h dark:16 h light, 18° C) 3 wk. Once a spawning coloration change (darkening) was observed in males, testes samples from a subset of the males were examined microscopically for spermatozoa motility. Spermatozoa from all male crappie were viable, and were successfully used to fertilize several wild-caught females. Only a single female exhibited gonadal development, but died due to intense behavioral interactions with conspecifics, likely males. Although the female died, a post-mortem examination revealed viable and well developed ovaries.

Conclusion

Crappie are economically important gamefishes, yet their culture and management are far from optimized. A survey of U.S. crappie hatcheries and commercial production data indicate substantial annual production in both public and private sectors. Currently, the most commonly used culture method is extensive pond spawning and juvenile rearing (< 70 mm), although some public and private facilities are using more advanced techniques, such as strip spawning and supplemental feeding of juveniles. Key research areas with potential to provide important advances in crappie aquaculture include studies on: identifying specific physiological tolerances, improving current pond production methods, determining nutrition and feed requirements, developing tank culture methods, examining the viability of sperm cryopreservation, examining fitness and growth benefits from triploidy and hybridization, refining induced spawning techniques, developing methods for out-of-season spawning and standardizing harvest

and transport protocols. Of these research needs, advances in feed-training, spawning induction and sperm cryopreservation would directly benefit investigations into out-of-season spawning and have immediate impacts on current aquaculture practices. Artificial feed training of crappie is essential for sustainable, long-term tank culture efforts, and would reduce both labor and costs associated with maintaining healthy populations of fish for crappie feeding. Controlled spawning through spawning induction hormones would reduce reliance on environmental conditions and provide greater flexibility during spring spawning efforts. The ability to preserve and extend the viability of sperm through cryopreservation techniques would eliminate the need for males to be held in captivity, reducing required space and resources. These advances would facilitate investigations into out-of-season spawning which ultimately has the greatest potential to expand the production capacity of public and private aquaculture facilities.

Tables

Table 1.1 Crappie *Pomoxis* spp. production at U.S. state hatcheries in 2014

State	Species	Mean Annual Production	Spawning Method
Alabama	BC	400,000	Pond
Arkansas	WC/BC	125,000 WC/450,000 BC	Pond
Colorado	BC	400,000	Pond
Illinois	BC	18,000	Pond
Mississippi	WC/TH	200,000 WC/ 100,000 TH	Strip spawning
Missouri	WC/BC	300,000 WC/190,000 BC	Pond
Nebraska	BC	60,000	Pond
Pennsylvania	WC/BC	35,000 WC/50,000 BC	Pond
Tennessee	WC/BC	85,000 WC/1,700,000 BC	Pond
Virginia	BC	30,000	Pond

Species abbreviations: black crappie *P. nigromaculatus* (BC); white crappie *P. annularis* (WC); triploid-hybrid crappie (TH).Data provided in table are from personnel communication with hatchery managers from each state (3 August – 12 August 2015): Alabama Dept. of Conservation and Natural Resources; Arkansas Game and Fish Commission; Colorado Parks and Wildlife; Illinois Dept. of Natural Resources; Mississippi Dept. Wildlife, Fish and Parks; Missouri Dept. of Conservation; Nebraska Game and Parks Commission; Pennsylvania Game Commission; Tennessee Wildlife Resources Agency; Virginia Dept. of Game and Inland Fisheries.

Table 1.2 Commercial crappie *Pomoxis* spp. aquaculture production in the U.S. (2013)

		Production (kg)		Sales		
	Farms	Live Wt. (kg)	Wt./Fish (kg)	Number Sold	Total ($)	Price by wt. (kg)
All	63	-	-	3,140,094	$558,725	-
Foodsize	10	909.09	0.27	3,000	$4,000	$3.92
Stockers	23	36,818	0.14	288,000	$198,000	$5.41
Fry/Fingerlings	31	-	-	1,203,000	$357,000	$652.59

Data summarized from USDA Census of Aquaculture 2013, National Agriculture Statistics Service.

Table 1.3 Summary of commercial crappie *Pomoxis* spp. production data by state (2005).

	Farms				Sales ($)			
	Total	Food	Fry	Stockers	Total	Food	Fry	Stockers
National	**73**	**12**	**35**	**24**	**$517,784**	**$53,153**	**$373,724**	**$85,692**
Alabama	1	1	-	-	-	-	-	-
Arkansas	1	-	1	-	-	-	-	-
California	1	1		-	-	-	-	-
Colorado	1	-	-	1	-	$22,618	-	-
Georgia	3	2	1	-	-	-	-	-
Idaho	1	1	-	-	-	-	-	-
Illinois	1	-	-	1	-	-	-	-
Indiana	1	-	-	1	-		-	-
Iowa	4	-	4	1	-	$3,344	-	-
Kansas	5	1	2	2	-	-	-	-
Kentucky	2	-	2	-	-	-	-	-
Louisiana	2	-	1	1	-	-	-	-
Michigan	2	1	-	1	-	-	-	-
Minnesota	8	-	5	2	$17,747	$52,439	$10,222	-
Mississippi	4	-	3	1	$21,500	$9,839	-	-
Missouri	2	-	2		-	$57,760	-	-
Nebraska	6	-	2	4	$11,104	$8,891	-	-
New York	4	1	2	1	-	-	-	-
N. Carolina	1	1	-	-	-	-	-	-
Ohio	6	-	4	2	$11,220	-	-	-
Oklahoma	2	1	1	-	-	-	-	-
Oregon	2	-	1	-	-	-	-	-
Pennsylvania	3	1	-	1	-	-	-	-
Texas	1	-	-	1	-	-	-	-
Wisconsin	9	1	4	4	$18,311	$42,860	-	$9,367

Data summarized from USDA Census of Aquaculture 2005, National Agricultural Statistics Service. Individual state production information not available from 2013 census

Table 1.4 Incubation variables for rearing crappie (*Pomoxis* spp.) eggs

Temperature (°C)	18-21
pH	7-8
Calcium (ppm $CaCl_2$)	0-10
Density (eggs/cm^2)	10-12
Tannic Acid Bath (5-10min)(ppm)	300

Tannic acid was used to remove the adhesive coating of eggs before incubation.

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CHAPTER II

CRYOPRESERVATION OF WHITE CRAPPIE *POMOXIS ANNULARIS* SPERM

Abstract

Crappie *Pomoxis* spp. are popular game fish throughout North America and are produced by public and private hatcheries. However, annual production potential is limited by a lack of information on tank culture and induced spawning methods. Sperm cryopreservation, a technique currently used for many intensively cultured fishes, provides greater flexibility and control of artificially-induced spawning, particularly for year-round production methods. Therefore, optimal techniques for preserving crappie sperm were examined. Sperm from adult wild white crappie *P. annularis* were used to determine optimal sperm buffer, cryopreserving agent and concentration, and cooling technique providing the highest percent sperm motility. Sperm motility was assessed using a computer assisted sperm analysis system. White crappie sperm were cryopreserved using treatment combinations of two buffer solutions (350 mOsmol/kg Hanks Balanced Salt Solution (350HBSS) and 350 mOsmol/kg Ca^{2+} free Hanks Balanced Salt Solution (Ca-free HBSS)) and two cryoprotectants (dimethyl-sulfoxide (DMSO) and methanol) at concentrations of 5, 10 and 15%, then cooled at four different rates: 5, 10, 20 and 40°C/min. Post-thaw sperm motility data indicate white crappie sperm can be cryopreserved, with the most effective cryopreservation method using either buffer solution (350HBSS and Ca-free 350HBSS), a 10%-methanol or 5%-DMSO

cryoprotectant and cooled at 40°C/min. Results from an overnight shipping experiment show no difference in sperm motility between un-cryopreserved, neat sperm and sperm cryopreserved with 10%-methanol, indicating that sperm buffered with 350HBSS can be shipped overnight with no decrease in sperm motility following cryopreservation.

Introduction

Artificial spawning of crappie *Pomoxis* spp. currently requires freshly excised testes for egg fertilization, obligating aquaculturists to collect live males for any spawning efforts (Gomelsky et al. 2000; 2002). Similarly, the culture of most fishes utilizes freshly milted sperm or excised testes, which is activated osmotically on contact with water (Brown and Brown 2011). However, sperm remain viable for only 1-5 minutes after activation, which constrains spawning and fertilization efforts to a short time and limits genetic diversification techniques (Brown and Brown 2011). Using systematic germplasm cryopreservation provides numerous benefits to fish reproductive efforts, such as: reducing interbreeding though maintaining large gene pools, increasing the availability of spermatozoa, decreasing impacts on endemic, wild populations, declining facility costs associated with maintaining broodstock, increasing reproductive and fertilization success by alleviating human errors and increasing consistency in gamete maturity (Hagedorn and Kleinhans 2011).

Currently, no information is available regarding the extension of crappie sperm cells. However numerous, more intensively cultured fishes (i.e., coppernose bluegill, *Lepomis macrochirus purpurescens;* sharp-tooth catfish, *Clarias gariepinus*; zebrafish, *Danio rerio*; rainbow trout *Oncorhynchus mykiss*; striped bass, *Morone saxatilis;* white bass, *Morone crysops*) sperm have been cryopreserved, from a few days to more than 1

yr, using semen extender-buffers and cryoprotectant agents (Bates et al. 2005; Perez-Cerezales et al. 2009; Brown and Brown 2011; Hagedorn and Kleinhans 2011; Urbanya et al. 2011; Viveiros 2011; Woods III 2011). Preservation techniques are variable between species, in terms of cryoprotectant agents and concentrations, sperm:extender ratios, extender solution composition, and cooling and thawing regimes (Hagedorn and Kleinhans 2011). Determining the optimal procedures for the cryopreservation of a given fish species requires several experimental phases which gradually narrow down the preferred process. Optimization consists of neat, fresh sperm concentration calculation and initial sperm motility estimations, buffer agent analysis, examination of acute toxicity to cryoprotectants, sperm motility estimations following variable cooling rates, sperm motility estimation following thawing, and direct comparisons of percent fertilization between fresh and frozen semen samples (Brown and Brown 2011; Lansteiner 2011). At each of these phases, a computer assisted sperm analysis system is commonly used to determine sperm motility, which is used to assess the effectiveness of treatments throughout each phase of the process (Wilson-Leedy and Ingermann 2007). Sperm cells from other North American teleost species, such as white and striped bass and coppernose bluegill, have been buffered with Hanks balanced salt solution and preserved using cryoprotectant agents (white and striped bass = 4%-dimethyl-sulfoxide (DMSO); coppernose bluegill = 10%-DMSO and 10%-methanol)(Bates et al. 2005; Brown and Brown 2011).

Developing a method to preserve and extend the viability of crappie sperm using extender buffers and cryopreservation for long-term frozen storage, offers important benefits. It will provide a means for aquaculturists to spawn crappie without the need for

live males, allowing greater flexibility of spawning procedures during normal spring spawning and facilitating out-of-season spawning without the need for live males. Therefore, the objectives of this study were to determine the preferred buffering agent that prevents sperm cell activation, identify suitable cryoprotectant agents for crappie sperm cryopreservation, and pinpoint the optimal cooling rate that maximizes percent sperm motility following thawing.

Methods

Fish Collection

Male white crappie *P. annularis* were collected from Enid Reservoir, in northern Mississippi, USA. Fish were electroshocked using a sampling boat and a 7.5 horse power, generator-powered electrofisher shocking unit set at 60 Hz direct current (Smith-Root Inc., Vancouver, WA), with 3 m.1m shocking poles extended out the front of the boat. For Experiment 1, 10 male white crappie were transferred, within one hour after collection, to a hauling tank supplied with compressed oxygen, and transported to the Aquaculture Research Station at the Louisiana State University Agriculture Center, Baton Rouge, LA for cryopreservation experiments. The hauling tank was filled with fresh reservoir water, and 3 ppt NaCl to reduce stress during transport. No mortality occurred during the 6-hr transport haul. For Experiment 2, white crappie, held in a recirculating aquaculture system (15°C; 3-5ppt salinity) for 4 wk following capture and testes were removed by dissection. The recirculating system's fiberglass trough tank (2,460 L) was divided into four slots (125 cm length x 91 cm width x 54 cm depth;0.61 m^3), using prefabricated metal slot dividers. The system's water quality and temperature were maintained using a 120-watt high output ultraviolent sterilizer (Emperor Aquatics,

Pottstown, PA), bead filter (DF3, Aquaculture System Technologies, New Orleans, LA) and a water chiller (1/2 HP, 115 volt Cyclone water chiller, Aqua Logic Inc., San Diego, CA). Water quality variables (temperature, dissolved oxygen, total ammonia nitrogen, salinity and pH) were measured daily throughout the acclimation phase using a YSI Professional Plus multi-parameter probe (Model #10102030,YSI Inc., Yellow Springs, OH)(Chapter 3: Table 1, Experiment 2). Excised testes were processed (Chapter 3: Spawning Protocol) and buffered at a ratio of 1 ml semen:10 ml in 350 mOsmol/kg Hanks Balanced Salt Solution (350HBSS). The buffer solution was then shipped overnight, on ice, to the Aquaculture Research Station at Louisiana State University Agriculture Center, Baton Rouge, LA for addition of cryopreservation experiments.

Experiment 1

Upon arrival at the Aquaculture Research Station, 7 male white crappie were placed into a 5600 L recirculating system (15°C) until they were needed for sampling (~13 hr).

Neat Sperm Concentration Calculation

A 500 µl sperm suspension was prepared for each male by diluting the neat sperm 1:50 with 350HBSS. This suspension was lightly vortexed to ensure proper mixing. A 10µl sample was then removed from the sperm suspension and placed on a Makler® counting chamber (SEFI Medical Instruments LTD, Distributed by: Irvine Scientific, Santa Ana, CA). The counting chamber was then viewed through a microscope (Olympus CX41RF, Japan) at 200x magnification. Each sample was counted in triplicate using the

standard Makler® counting protocol. The counts were averaged to obtain the sperm concentration per ml for each male.

Motility estimation

Sperm motility was estimated by placing 5 µl of sperm on the Makler® counting chamber and then adding 20 µl of water for activation. The chamber was then immediately placed on a microscope (Olympus CX41RF, Japan) at 200x magnification and quantified using computer-assisted sperm analysis (CASA)(Hamilton Thorne, Inc., Beverly, MA; CEROS model). The CASA system, which quantifies sperm movement using computer-calculated motility characteristics, is currently the most objective and comprehensive method available for sperm quality assessment (Wilson-Leedy and Ingermann 2007). The variable settings were: minimum contrast, 60; minimum cell size, 2; number of frames for recording, 100; average-path velocity (VAP) cut off, 25 µ/s; straight-line velocity (VSL) cut off, 1µ/s. For each sample, at least three measurements of different viewing fields were performed, and the average was used as the motility for that sample. These variables were verified using the "playback" function of the software.

Acute Toxicity Trials

Sperm samples from three white crappie males were used in the acute toxicity trials. From each male, two sperm suspensions with concentrations of 5.0 x 10^8 sperm per ml were made by diluting 1:1 (sperm:extender) with the appropriate extender (350HBSS and 350 mOsmol/kg or Ca^{2+} free Hanks Balanced Salt Solution (Ca-free HBSS))then adjusted to concentrations of 5.0x10^8 sperm per ml. Two cryoprotectants (Methanol, DMSO) with final concentrations of 5%, 10%, and 15% (v/v) were chosen for this study.

Each cryoprotectant was further subdivided by dilution with 350HBSS or Ca-free 350HBSS to reach twice the desired target concentrations. The cryoprotectant solution was then mixed with the buffered sperm solution to reach the targeted treatment concentration.

Cryopreservation

The cryoprotectants (methanol and DMSO) were prepared in 350HBSS at twice the target concentrations, and were mixed 1:1 (v/v) with the sperm samples to yield the desired final concentration of cryoprotectant (5%, 10%, and 15%) and sperm concentration of $2.5x10^8$ sperm/ml. Upon mixing with the cryoprotectant, the sperm samples were placed in a Quattro Minitube system (Verona, WI) and straws were filled, sealed, and labeled using a proprietary computer program (Minijet ver. 4.00.01, Verona, WI). Samples were drawn into 0.5 ml French straws by vacuum pressure applied to the cotton end of the straw. The straws were continuously transferred to the sealing platform and sealed on one end by application of a 158˚C heat clamp. Afterwards, straws were dropped onto a conveyor belt where they were labeled with alphanumeric information and a barcode by an ink jet printer (A-series plus, Domino, IL, USA) before being transferred to the collection area for label verification. Straws were arrayed on horizontal racks (40 straws/rack) and placed in a commercial-scale programmable freezer (Mico Digitcool, IMV, France) with a capacity of 280 straws per cycle. Thermal mass was not equalized at every cooling cycle (e.g. by adding "dummy" straws to fill the freezer) and the number of straws in each freezing cycle ranged from 113 to 221 straws. Between 15 and 19 min after the cryoprotectant was added to the sperm suspension, the cooling program was initiated. Based on results from the acute toxicity trials, three

cryopreservation agent solution combinations (5% methanol; 5% dimethyl-sulfoxide (DMSO); 10% methanol) were selected for cooling experiments because these treatments yielded the highest percent of motile sperm. Four different cooling rates (5, 10, 20 and 40°C/min)(n = 27 sperm sample straws/cooling rate) were used to determine the optimal rate which maximizes sperm survival.

Thawing and motility assessment

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

Experiment 2

To assess sperm survival through short-term buffering and shipping (24 hr), a second experiment was conducted. Sperm samples were acquired from seven white crappie males, which were held in a recirculating tank system (15°C; 3-5 ppt salinity) for approximately 4 wk.

Sperm Sample Buffering

Males were netted and placed in a portable electroshocking unit (Portable Electroshocking System, Smith-Root Inc., Vancouver, WA) and stunned using a 60 Hz, 300 volt shock for 3-4 sec. Once shocked, the testes were excised from the male, placed on a sterile petri dish, weighed and then minced with a scalpel. A small sample

(approximately 100 µl) of fresh sperm was activated by the addition of 1-2 ml water and observed under a dissecting microscope (Model 162-P; National, Schertz, Texas) at 10x magnification to confirm sperm motility before buffering. Testes samples were then squeezed through a standard aquarium net (Aqueon, Franklin, WI) into a 50 ml sample vial, to filter the semen and remove large tissue pieces prior to buffering. 350HBSS was then added to the neat semen samples at a ratio of 10 ml:1 g testes, based on results from acute buffer toxicity trials of Experiment 1. Vials were gently mixed, with vial caps loosely tightened to allow for ample oxygen exchange (Tiersch et al. 2011), and placed in an insulated container with ice (4 ˚C) and shipped to the Aquaculture Research Station at Louisiana State University, Baton Rouge, LA for post-transport motility and post-cryopreservation motility assessments.

The cooling rate was programmed at 40 ˚C/min based on cooling trail results from Experiment 1. Once the final target temperature of -80˚C was reached the frozen samples were held at this temperature for 5 min and then placed into liquid nitrogen (-196˚C). Individual straws were sorted in liquid nitrogen and placed into storage containers (Daisy goblets, reference number: 015144, Cryo Bio Systems) for long term storage in liquid nitrogen. Thawing and motility assessment methods were identical to Experiment 1.

Statistical Analysis

All data were analyzed using program R (R Foundation for Statistical Computing, Vienna, Austria). Normality was tested for using the Shapiro-Wilk normality test ($\alpha = 0.05$). The Bartlett test was used to determine equal variance between treatments, where $\alpha = 0.05$. A one-way analysis of variance (ANOVA) was used to determine if a difference existed between hormone treatments, and Tukey's honestly-significant-difference post

hoc test was used to identify significant differences between treatment means, where $\alpha = 0.05$.

Results

Acute Toxicity

There was no significant difference between buffers (350HBSS and Ca-free 350HBSS), indicating either can be effectively used as a short-term sperm extender solution (Figure 2.1). There were differences between cryoprotection agent type (Methanol or DMSO) and concentration (5, 10 or 15%) across all time points (10, 20 and 30 min post-mixing). Based on these results, 5%-methanol (mean ± S.E. %-sperm motility at 30 min post-mixing: 37.80 ± 7.56%), 10%-methanol (24.60 ± 4.80%) and 5%-DMSO (18.88 ± 4.18%) were selected as the three best treatment combinations to be used for the next phase of cryopreservation experiments, determination of cooling rate.

Cooling Rate

There was a difference in %-sperm motility among cooling rates (5, 10, 20 and 40 ˚C/min), with 40˚C/min having the highest overall %-sperm motility (24-28%) after thawing, while the lowest %-sperm motility was 5˚C/min (Figure 2.2). There was no significant difference between 5%-methanol, 10%-methanol and 10%-DMSO at a cooling rate of 40˚C/min.

Thawing Experiment

A significant difference occurred between 5%-methanol and 5%-DMSO and 10%-methanol, where 5%-methanol resulted in lower percent sperm motility following thawing (Figure 2.3). No difference was found between 5%-DMSO and 10%-methanol.

Shipping Experiment

A difference occurred between un-frozen sperm samples (neat) and 5%-methanol and 5%-DMSO, where neat samples had higher %-sperm motility, however there was no difference between neat and 10%-methanol and no difference between 5%-DMSO and 10%-methanol (Figure 2.4).

Discussion

The present study examined the potential for white crappie sperm to survive toxicity buffering, cryopreservation and thawing. Results from computer assisted sperm analysis indicate white crappie sperm can be cryopreserved using either 350HBSS or Ca-free HBSS as a buffer, and 5% DMSO or 10% methanol as a cryoprotectant. A 40˚C/min cooling rate was the best method for preserving white crappie sperm, with both 5% DMSO and 10% methanol having similar %-sperm motility (24-28%) following thawing. Further, short-term (< 24 hr) buffering of semen samples, with a 1:1 ratio of 350HBSS and stored at 4°C, maintained sperm survival during transportation, indicating this is a useful method for storage of crappie semen samples while in the field.

Limited cryopreservation experiments have been conducted with centrarchid species, with most extant data on coppernose bluegill. This study found white crappie semen has similar buffering osmolality requirements (300-350 mOsmol/kg HBSS) to inhibit sperm cell activation as coppernose bluegill (Bates et al. 2005). Similarities in sperm extension methods used for other freshwater fishes also exist. In terms of toxicity buffers, a 1:1 semen:extender ratio is recommended for white bass (Brown and Brown 2011), equal to the ratio used for white crappie in Experiment 1. Buffering methods for the razorback sucker *Xyrauchen teaxnus* also recommend a 1:1 semen:extender ratio with

Ca-free HBSS, and found sperm motility decreased across time, where 73% was achieved after 1 d, 60% after 5 d and a sharp decrease (2%) by 8 d (Tiersch et al. 2011). A semen:extender ratio of 1:3 is recommended for striped bass and rainbow trout sperm (Lahnsteiner 2011; Woods III 2011). Lahnsteiner (2011) also reported sperm concentration in salmonid fishes should not exceed 2.5 x 10^9 sperm cell/ml diluent due to a decrease in post-thaw fertility, likely caused by limited intercellular space or cell compression in storage. Additional white crappie cryopreservation studies may benefit from examining the effect of higher dilution ratios (1:3, 1:5 and 1:7) on sperm motility.

Similar CPA concentrations and cooling rates have been used by previous freshwater fish, sperm cryopreservation studies. Coppernose bluegill can be cryopreserved for long term storage using either 10%-DMSO or 10%-methanol (Bates et al. 2005). Brown and Brown (2011) used a 4% DMSO concentration for both white and striped bass, with the addition of 10% trehalose at 100 mg/ml which increased motility. However, Woods III (2011) recommended a 7.5% DMSO concentration for striped bass. These experiments found that a 5% DMSO was an effective CPA and concentration for white crappie sperm cryoprotection, which is in a similar range to striped bass studies.

Both DMSO and methanol, at 5 and 10% concentration, have been selected as recommended CPAs in fertilization experiments in other freshwater fish species, indicating cryopreservation methods and %-fertilization rates are largely species dependent (Tiersch et al. 2011; Mims et al. 2011; Viveiros 2011; Woods III 2011). Fertilization experiments conducted with coppernose bluegill using sperm in 10%-DMSO found that %-fertilization was 15% versus 95% for freshly excised, undiluted semen. The 10%-methanol CPA, however, resulted in much higher post-thaw %-fertilization than

10%-DMSO (%-fertilization: methanol = 50; DMSO = 15)(Bates et al. 2005). Coppernose bluegill %-sperm motility following thawing and activation was higher than the post-thawing motility of crappie semen reported in this study for the 10%-methanol treatment (mean coppernose bluegill post-thawing %-sperm motility = 51%; mean white crappie post-thawing %-sperm motility = 26%)(Bates et al. 2005). Since post-thawing %-fertilization assessments could not be made in the present study due a lack of available males during spawning experiments, insights cannot be made to the fertilization potential of cryopreserved crappie sperm cells, however a similar decrease in %-fertilization between fresh and cryopreserved semen may be observed. A 70-80% post-thaw sperm motility has been achieved with common carp using 10% DMSO, however fertilization rates were low (30 - 40% fertilization)(Cognie et al. 1989). Following buffering, a 10% methanol CPA, frozen instantly in liquid nitrogen without a freezing gradient, provided the highest post-thaw sperm motility (24%) in the razorback sucker (Tiersch et al. 2011). In rainbow trout the highest %-fertilization (82%) post-thawing (25°C for 30 sec) was 5% methanol (Lahnsteiner 2011). A 40°C/min cooling rate, equal to the recommended rate suggested by Lahnsteiner (2011) and Woods III (2011) for rainbow trout and striped bass, produced the highest white crappie %-sperm motility following thawing.

Buffering and refrigerated storage of sperm samples is a useful tool for gamete collection in the field, where cryopreservation cannot be accomplished (Tiersch et al. 2011). Results from Experiment 2 indicate overnight shipping of white crappie sperm, using a 1:10 dilution with 350HBSS, is a useful technique for transporting sperm samples to a facility where cryopreservation can be accomplished. A recent cryopreservation experiment in common carp suggests oxygen supply during transportation and short-term

storage strongly influences sperm quality (Magyary et al. 2011). Other studies of cryopreservation observed highest post-thawing sperm motility rates when sperm samples were stored and transported in thin layers, using Petri dishes instead of glass or plastic vials, to maximize oxygen exposure and ATP recovery (Saad et al. 1988; Billard et al. 1995). Magyary et al. (2011) found sperm motility following transportation can be improved by 30 min of supplemental oxygen exposure, indicating ATP recovery is possible in spermatozoa. Further shipping experiments should be conducted to compare transportation methods (plastic tubes versus Petri dishes) and examine the potential of post-transportation ATP recovery through oxygen supplementation.

This study shows white crappie sperm cells can survive both short-term buffering and long-term cryopreservation using commonly used buffering agents and CPAs. Since a wide variety of post-thawing %-sperm motility exists among different fish species (~15 - 50% post-thawing sperm motility), no direct evaluation of cryopreservation quality can be made to other cryopreservation publications. Cryopreservation may be a viable alternative to harvesting testes from wild-caught male crappie in future spawning efforts, with potential utility for out-of-season spawning efforts. Further studies are needed to compare the fertilization efficacy of cryopreserved sperm compared to fresh sperm, examine the effect of transportation methods (tubes versus Petri dishes) and post-transportation oxygen supplementation on sperm motility, determine optimal dilution ratios which maximize sperm survival through preservation, and examine the effects of short-term buffered semen (1-5 d; 4˚C) and long-term storage (1-2 yr; -196˚C) on cryopreserved sperm motility across time. This study validates the potential for sperm cryopreservation to be used as a production tool for crappie spawning efforts, with

highest sperm motility obtained using a buffer of 350HBSS, a CPA of either 5%-DMSO or 10%-methanol, and a cooling rate of 40˚C/minute.

Figures

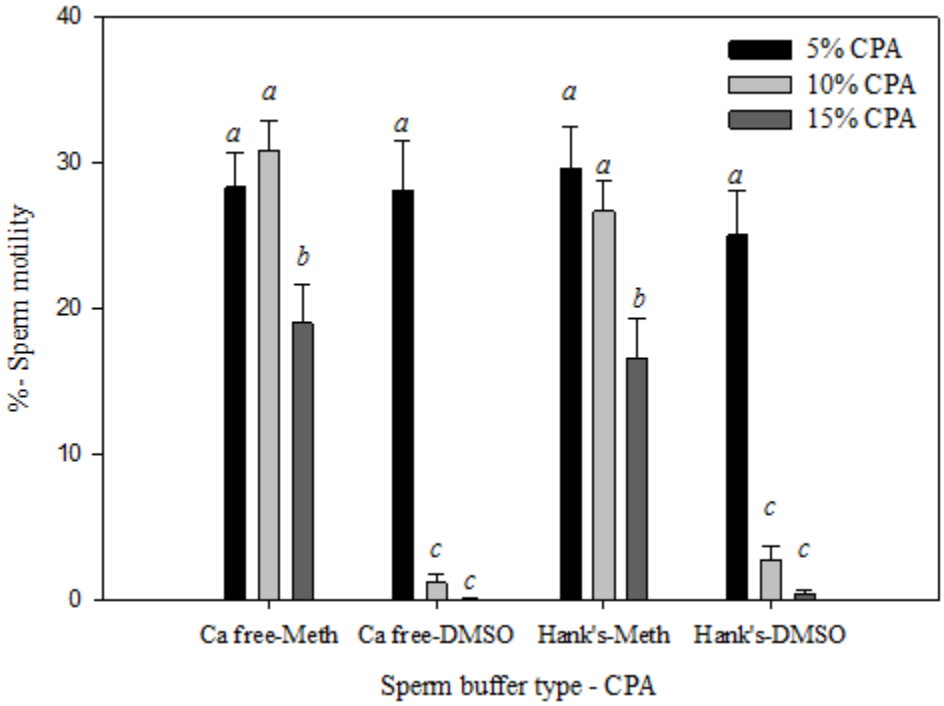

Figure 2.1 White crappie *Pomoxis annularis* percent sperm motility (mean ± S.E.) following a 10 min acute exposure to a cryopreservation agent (CPA)

Cryopreservation agent treatments of 5, 10 and 15% methanol (Meth) or dimethyl-sulfoxide (DMSO) mixed with either of two sperm buffering solutions: Ca free Hank's balanced salt soln. (Ca free) or standard Hanks balanced salt soln. (Hank's)(n = 7 fish/treatment) were examined. The letters beside each treatment represent significant differences ($p < 0.05$) using a one-way ANOVA and Tukey's honest-significant-difference test.

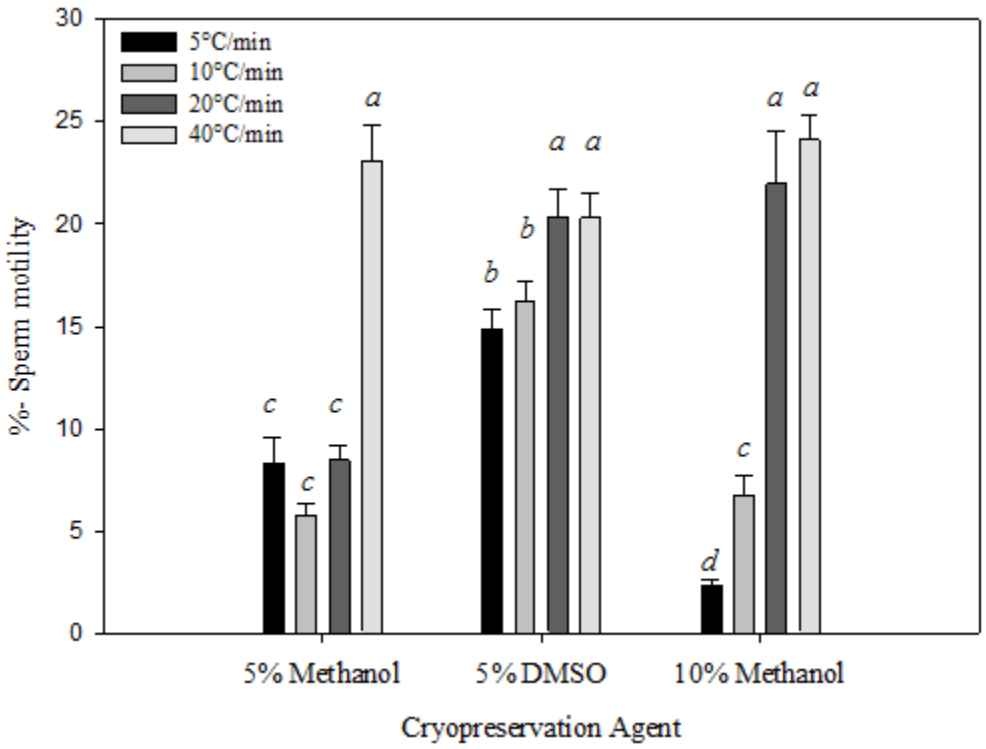

Figure 2.2 Comparison of mean percent sperm motility (±S.E.) of white crappie *Pomoxis annularis* sperm following four different cooling gradients.

Treatment cooling rates (5, 10 ,20 and 40°C/min) (n = 27 sperm sample straws/cooling rate) from three cryopreservation agent solution combinations (5% methanol; 5% dimethyl-sulfoxide (DMSO); 10% methanol) after being thawed at 40°C for 8 sec were examined. The letters above each treatment represent significant differences ($p < 0.05$) using a one-way ANOVA and Tukey's honest-significant-difference test.

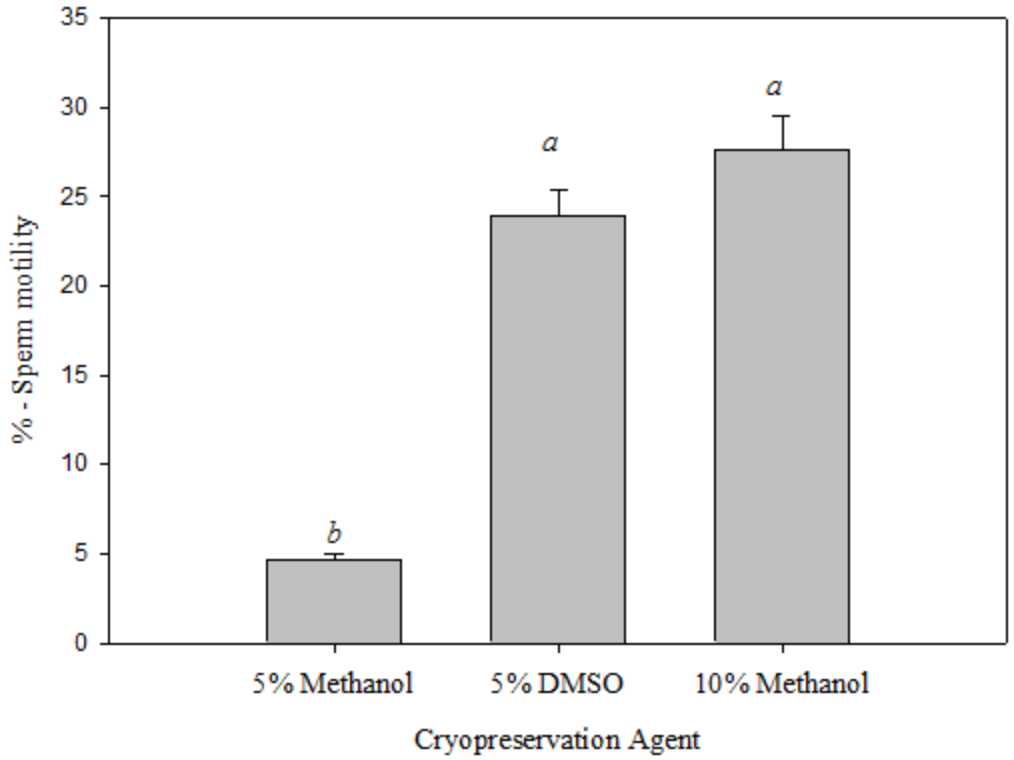

Figure 2.3 White crappie *Pomoxis annularis* mean %- sperm motility (± S.E.),following thawing at 40°C for 8 sec.

Treatments were three cryopreservation agent solutions; 5% methanol, 5% dimethyl-sulfoxide (DMSO), 10% methanol. Lowercase letters indicate significant differences ($p < 0.05$) using a one-way ANOVA and Tukey's honest-significant-difference test (n = 7)

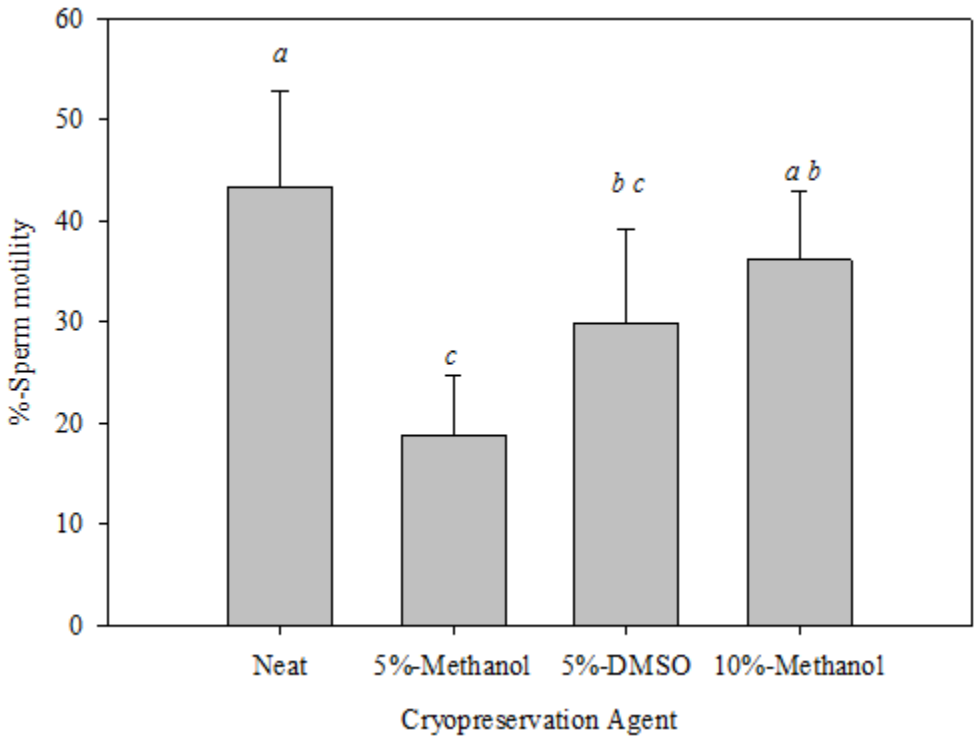

Figure 2.4 White crappie *Pomoxis annularis* mean %- sperm motility (± S.E.), thawed at 40°C for 8 sec following overnight shipment.

Treatments were sperm that was not cryopreserved (Neat) or three cryopreservation agent solutions; 5% methanol, 5% dimethyl-sulfoxide (DMSO), 10% methanol. Lowercase letters indicate significant differences ($p < 0.05$) using a one-way ANOVA and Tukey's honest-significant-difference test (n = 7)

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CHAPTER III

WHITE CRAPPIE *POMOXIS ANNULARIS* INDUCED SPAWNING IN TANK AQUACULTURE SYSTEMS

Abstract

Although white crappie *Pomoxis annularis* have been extensively cultured for decades due to their popularity as a recreational gamefish, a lack of broodstock management and spawning methods limits hatchery production. Advanced aquaculture techniques, such as using recirculating tank systems and hormone-induced spawning, have potential to stabilize and enhance annual production rates. However, wild-caught adult crappie are prone to bacterial disease following tank transfer without proper stress management. Therefore, acclimation methods were developed using chronic exposure to low concentrations of salt (3-5 ppt) and cool water temperatures (16° C) to reduce mortality following capture and tank holding. Following acclimation, induced spawning in white crappie was evaluated using four spawning induction hormones: luteinizing hormone releasing hormone analogue (LHRHa), human chorionic gonadotropin (HCG) and salmonid gonadotropin releasing hormone analogue + domperidone (GnRHa) and LHRHa + domperidone (LD) in two subsequent years. White crappie were collected during pre-spawning (March-April) and transferred to freshwater, flow-through tanks. Fish were injected with LHRHa, HCG, GnRHa or were not injected as a control (10% priming dose, 90% resolving dose (RD) 12 hr later), strip spawned upon ovulation (~30

hr post-RD), and fertilization success was determined 24 hr post-fertilization. Spawning occurred in all three hormone treatments (spawning success (%-females spawned): LHRHa = 65%; GnRHa = 50%; HCG = 25%), but not the control. GnRHa produced the highest fertilization rates (68.3 ± 8.8%), while LHRHa had the lowest fertilization rates (12.4 ± 4.8%). The HCG treatment was associated with mortality before spawning (54.2%) and highly variable mean fertilization rates (28.6 ±12.1%). A second experiment evaluated adding domperidone, a dopamine antagonist already present in GnRHa, to LHRHa. Spawning occurred in all three hormone treatments (spawning success: LHRHa = 64%; LD = 69%; GnRHa = 50%), and there were no differences in %-fertilization among treatments (%-fertilization: LHRHa = 34.1% ± 29.0; LD = 34.3% ± 33.9; GnRHa = 34.1% ± 37.4). These results indicate crappie can be held in tank systems with low mortality, domperidone is not a contributing factor to overall spawning success, and GnRHa and LHRHa are both effective in white crappie spawning induction.

Introduction

White crappie *Pomoxis annularis* and black crappie *P. nigromaculatus* are popular recreational gamefish and provide a substantial source of income to local, regional and state economies throughout the U.S. (Dorr, Munn and Meals 2002; Dudenhoeffer et al. 2014). However, crappie populations fluctuate, particularly in smaller impoundments (< 20 ha) and annual recruitment often varies from year-to-year, with a dominating single year-class produced every 2-5 yr and numerous stunted year-classes occurring in between (Busack and Baldwin 1988; Mitzner 1991; Cuevas-Uribe et al. 2009). This high-variability in annual recruitment and diminished growth and harvest potential have been linked to fluctuating environmental conditions impacting: water

levels, lake temperature, habitat availability and food availability (Swingle and Swingle 1967; Boxrucker and Irwin 2002; Dubuc and DeVries 2002; Miranda et al. 2013). As an alternative to classical fisheries management strategies, crappie aquaculture production may provide a solution to this problem. Recent research has focused on monosex or sterile black crappie production, pond culture techniques and larval rearing methods (Al-ablani and Phelps 1997, Gomelsky et al. 2000; Arslan and Phelps 2004; Cuevas-Uribe et al. 2009 and Dudenhoeffer et al. 2014). However, research on adult crappie tank culture, broodstock management and in-tank reproduction techniques is needed to facilitate reliable production.

While broodstock culture and induced spawning methods of several other centrarchids, such as largemouth bass, *Micropterus salmoides* (Willis and Flickinger 1981) and bluegill, *Lepomis macrochirus* (Mischke and Morris 1997), have been examined and are currently being used in the public and private sectors, limited research has been conducted in these areas with crappie (Morris and Clayton 2009). Several technological advances in aquaculture over the past few decades have facilitated indoor centrarchid culture in recirculating tank systems allowing for year-round production (Willis and Flickinger 1981; Mischke and Morris 1997). Advances have included feed-training of larval fish allowing for use of formulated diets with white and black crappie (Dudenhoeffer et al. 2014) and the artificial manipulation of reproductive timing to successfully spawn fish out-of-season (Mischke and Morris 1997; Matthews and Stout 2013). Research on crappie reproduction methods still remains limited due to adult crappie proclivity for bacterial diseases once transferred to tank systems (Smeltzer and Flickinger 1991; Parsons 1992). Smeltzer and Flickinger (1991) demonstrated increasing

salinity to 3-5 ppt and maintaining cool water temperatures (< 15° C) will reduce disease and improve survival during short-term transport, handling and pond harvest of juveniles. However, these stress mitigation methods have not been tested in longer-term holding conditions in recirculating tank systems, nor with wild-captured adult crappie.

Two commonly used spawning hormones for finfish, luteinizing hormone releasing hormone analogue (LHRHa) and human chorionic gonadotropin (HCG), have been used to artificially induce black crappie to spawn in tanks. Gomelsky et al. (2000) induced female black crappie using 100 μg LHRHa/kg, with a 10% priming dose and 90% resolving dose 12 hr later. Studies by Al-ablani and Phelps (1997), Arslan and Phelps (2004) and Cuevas-Uribe et al. (2009) used HCG for spawning crappie using a 10% priming dose and 90% resolving dosage at a rate of 1000 IU/kg for females and 500 IU/kg for males. For these studies, ovulation following hormone inoculation ranged from 24 – 48 hrs, and Al-ablani and Phelps (1997) reported 86% spawning success using HCG at 24° C. These experiments demonstrate black crappie can be effectively spawned in tanks using hormone induction.

Research investigating adult tank acclimation, maturation assessment techniques and optimizing hormone induced spawning methods is needed to improve crappie hatchery production. These advances would provide numerous benefits such as: controlled spawning periodicity during spring spawning efforts, accurate adult crappie number estimations needed for spawning, female size and maturation selection, decreased reliance on environmental conditions to induce reproduction, increased health and thus reproductive potential of collected crappie through tank culture. To develop crappie reproduction methods in tank aquaculture systems, tank acclimation methods and

four spawning induction hormones were investigated (LHRHa, HCG, salmonid gonadotropin releasing hormone analogue + domperidone (GnRHa) and luteinizing hormone releasing hormone analogue + domperidone (LDOM)).

Methods

Fish Collection

White crappie were collected from Enid and Grenada Lakes in northern Mississippi, USA on 1 April 2014 and 3 April 2014from tributary arms of the main reservoir. Crappie were located at 1.2-1.8 m depth and 17° C, concentrated along submerged streambeds where a large, acute depth change occurred from 1.2 - 1.8 m to 3.0 - 3.6 m. Fish were electroshocked using a sampling boat and a 7.5 hp, generator-powered electrofisher shocking unit set at 60 Hz direct current (Smith-Root Inc., Vancouver, WA), with 3 m shocking poles extended out the front of the boat. Upon collection, crappie were placed into a 370 L rectangular tank on the boat supplied with continuously circulating surface water until electroshocking collection was completed (~1 hr). Crappie were then hauled to a recirculating system at the Mississippi Department of Wildlife, Fisheries and Park's North Mississippi Fish Hatchery (NMFH). The truck's hauling tank was filled with fresh lake water with 3 g/L NaCl to reduce physiological stress associated with collection, transport and stocking. Crappie were then transferred to an acclimation tank system and acclimated for 1 mo to tank conditions before experimentation.

Tank Acclimation

Before sampling, pilot studies were conducted over 6 mo on conditions necessary for tank acclimation. Initially, adult mortality was high from wild fish placed into indoor recirculating tank systems with *Flavobacterium columnare* determined by veterinary diagnosis to be the primary cause of mortality. Salinity and temperature were then manipulated, as increased salinity (> 3 ppt) and decreased temperature (< 20° C) significantly decreases *Flavobacterium columnare* survival (Plumb 1999). Crappie acclimated to tanks with 3-5 ppt marine salt (Instant Ocean Aquarium Sea-salt mixture, Instant Ocean, Blacksburg, VA) and 16° C had low mortality (2.5% mortality in all acclimation phases of all experiments pooled, n = 280). Therefore these tank conditions were maintained for approximately 2 wk after initial capture and stocking.

For acclimation, a rectangular fiberglass tank (2,460 L) was divided into four equal areas (125 cm length x 91 cm width x 54 cm depth;0.61 m^3), using prefabricated metal dividers. The system's water quality and temperature were maintained using a 120-watt high output ultraviolent (UV) sterilizer (Emperor Aquatics, Pottstown, PA), bead filter (DF3, Aquaculture System Technologies, New Orleans, LA) and a water chiller (1/2 HP, 230 volt Cyclone water chiller, Aqua Logic Inc., San Diego, California). During acclimation (2-3 wk), the system's temperature was maintained at 16-17° C to prevent gonad maturation and spawning. Temperature in the acclimation system was held until 1 wk before experimental stocking, where the temperature was raised 1° C/day until reaching ideal spawning temperature (21° C) to provide the temperature cue needed to induce final gonad development before spawning. Water quality variables were measured using a multi-parameter probe (YSI Professional Plus Model #10102030, YSI Inc.,

Yellow Springs, Ohio). Temperature, dissolved oxygen, total ammonia nitrogen, salinity and pH were measured daily throughout the acclimation phase.

Busack and Baldwin (1988) and Parsons (1992, 1996, and 1999) suggested white and black crappie males and females should be held in separate tanks to prevent male aggressive behavior towards females. Therefore, crappie were divided by sex into different tank sections to prevent behavioral mortality. A total of 54 males and 42 females were stocked into the acclimation tank 1 mo before spawning experiments, and no mortality occurred during acclimation.

Experiment 1

The first induced spawning experiment was conducted from 22 April 2014 to 25 April 2014 and was designed to compare the effectiveness of two commonly used fish spawning induction hormones, HCG and LHRHa. Nine large fiberglass, rectangular tanks (2,460 L each) containing four, equally divided areas (125 cm length x 91 cm width x 54 cm depth;0.61 m^3) and six small fiberglass troughs (792 L) containing two, equally divided areas (125 cm length x 60 cm wide x 30.5 cm depth; 0.23 m^3) were used during the experiment. All tanks used flow-through surface water from the NMFH holding pond which receives water from directly from Enid Lake. Water quality variables were measured using a multi-parameter probe (YSI Professional Plus Model #10102030, YSI Inc., Yellow Springs, Ohio). Temperature, dissolved oxygen (DO), total ammonia nitrogen, salinity and pH were measured 3 times per day throughout the experiment (Table 1). Four male and four female crappie were placed into each of the large tanks, with two fish of the same sex per divided tank section. Male and female slots were alternated to ensure stocking uniformity in all tanks. Two male crappie and two female

crappie were placed into each of the small tanks, also with two fish per divided slot. Each fish was weighed to the nearest gram using an Ohaus platform scale (Model CW11-2EO, Ohaus Corporation, Parsipphony, New Jersey) and measured to the nearest millimeter on a fish measuring board during initial transfer to determine condition factor and hormone dosage rates. Final experimental design included 3 large tank replicates with 4 male and 4 female crappie (2 either male or female per divided tank section) and 2 small tank replicates with 2 male and 2 female crappie per treatment (LHRHa, HCG, control), for a total of 36 fish per treatment.

Hormone Administration

Dosage rates of HCG and LHRHa were based on crappie spawning studies conducted by Al-ablani and Phelps (1997), Arslan and Phelps (2004), Cuevas-Uribe et al. (2009) and Gomelsky et al. (2000, 2002 and 2005). Females in the LHRHa treatment received a 10% priming dose of LHRHa (Syndel Laboratories, Qualicum Beach, British Columbia, Canada)(10 µg LHRHa/kg) intramuscular (IM) upon initial stocking, followed by a 90% resolving dose (90 µg LHRHa/kg) 12 hr later. Males in the LHRHa treatment received a single dose of 50 µg LHRHa/kg when the resolving dose was given to the female crappie. The HCG treatment crappie received a 10% priming dose (Chorulon®, Intervet Inc. Millsboro, DE)(100 IU HCG/kg fish weight) followed by a 90% resolving dose (900 IU HCG/kg fish weight) 12 hr later, regardless of sex. Following hormone inoculation, all fish were closely monitored for spawning activity and ovulation, determined by eggs released onto the tank bottom, every 3 hours for 48 hr, and dead crappie were removed from tanks immediately.

Release of eggs following gentle abdominal pressure was used as the indicator for spawning readiness in females. When eggs were easily released from the females' urogenital opening following palpation, they were strip spawned using the methods described by Baldwin et al. (1990). However, fish spawned in this manner resulted in broods with poor fertility. Therefore, the indicator for ovulation and spawning readiness was changed to the observation of eggs on the bottom of tanks. This resulted in larger volumes of eggs spawned and higher fertilization rates. Males were also palpated, but no flowing semen was observed. It was concluded testes development and spawning condition could not be assessed using this method. Therefore, male spawning was conducted by excising the testes. Males used for spawning were placed in a portable electroshocking unit (Portable Electroshocking System, Smith-Root Inc., Vancouver, WA) and stunned using a 60 Hz, 300 volt shock for 3-4 sec. After stunning each male using electro-narcosis, each male was placed on a table, dried with a towel, and dissected using a fillet knife from the back of the pelvic fin down past the dorsal fin to expose the testes for removal. Using forceps and a scalpel, the whole, intact testes were removed from the connected intestinal tissues.

Spawning Protocol

When a female was determined to be ready to spawn, the following spawning protocol was used based on Parsons (1999). The ovulated female and a male from the same treatment and same replicate, rectangular tank were netted and stunned using the electro-narcosis methods described previously. Once shocked, testes were excised from the male, placed on a sterile petri dish, weighed and minced with a scalpel. A small sample (approximately 100 µl) of fresh sperm was activated by adding 1-2 ml water and

observed under binocular dissecting scope (Meiji Techno America, San Jose, California) at 10x magnification to confirm sperm motility before spawning. The female eggs were then stripped, by applying strong, downward pressure to the lower abdomen, into a plastic bowl, where they were mixed with fresh semen squeezed through a standard aquarium net (Aqueon, Franklin, WI) to filter the semen and prevent large tissue pieces from mixing with the eggs. The eggs and aquarium net were then immediately rinsed with 25-50 ml salt-urea solution (11.34 g Urea/3.785 L H_2O and 15.12 g NaCl/3.785 L H_2O) and gently mixed with a turkey feather for 2 min. After 2 min, an additional 50-100 ml salt-urea solution (depending on egg quantity) was added and gentle mixing with a turkey feather continued for 10 min more. Eggs were then slowly rinsed 2-3 times with fresh water until free from floating debris and broken egg membranes, at which point the eggs settled to the bottom and a clear solution remained in the bowl.

The eggs were then poured into a 50 or 100 ml graduated cylinder depending on egg volume, allowed to settle for 1 min, and a volumetric measurement was taken. Pre and post fertilization egg measurements in graduated cylinders indicated the eggs swell to nearly double in size after the spawning procedures were completed. The fertilized eggs were then placed into a vigorously aerated McDonald-type hatching jar (Pentair-Aquatic Ecosystems, Apopka, FL) filled with 3 L tannic acid solution (1 g $C_{76}H_{52}O_{46}$/ L H_2O) and allowed to mix for 2 minutes to remove the adhesive coating on the eggs. After the tannic acid bath, the hatching jars were set up on an incubation table and supplied with a slow, continuous flow of surface water until hatching was completed.

Spawning and Fertilization Assessment

Spawning success (spawned females/total females) and mean %-fertilization were used to assess the overall effectiveness of each hormone treatment. To determine percent fertilization, egg samples (n > 150) were collected from each hatching jar at 12 and 24 hr post-fertilization and placed on a 5 mm ruled microscope slide, 1 mm^2 grid (Neo Science Corp., Nashua, NH), which allowed the eggs to be counted on a grid. A binocular dissecting microscope (EMT-4Meiji Techno America, San Jose, California) at 10x magnification was used for all samples. Total and viable egg counts were taken 3 times per spawning pair per time period to determine mean fertilization over time. Viable eggs were defined as having an intact chorion and vitelline membrane, opaque coloration and were free of parasites (Figure 4).

Experiment 2

A second induced spawning experiment was conducted from 5 May 2014 to 7 May 2014 to assess the effectiveness of GnRHa (Ovaprim®, salmonid GnRHanalogue 20 µg/ml + domperidone 10 mg/ml)(Syndel Laboratories, Qualicum Beach, British Columbia, Canada) and LHRHa hormones for inducing crappie to spawn. LHRHa was chosen for the second experiment due to successful spawning results from Experiment 1. Tricaine-methanesulfonate (MS-222) was chosen as an alternative sedation method to electroshocking for Experiment 2 to determine if MS-222 caused less sedation and handling stress, and thus improved survival rates during spawning.

For the second experiment a total of 32 crappie were used, with 8 males and 8 females for each treatment (LHRHa and GnRHa) placed into two of the same, large flow-through tanks described in Experiment 1. Before tank transfer, each fish was anesthetized

using 50 mg/L MS-222, weighed to the nearest gram using an Ohaus Model CW11-2EO platform scale (Ohaus Corporation, Parsipphony, New Jersey), and measured to the nearest millimeter on a fish measuring board during initial stocking to determine hormone dosage rates. Water quality variables were measured using a multi-parameter probe (YSI Professional Plus Model #10102030, YSI Inc., Yellow Springs, Ohio). Temperature, dissolved oxygen, total ammonia nitrogen, salinity and pH were measured 3 times per day throughout the experiment (Table 1). A control treatment was not included in this experiment because no spawning occurred for this treatment in Experiment 1.

The dosage rates and injection timing for the LHRHa treatment remained the same as Experiment 1. Dosage for GnRHa was set based upon the manufacturer's recommendation of 0.5 ml GnRHa/kg, with a 10% priming dose and a 90% resolving dose for females 24 hr post priming dose, and a single dosage of 0.5 ml GnRHa /kg for males at the same time as the female resolving dose. All tank slots were visually inspected every 4 hr following hormone injection for the presence of eggs on the tank bottom. No mortality occurred during the experiment. Fish were strip spawned using the methods described previously.

Experiment 3

A third induced spawning experiment was conducted from 13 April 2015 to 17 April 2015, during the natural crappie spawning season of 2015, to further investigate the effectiveness of GnRHa and LHRHa using larger sample sizes, and to determine if the addition of domperidone (a dopamine blocker) (10mg/ml; $C_{22}H_{24}ClN_5O_2$)(Tocris Bioscience, Avonmouth, UK) to LHRHa increases spawning success and %-fertilization.

The GnRHa used already contains domperidone at 10 mg/ml. Latency period, the time from resolving dose to the observation of in-tank ovulation, was also quantified for each spawning female during this experiment. Adult white crappie (n = 109) were collected over a two week period (23 March 2015 to 3 April 2015) from Enid Lake, Mississippi using the same equipment and methods described above. Following transport to the NMFH, Enid, MS, fish were acclimated for > 2 wk using the same acclimation methods as previous experiments. Water quality was monitored daily throughout acclimation and three times per day during the induced spawning experiment using a multi-parameter probe (YSI Professional Plus Model #10102030, YSI Inc., Yellow Springs, Ohio).

White crappie were transferred into the same nine flow-through trough tanks described in Experiment 1 and received surface water from Enid Lake (Table 2). Three replicate tanks were used for each hormone treatment (GnRHa, LHRHa and LDOM), where 3 crappie, either male or female, were randomly transferred into each of the four divided sections of each tank for a total of 6 potential spawning pairs/replicates and a total of 18 males (mean Wt. = 557 g ± 148 g, mean TL = 328 ± 29 mm) and 18 females (mean Wt. = 653 ± 228 g, mean TL= 346 ± 35 mm) per treatment. Before tank transfer, crappie were anesthetized using MS-222 (50 mg/L) until lethargic (~5 min), then measured and weighed.

Hormone injection methods remained the same as those used in Experiments 1 and 2, where an initial priming injection, 10% total dose, was given to all females following sedation and measurement, and a 90% resolving dose was given to all females 24 hr post priming dose. All males received a 100% dose at the same time as the resolving dose was administered to females. Dosage rates for GnRHa and LHRHa

remained the same as those used in Experiments 1 and 2. LDOM was prepared by dissolving domperidone (10 mg/ml) into 0.5 mg/ml LHRHa. Four females died during the experiment, all showing signs characteristic of acute bacterial septicemia and water mold infection (*Saprolegnia* spp.), such as epidermal ulceration, fin and gill necrosis, fin base hyperemia and the presence white cottony growth around the caudal peduncle and fin. Upon the observation of eggs on the tank bottom or eggs releasing from the female urogenital opening, crappie were spawned using the methods described above.

Statistical Analysis

All data were analyzed using program R (R Foundation for Statistical Computing, Vienna, Austria). Equal variance between treatment means was tested for using the Bartlett test and normality of data was confirmed using the Shapiro-Wilk normality test. A one-way analysis of variance (ANOVA) was used to determine if a difference existed between hormone treatments. Tukey's honestly-significant-difference test was used to identify significant differences between treatment means, where $\alpha = 0.05$.

Results

Tank Acclimation

Tank acclimation survival throughout Experiments 1 and 2 was 100% (n = 96). Only 2 fish died during the acclimation phase of Experiment 3, where both occurred within 48 hr following wild capture and tank transfer (n = 109). There were no differences in water quality variables (temperature, pH, DO and total ammonia nitrogen) among treatments during each experiment (Tables 1 and 2). A total of 10 crappie died

due to bacterial disease during Experiments 1, 2 and 3, following transfer into the flow-through experimental tanks.

Experiments 1 and 2

Spawning occurred in all three-hormone treatments from Experiments 1 and 2 (spawning success: LHRHa = 65%; GnRHa = 50%; HCG = 17%), but none of the crappie in the control treatment spawned. GnRHa produced the highest fertilization rates at 24-hr post-spawn (mean ± standard error (SE); 68.33 ± 8.78%; n = 4), while LHRHa had the lowest fertilization rates at 24 hr post-spawn (12.36 ± 4.79%; n = 7)(Figure 1). The HCG treatment experienced high mortality before spawning during Experiment 1 (54.17%), and variable mean fertilization rates (28.64 ± 12.06%, n = 3) from the three-crappie pairs that did spawn. There was 77% male mortality in the HCG treatment within 12 hr of HCG resolving injections, while only 11% of the males in the LHRHa treatment and 5% of the males in the control treatment died throughout the entire experiment. A difference occurred between GnRHa and both LHRHa ($p < 0.001$) and HCG ($p = 0.051$) treatments, where GnRHa had the highest %-fertilization (Figure 2). There was no difference between LHRHa and HCG ($p = 0.442$).

Experiment 3

During the 2015 induced spawning experiment, there was no difference in water quality variables (temperature, pH, D.O. and total ammonia nitrogen) among tanks at each time point (Table 2). Spawning occurred in all three hormone treatments (spawning success: LHRHa = 64%; LDOM = 69%; GnRHa = 50%) with no difference in success among treatments. No difference was found in latency period among treatments (latency

period (hr): LHRHa = 44.7 ± 6.8; LDOM = 43.8 ± 8.3; GnRHa = 43.9 ± 8.6)(p=0.74). In terms of egg volume produced by each spawning female, there was no difference among hormone treatments (egg volume (mm): LHRHa = 32.6 ± 19.4; LD = 26.9 ± 20.6; GnRHa = 46.4 ± 40.1). Also, there was no difference in %-fertilization among hormone treatments (%-fertilization: LHRHa = 34.1% ± 29.0; LDOM = 34.3% ± 33.9; GnRHa = 34.1% ± 37.4)(Figure 3). No correlation was found between female body weight and %-fertilization ($r^2 < 0.01$), nor egg volume produced ($r^2 < 0.17$). Female body weights were different between LHRHa and GnRHa ($p = 0.02$), however body weight was not different between LHRHa and LDOM ($p=0.08$), nor between LDOM and GnRHa ($p = 0.58$). There was no difference in non-spawning female GSIs among hormone treatments, and no difference in female body weights.

Discussion

Knowledge of adult crappie tank acclimation and controlled spawning methods, using hormones to induce ovulation in tanks, is limited and hampers annual hatchery crappie production. These studies demonstrate adult white crappie can be acclimated to tank systems using a salinity of 3-5 ppt and cool water temperatures (16° C), resulting in low mortality (< 5%). Following acclimation (2-3 wk), adult crappie gonad maturation and ovulation can be induced in tanks using GnRHa or LHRHa, with fertilization rates of approximately 40% following strip-spawning.

The lack of effective tank culture methods for adult crappie limits development of reproduction and culture techniques. In this study, crappie were acclimated to tanks using low temperature (~16° C) and low salinity (3-5 ppt). These conditions improved adult survival in tanks presumably by reducing metabolic rates and preventing bacterial

growth. Cool water temperatures and sodium chloride have been used by aquaculturists for decades to prevent bacterial pathogens from inducing disease in freshwater fish reared in high-density pond and tank environments (Smeltzer and Flickinger 1991; Plumb and Shoemaker 1995). *Flavobacterium columnare* was the primary cause of mortality in this study. Notably, growth and survival of *F. columnare* is reduced at temperatures < 20° C and at salt concentrations > 3 ppt (Plumb 1999), which is presumably why using these acclimation conditions greatly improved survival. Some mortality (< 10%) did occur during the spawning experiments following transfer into flow-through experimental tanks, which had 0 ppt salinity and no UV sterilization. Therefore, maintaining crappie in a controlled recirculating system, where salinity and UV sterilization can be maintained, may further enhance adult survival.

Literature on spawning methods for crappie is limited. There are no known published experiments comparing the efficacy of different hormones for induced spawning nor any reproduction data on %-fertilization or spawning success rates. Although studies using LHRHa and HCG to spawn crappie have been conducted (Al-ablani and Phelps 1997; Gomelsky et al. 2000; Arslan and Phelps 2004; Cuevas-Uribe et al. 2009) these experiments focused on monosex crappie production. While these publications demonstrated LHRHa and HCG can effectively induce crappie to spawn in tanks, methods for hormone administration and spawning were not presented. Gomelsky et al. (2000) induced crappie to spawn in tank systems, during the natural spawning season, using a similar dosage of LHRHa to that administered in this study (100 µg LHRHa/kg). Additional monosex production experiments used HCG to spawn black crappie at a rate of 1000 IU/kg for females and 500 IU/kg for males, although spawning

data were not presented (Al-ablani and Phelps 1997; Arslan and Phelps 2004; Cuevas-Uribe et al. 2009; Gomelsky et al. 2000). However, in this study, the HCG treatment, with an equal dosage rate, experienced high mortality (54%) in Experiment 1, and produced poor fertilization rates (29%) and spawning success (spawning females/total females) rates (17%). The cause of mortality following HCG administration is unknown. In comparison, mortality was low (< 5%) in LHRHa, GnRHa and control treatments during all experiments.

Domperidone has been shown to increase spawning success rates and to decrease latency period variability in other fishes (Sahoo et al. 2005; Wang et al. 2010). In this study spawning success rate and %-fertilization did not change with the addition of domperidone between LHRHa and LDOM treatments. Further, latency period was also not affected by domperidone. Previous induced spawning studies have found both positive (Wang et al. 2010) and neutral results (Copeland and Thomas 1989; Van Eenennaam et al. 2008) from adding dopamine antagonists, suggesting that the efficacy of domperidone may be species dependent. For crappie, this study suggests domperidone does not affect spawning success, %-fertilization, or latency period.

Variability in %-fertilization between experiments may be due to several factors, any of which could have resulted in greater variability in gonadal development between individuals. Results from Experiment 3 show LHRHa and GnRHa were not different in terms of spawning success or %-fertilization, however in Experiments 1 and 2 GnRHa %-fertilization was higher than LHRHa, (GnRHa= 68.3%; LHRH= 12.4 %). One factor that may be attributed to this difference in %-fertilization was the approximately 1° C difference in system water temperature between 2014 (20.3° C) and 2015 (19.4° C)

experiments. Water temperature is known to influence gonadal investment and maturation timing in iteroparous fishes (Rideout et al. 2005), and field evidence suggests temperature controls white crappie gonadal development and spawning timing (Bunnell et al. 2007). Two other factors were greater environmental variability and differences in collection duration between experimental years. In 2015 (Experiment 3), a strong cold front at the beginning of the natural spawning season resulted in sharp decline in water temperature (2-3° C) over 3 d, reducing the broodstock collection time to 1 wk, and also resulting in a poor recruitment year for the lake. In 2014 (Experiments 1 and 2), crappie were collected over a longer time (3 wk) during the natural spawning season encompassing a broader range of lake temperatures and weather conditions. These conditions may have led to more synchronous development for females in Experiment 3 and greater differences in gonadal development between females for Experiments 1 and 2, although this was not assessed to reduce stress to the fish before spawning. In these studies, maintaining broodstock health and minimizing stressors, such as capture, aerial exposure, sedation and cannulation required for maturation assessment, were paramount as these were the first experiments conducted to induce spawning in white crappie in tanks.

These experiments demonstrate adult white crappie can be maintained in recirculating tank systems through an initial 2 wk acclimation manipulating salinity and temperature. Following acclimation, LHRHa and GnRHa both effectively induced final gonadal maturation and ovulation in tanks, with GnRHa performing more consistently across all spawning experiments (mean spawning success = 50% in Experiments 1, 2 and 3; %-fertilization rates: Experiments 1 and 2 = 68.3%; Experiment 3 = 34.1%). Future

research may benefit from a focus on: varying dosage levels of GnRHa and LHRHa to further enhance spawning success and %-fertilization, manipulating tank water temperatures to determine optimal spawning conditions, and developing maturation assessment techniques to determine the extent of seasonal maturation synchronicity in white crappie.

Tables

Table 3.1 Mean water quality variables during acclimation and Experiments 1 and 2

	Acclimation			Experiment 1			Experiment 2		
	Mean	SE	*n*	Mean	SE	*n*	Mean	SE	*n*
Temperature (°C)	17.10	0.42	14	20.10	0.03	13	20.54	0.01	9
Dissolved Oxygen (mg/L)	6.68	0.84	14	7.27	0.10	13	6.01	0.21	9
pH	7.79	0.38	14	6.98	0.00	13	6.67	0.01	9
Total Ammonia (mg/L)	0.40	0.23	14	0.00	0.03	13	0.02	0.01	9
Salinity (ppt)	4.77	1.09	14						

Temperatures from 3-4 d of increasing temperatures during acclimation phases were not included in the mean temperatures presented in the acclimation phases. In each period, *n* represents the daily mean from all experimental tanks. There were no significant difference between experiment water quality variables within each experiment ($p < 0.05$)

Table 3.2 Mean (± SE) water quality variables during acclimation and Experiment 3

	Acclimation			Experiment 3		
	Mean	SE	*n*	Mean	SE	*n*
Temperature (°C)	15.36	0.93	15	19.36	0.12	16
Dissolved Oxygen (mg/L)	6.72	0.57	15	6.53	0.49	16
pH	7.96	0.42	15	7.31	0.29	16
Total Ammonia (mg/L)	0.03	0.03	15	0.01	0.01	16
Salinity (g/L)	4.26	0.84	15			

Temperatures from 3-4 d of increasing temperatures during acclimation phases were not included in the mean temperatures presented in the acclimation phases. In each period, *n* represents the daily mean from all experimental tanks at that single time point. There was no significant difference between tank water quality variables within each experiment ($p < 0.05$).

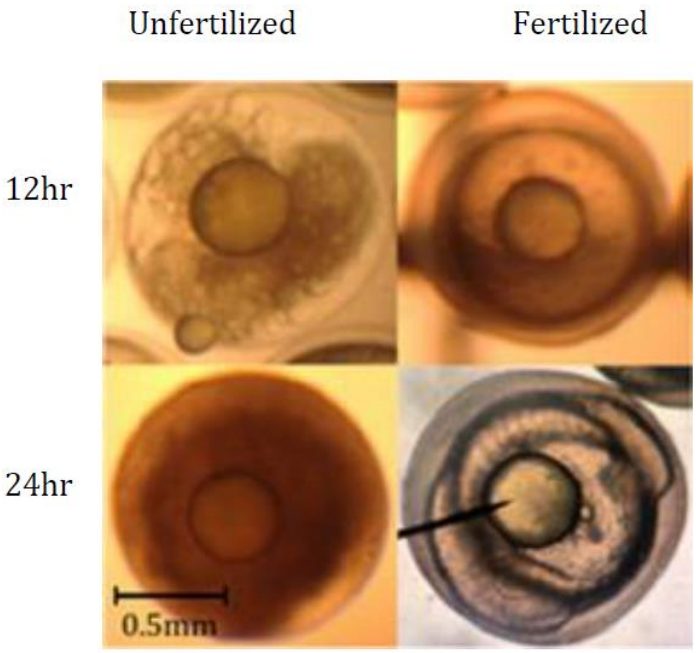

Figure 3.1 Representative unfertilized and fertilized white crappie *Pomoxis annularis* eggs at 12 and 24hr post-fertilization

The needle in the 24hr post-fertilization photograph indicates the yolk-sac.

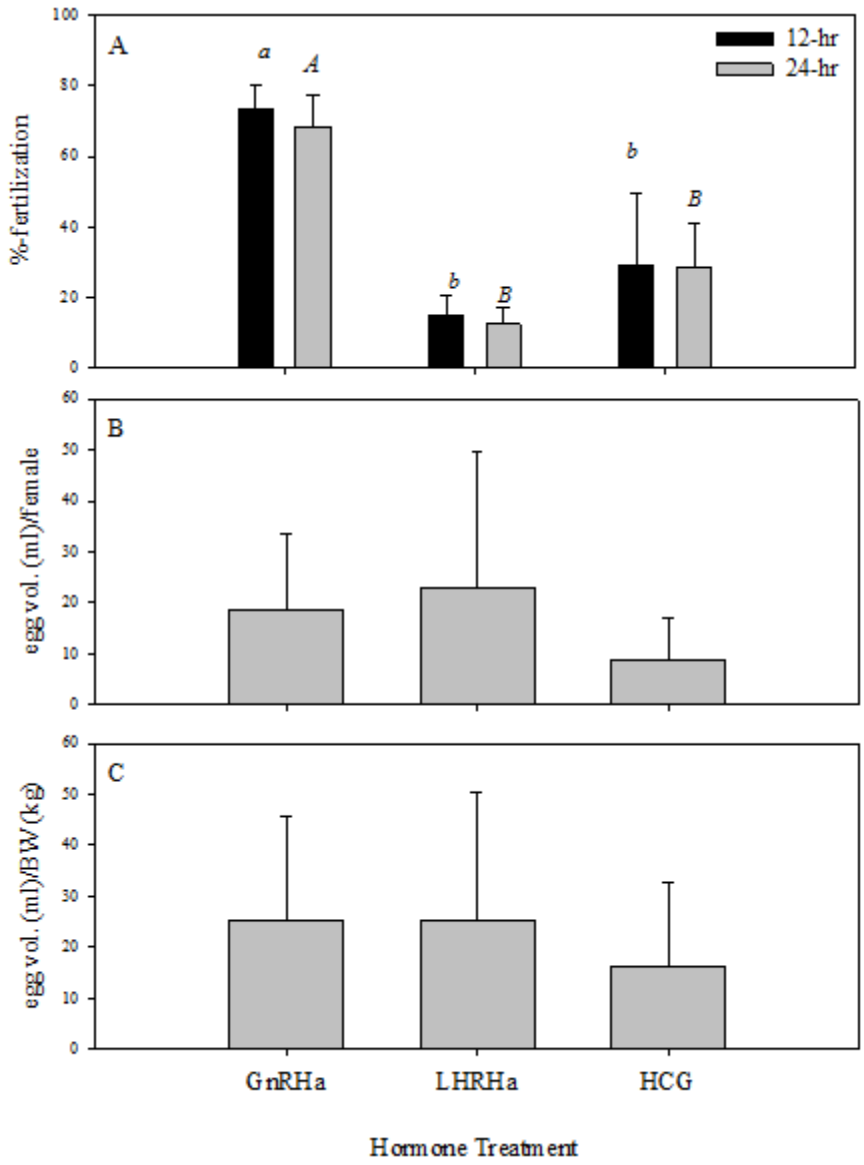

Figure 3.2 Comparison of mean ± SE %-fertilization (A), total volume of eggs (hydrated and fertilized) spawned/female (B) and egg volume (ml)/kg body weight (BW)(C) of spawning white crappie *Pomoxis annularis* between hormone treatments

Hormone treatments (salmonid gonadotropin releasing hormone analogue (GnRHa), *n*=4; luteinizing hormone releasing hormone analogue (LHRHa), *n*=7; human chorionic gonadotropin (HCG), *n* =3) were examined in Experiments 1 and 2. Letters beside each treatment represent differences in a two-way ANOVA ($p<0.05$) using Tukey's honest-significant-difference test.

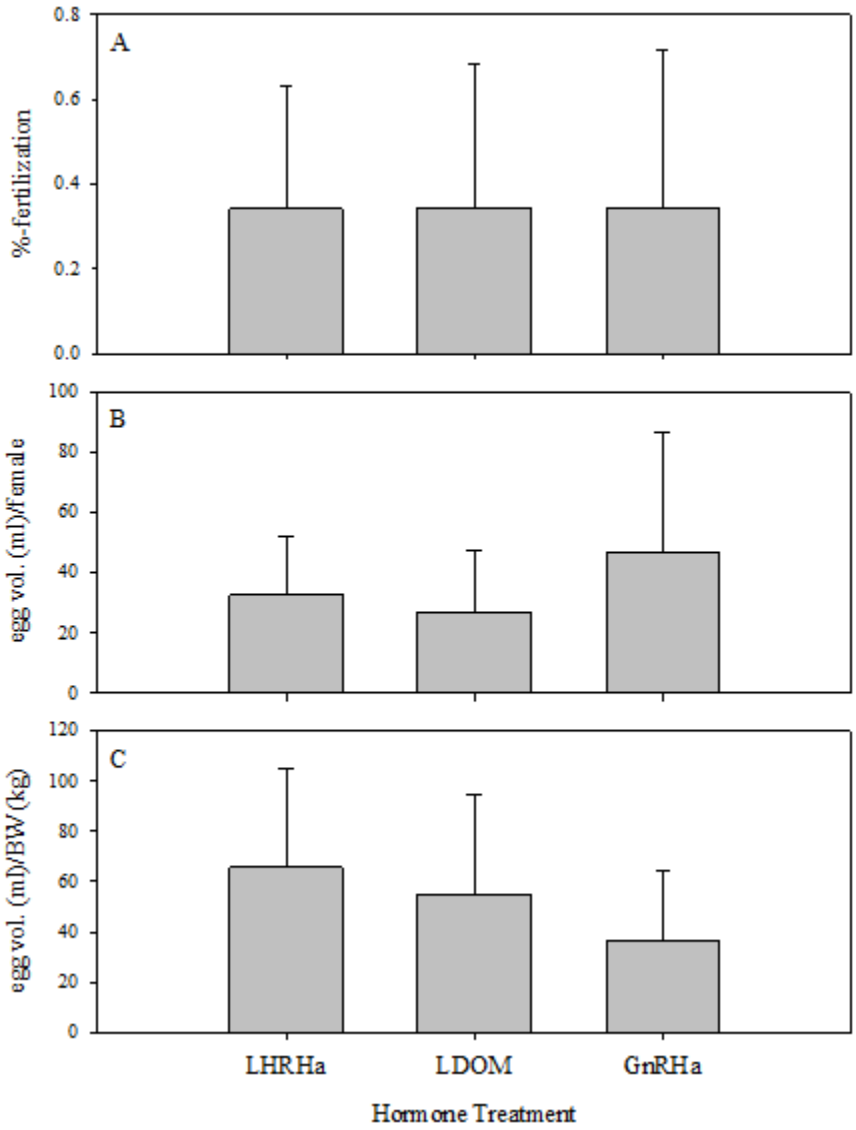

Figure 3.3 Comparison of mean ± SE %-fertilization (A), total volume of eggs (hydrated and fertilized) spawned/female (B) and egg volume (ml)/kg body weight (BW)(C) of spawning white crappie *Pomoxis annularis* between hormone treatments

Hormone treatments (luteinizing hormone releasing hormone analogue (LHRHa), n=9; luteinizing hormone releasing hormone analogue + domperidone (LD), n=11; salmonid gonadotropin releasing hormone analogue (GnRHa), n=7) were examined in Experiment 3. No significance differences ($p<0.05$) were identified using a one-way ANOVA

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CHAPTER IV

WHITE CRAPPIE *POMOXIS ANNULARIS* TANK CULTURE AND OUT-OF-SEASON SPAWNING

Abstract

While extensive crappie culture has been practiced for decades, limited knowledge of crappie aquaculture methods has hampered white crappie *Pomoxis annularis* production potential to a small timeframe during the natural spawning season which occurs only once annually. Out-of-season spawning of white crappie could greatly enhance annual production potential. Therefore, white crappie out-of-season spawning experiments were conducted from November-February in recirculating tank systems. Following a 2 wk winter (10° C, 8 hr light), photoperiod and temperature were increased to spring (22° C, 16 hr light) conditions over 3 wk (1° C and 30 min increase every 2 d) and 6 wk (1° C and 30 min increase every 4 d). Fish were injected with gonadotropin releasing hormone analog (GnRHa) (100%-priming dose, 100%-resolving dose; 24 hr and 48 hr later) and strip-spawned upon ovulation. The 3-wk treatment (11females:10 males) induced 1 female to spawn with 11% fertilization rate. The 6-wk treatment (12 females:10 males) induced 2 females to spawn with fertilization rates of 31.5% and 79.2%. Gonads of unspawned females were sampled at 96 hr post-resolving dose to assess reproductive maturity. Gonadosomatic index (GSI) and egg diameter (ED) from un-spawned females (3-wk GSI = (mean ± SE) 0.02 ± 0.01; ED = 0.71 ± 0.22, 6-wk GSI

= 0.03 ± 0.02; ED = 0.72 ± 0.10) were compared with wild females sampled during December (Winter; GSI = 0.02 ± 0.01; ED = 0.53 ± 0.07) and April (Spring; GSI = 0.04 ± 0.01; ED = 0.90 ± 0.12). No difference between 6-wk GSI and Spring GSI was detected; however, ED was smaller for 3-wk and 6-wk treatments when compared to Spring. Therefore, out-of-season spawning of white crappie, through using recirculating tank systems and hormone-induced spawning is possible, but a longer seasonal shift regime is needed to enhance spawning success.

Introduction

Despite the popularity and socioeconomic value of crappie fisheries (Miranda et al. 2013), crappie population management has provided a difficult challenge to fisheries biologists for decades. Boxrucker and Irwin (2002) provided a synthesis of the fisheries research and challenges hindering crappie fisheries management. Typically, both white crappie *Pomoxis annularis* and black crappie *P. nigromaculatus* overpopulate their environments, particularly in small impoundments (< 20 ha) limiting crappie fisheries to large lakes and reservoirs (Busack and Baldwin 1988; Mitzner 1991; Allen and Miranda 2001). Crappie populations often fluctuate, with a dominating single year-class produced every 2-5 yr with low recruitment and stunted year-classes occurring in between (Swingle and Swingle 1967; Busack and Baldwin 1988; Parsons 1996; Miranda et. al. 2013).

To address population fluctuations and enhance hatchery production potential, studies on indoor crappie culture including tank acclimation, feeding, and spawning methods have been conducted, but techniques are not well-established. Hatchery research advances in crappie aquaculture over the past few decades indicate juvenile crappie can be cultured in high-density indoor systems (Willis and Flickinger 1981; Parsons 1999;

Dudenhoeffer et al. 2014). Limited research has been conducted, mainly focusing on production of infertile, monosex or triploid crappie for stocking in small water bodies (Baldwin et al. 1990; Al-ablani and Phelps 1997; Parsons 1999; Arslan and Phelps 2004 and Cuevas-Uribe et al. 2009). Studies using adult crappie in tank culture systems have been hindered by disease-related mortality. Disease outbreaks have been attributed to bacterial infections, notably *Flavobacterium columnare* and *Aeromonas* spp., which commonly affect adult crappie collected from natural systems in the first 2 wk following transfer to tanks (Smeltzer and Flickinger 1991; Culpepper and Allen Chapter 3). Recent advances indicate acclimation conditions can minimize disease through using cool temperatures (~15° C) and low salinity (3-5 ppt) (Culpepper and Allen Chapter 3).

Feed training adult crappie is necessary to any induced spawning experiment, because of the direct link between nutritional status and gonadal resource investment and fecundity in iteroparous fishes (Rideout et al. 2005). Feed training juvenile crappie has been investigated in both tanks and raceways (Smeltzer and Flickinger 1991; Thomas 1995; Hayward and Arnold 1996; Parsons 1999; Dudenhoeffer et al. 2014). However, no experiments have been conducted in adult crappie feed training with either live or formulated diets. Anecdotally, adult black crappie have been successfully feed-trained in large recirculating aquaria (tank volume = 567,750 L) starting with fresh, chopped smelt *Osmeridae* spp., and eventually weaning adults to a diet of mixed, frozen smelt, cod *Gadus morhua*, whiting *Merluccius bilinearis* and haddock *Melanogrammus aeglefinus* (Rob Mottice, Manager, Tennessee Aquarium, personal communication). Adult black crappie broodstock have also been maintained in ponds (0.4 ha) at low density (~0.0015 fish/m^3) by providing large numbers of small, (~5cm total length (TL)) live koi *Cyprinus*

carpio (Charles Silkwood, Mississippi Dept. of Wildlife, Fisheries and Parks, personal communication). Notably, there is no documented acceptance by adult crappie of live feed in tanks or formulated feed, which hinders long-term tank culture efforts.

Several publications indicate black crappie can be induced to spawn using luteinizing hormone releasing hormone analogue (LHRHa) and human chorionic gonadotropin (HCG) either volitionally in tanks (Al-ablani and Phelps 1997; Arslan and Phelps 2004; Gomelsky et al. 2005; Cuevas-Uribe et al. 2009) or strip spawned following the observation of spawning behavior (Gomelsky et al. 2000 and 2002). However, these studies did not conduct comparative experiments to determine the culture conditions and most effective hormone for inducing spawning in adult crappie. A recent spawning experiment on white crappie found ovulation can be induced in tanks using salmonid gonadotropin releasing hormone analogue (GnRHa)(0.5ml GnRHa/kg) or LHRHa (100 µg LHRHa/kg) (Culpepper and Allen, unpublished). Collectively, these studies facilitate the investigation of spawning crappie out-of-season.

Out-of-season spawning has been practiced in the aquaculture industry for decades (Morris and Clayton 2009), and has been successfully employed in numerous fish species (Eurasian perch *Perca fluviatilis*, pike perch *Sander lucloperca*, common carp, channel catfish *Ictalurus punctatus*, and red sea bream *Pagrus major*)(Lang and Tiersch 2007; Ronyai 2007; Kucharczyk et al. 2008; Biswas et al. 2010; Targonska et al. 2014). While techniques for out-of-season spawning vary depending on the specific spawning needs of a given fish species, the overall goal is the artificial simulation of winter to spring environmental conditions. This is achieved through precise control of photoperiod and/or temperature to induce gonad maturation and the application of

induced spawning hormones, depending on species (Tate and Helfrich 1998; Morris and Clayton 2009; Matthews and Stout 2013).

A few studies have shown out-of-season tank spawning of centrarchids is possible, yet research on these same production techniques have not been applied to crappie (Morris and Clayton 2009). Both bluegill *Lepomis macrochirus* and largemouth bass *Microterus salmoides* have been successfully spawned out-of-season (Mischke and Morris 1997; Matthews and Stout 2013). Mischke and Morris (1997) spawned bluegill in tanks through photoperiod and temperature manipulation without using reproductive hormones, providing a foundation for in-tank, out-of-season spawning techniques in centrarchids. Winter (16 h light; 15° C) conditions were maintained for 1 mo, followed by gradual change in photoperiod and temperature to spring spawning conditions (14 h light; 24° C) where fish volitionally spawned after 2 wk (Mischke and Morris 1997). Experiments on largemouth bass followed similar methodology, gradually adjusting photoperiod and temperature from winter (8 h light;10° C) to spring (14 h light; 23° C) over a 90 d (Matthews and Stout 2013). Similar to bluegill, largemouth bass readily spawned in-tanks without the need for artificial spawning hormones (Matthews and Stout 2013). However, bluegill are more easily cultured than crappie and have different feed and density requirements, even in pond aquaculture (Morris and Clayton 2009). For crappie, a single out-of-season spawning pilot experiment on black crappie manipulated photoperiod and temperature from winter (8 h light; 10° C) to spring spawning conditions (16 h light; 18° C) over 3 wk, however, only one female exhibited gonadal development, but died before spawning (Parsons 1992).

Increasing crappie annual production through out-of-season spawning techniques would have many potential benefits, such as the stabilization of populations through stock enhancement programs and expanding the potential for crappie to be further developed into a food fish (Arslan and Phelps 2004; Cuevas-Uribe et al. 2009 and Dudenhoeffer et al. 2014). Previous research on out-of-season spawning of other centrarchids and recent advances on tank acclimation and induced spawning methods in crappie (Culpepper and Allen unpublished) indicate out-of-season spawning of crappie may be possible. Therefore, the purpose of this study was to develop methods for long-term holding in tanks and the induction of out-of-season spawning in adult white crappie.

Methods

Fish Collection

Adult white crappie were collected from Grenada Lake, Mississippi, on 11 November 2014 and 21 November 2014 from tributaries and the headwaters of the main reservoir. Crappie were collected and transported as described previously (Culpepper and Allen unpublished). Before tank transfer, each fish was weighed to the nearest g, TL was measured to the nearest mm, and sex was determined based on visual cues. Literature related to crappie spawning does not describe non-lethal methods for determining sex and gonad maturation state. Observations from previous experiments indicated visual cues (i.e., morphology and color) were helpful in determining sex (Culpepper and Allen unpublished). Notably, the relative size of the urogenital opening compared to the anus was an indicator to differentiate males from females. Males generally had similar sized urogenital opening and anus, while females generally had an enlarged urogenital opening compared to the anus, particularly when in spawning condition (based on personal

observations). Body morphology was also an indicator, with males being generally thinner below the lateral line than females and darker in pigmentation. These cues were used to guide sorting fish collected out of season, but were not as pronounced as during the normal spawning season. Similarly, although insertion of a catheter into the urogenital opening for collection of eggs or sperm samples was successful during the spawning season, it was not useful for sorting crappie collected outside the spawning season.

A total of 48 white crappie (24 males, 24 females) were placed into two separate, identical recirculating aquaculture systems (RAS) (Table 4.1). Each RAS contained ten, 330-L, fiberglass, circular tanks stocked with 2-3 male or female crappie per tank. Crappie were sorted by visual cues and weight, and stocked equally between systems and treatment tanks. Tank biomass, in terms of kg fish/tank, and treatment population, in terms of males and females/treatment system, were kept as uniform as possible among tanks and treatments. One RAS (~9000 L total volume) was used for each temperature and photoperiod treatment, and included an 85-L bead filter with mixing impellor (PBF-3, Aquaculture System Technologies, New Orleans, LA), an 80-watt high output ultraviolent sterilizer (Emperor Aquatics, Pottstown, PA), a heat exchanger (Titan HP-7 in-line water heater, Aqua Logic, San Diego, CA), two in-line water heaters (Elecro EHE25T, Elecro Engineering, Stevenage Hertfordshire, UK) and a bio-media sump (1022-L).

Tank Acclimation

Previous experiments on tank culture systems demonstrate controlling salinity and temperature are important for preventing disease related mortality in crappie during the

first 2-3 wk after being collected from natural water bodies (Culpepper and Allen unpublished). During the 2-wk acclimation, crappie were held at 3 ppt salinity (Instant Ocean aquarium sea salt mixture, Spectrum Brans, Inc., Blacksburg, VA) and 12° C. Temperature, dissolved oxygen and salinity were monitored once daily throughout the experiment using a multi-parameter probe (YSI Model 85, YSI Inc., Yellow Springs, Ohio)(Table 4.2). Total ammonia nitrogen (TAN) and nitrite were checked 3 times per wk from each system using a colorimeter (HACH DR-890, HACH Co., Loveland, Colorado)(Table 4.2). Each system's pH was measured four times per wk using a pH meter (pH10a, YSI Inc., Yellow Springs, Ohio). Unionized ammonia was calculated each time TAN was taken using TAN, pH and temperature data from each system.

Feeding

Crappie were fed live koi (mean individual wt. ± SE; 1.70 ± 0.36 g) and silver shiners *Notropis photogenis* (mean individual wt. ± SE; 2.34 ± 0.22 g) to satiation throughout the experiment. During several pilot feeding experiments, before out-of-season spawning efforts, white crappie were offered numerous types of feed, including: several sinking and floating formulated feeds, red worms *Eisenia fetida*, fresh-chopped gizzard shad *Dorosoma cepedianum*, and frozen squid and shrimp. However, only small, live fish (koi and shiners) were consumed by the adult crappie. Each tank was supplied with 2 feeder fish per crappie per day. If all fish were consumed by the next feeding (24 h later), the tank's feed rate was increased by 1 feeder fish per crappie. When feeder fish were found left over at the next day's feeding, the feed density was maintained at the current feed rate by adding only replacement fish, ensuring feeder fish were always available. During the last 30% of the experiment, silver shiners were used instead of koi

because adequate numbers of koi could not be obtained. Shiners were on average 0.64 g larger than the koi used for the majority of the 3-wk treatment. Food consumption was recorded daily for each tank throughout the experiment to determine overall feed rates. Percent body weight consumed/day (%BW/d) was based on mean tank consumption day^{-1} divided by number of crappie in a tank, assuming equal consumption within a tank.

Photoperiod and Temperature Manipulation

Photoperiod and temperature were controlled in each system throughout the experiment. All tanks used were housed in an enclosed room where photoperiod could be controlled. A 0.51mm reinforced plastic sheet (Yunker Plastics Inc., Elkhom, Wisconsin) was placed between the two treatments to enable two separate photoperiod regimes within the same enclosed room. Light intensity/temperature data loggers (Model #: UA-002-64, Oneset Computer Corp., Bourne, Massachusetts) were installed in four tanks, with tank locations evenly distributed in each treatment system to monitor light intensity (lux) and temperature (°C) every 5 min throughout the experiment (Figure 4.1). Light was provided by 121.9 cm, T8 florescent bulbs distributed evenly across all tanks at an average of (mean ± SE) 98.53 ± 13.37 lux (Figure 4.1). Temperature was maintained within ± 1° C using the RAS in-line heater exchanger (Elecro EHE25T, Elecro Engineering, Stevenage Hertfordshire, UK) and two in-line water heaters (Titan HP-7 in-line water heater, Aqua Logic, San Diego, CA), which controlled water temperature via a digital thermostat. On 12 December 2014, following a 2-3 wk acclimation, winter phase (10° C, 8 hr light) began for both treatments and was maintained for 2 wk. For the 3-wk treatment, temperature was raised 1° C and photoperiod was increased by 30 min every other day until spawning conditions were reached (22° C and 16 hr light; 24 d).

Photoperiod and temperature in the 6-wk treatment were increased every four days by the same interval, 1° C and 30 min respectively, until also reaching spawning conditions (48 d).

Hormone Induction

Previous experiments identified GnRHa (Ovaprim®, salmonid GnRHanalogue 20 µg/ml + domperidone 10 mg/ml)(Syndel Laboratories, Qualicum Beach, British Columbia, Canada) as an effective spawning induction hormone for white crappie (Culpepper and Allen unpublished). Therefore, GnRHa was used for all out-of-season spawning injections (25 gauge; 1.59 cm needle) at a dosage rate of 0.5 ml GnRHa/kg . A 100% dose was given intramuscularly (IM) approximately 2 cm below the dorsal fin to both males and females in the 3-wk treatment on 12 January 2015, following the seasonal shift. Fish were netted and gently held in the water against the tank wall for injections to reduce stressors, such as aerial exposure, net transportation and sedation. Tanks were monitored every 4-5 hr following hormone injections for the presence of eggs on the tank bottom or what was considered to be female spawning cues, consisting of: pale pigmentation, lethargy, and a positively angled orientation (i.e., where the head is oriented higher than the caudal fin and fish suspend within the water column instead of remaining on the tank bottom where most adult crappie preferred to locate). These spawning cues were based on observation and may not correlate directly with female maturation. Several studies have confirmed the viability of using repeated hormone injections to induce spawning in fish (Watanabe and Carroll 2001; Mylonas et al. 2003 and 2010). Therefore, after the first 48 hr, another 100% dose of GnRHa was given intraperitoneally (IP) 2 cm anterior to the urogenital opening to all un-spawned females.

Fish in the 6-wk treatment were injected using the same methods as the 3-wk treatment on 2 February 2015 and again 48 hr later. Spawning procedures and fertilization assessment followed previously described methods (Culpepper and Allen unpublished). Out-of-season spawning experiments concluded at 96 hr post initial hormone injection.

Gonad Sampling

All crappie that failed to spawn at the end time point (96 hr post initial hormone injection) were anesthetized (MS-222, 250 mg/L) for gonad removal and reproductive maturation assessment (gonad weight, egg diameter assessment and gonadosomatic index). Gonadosomatic index (GSI), an index of the gonad weight relative to fish weight was calculated, according to Ricker (1975):

$$GSI = \frac{gonad\ wt\ (g)}{fish\ body\ wt\ (g)} \text{ x } 100 \qquad (4.1)$$

Each crappie was weighed, measured and gonads were removed by dissection. Ovaries and testes were weighed and preserved in 10% neutrally buffered formalin (100 ml/L, 37-40% formaldehyde; 900 ml/L deionized water; 4.0 g/L sodium phosphate monobasic; 6.5 g/L sodium phosphate dibasic). Twelve additional white crappie gonad samples were collected from Eagle Lake, Vicksburg, Mississippi on 17 December 2015 to compare GSIs during winter and spring seasons.

Following fixation, tissue samples were processed at Mississippi State University College of Veterinary Medicine's histology laboratory to determine mean oocyte size in different treatments. Each tissue sample was infiltrated and embedded in paraffin wax, sectioned to 5 µm and stained with hematoxylin and eosin. Two slides were prepared from each female gonad sample, each slide from a separate tissue sample of the gonad.

Mature egg size for white crappie (Stage III, 0.34 - 0.64 mm egg diameter and IV, 0.58 - 1.02 mm egg diameter; described by Thomas and Kilambi (1981)) were used to estimate fecundity in all female ovary samples. Twenty intact eggs within Stage III and IV (0.34 - 1.02 mm, with an intact chorion and eosinophilic staining) were measured at 10x magnification (Illuminator microscope Model # B-6742DV, Bosch and Lomb, Rochester, NY) to the nearest 0.02 mm from each tissue sample to determine if a difference in mean egg size existed between the reproductive stages sampled. All experiments were conducted under Mississippi State University animal care protocol 13-111.

Statistical Analysis

All data were analyzed using program R (R Foundation for Statistical Computing, Vienna, Austria). Equal variance between treatment means was tested for using the Bartlett test and normality of data was confirmed using the Shapiro-Wilk normality test. A one-way analysis of variance (ANOVA) was used to determine if differences existed among treatments for feeding rate between sexes, body weight of males and females and photoperiod and temperature between systems. ANOVAs were also conducted on spawning success, GSI and egg diameter data. Tukey's honestly-significant-difference *post hoc* tests were used to identify significant differences among treatment means, where $\alpha = 0.05$. A student's t-test was used to determine differences in feed consumption between 3-wk and 6-wk treatments. Data which did not meet assumptions of normality and homogeneity of variance were analyzed using non-parametric Kruskal-Wallis rank sum tests followed by Nemenyi *post hoc* tests.

Results

Feeding and Spawning

No difference in body weight ($p = 0.67$) was found among treatments. There was no difference in feed consumption between 3-wk and 6-wk treatments(mean ± S.E.)(3-wk: 0.33 ± 0.27 %BW/d; 6-wk: 0.41 ± 0.31 %BW/d)(Figure 4.2). A single spawn occurred in the 3-wk treatment at 96 hr after the first GnRHa injection (48 hr after the second injection). The spawning female (wt = 540 g, TL = 329 mm) produced 0.75 ml of eggs, a low spawning volume compared to egg volume produced from females induced to spawn using GnRHa during the natural spawning season (mean ± SE: 11.13 ± 9.83 ml; Culpepper and Allen unpublished), suggesting only partial gonadal maturation occurred in this female. At 24 hr post fertilization, only a small proportion of the eggs produced were fertilized (10.51 ± 2.42%, n = 33 total eggs sampled).

The 6-wk treatment produced two spawns, both at 48 hr post first injection, without the need for an additional dosage of GnRHa. No other spawns occurred following the second hormone injection (0.5ml GnRHa/kg fish wt at 48 hr post initial injection). The first spawning female (wt = 770 g, TL = 368 mm) produced 12 ml of eggs. At 24 hr post fertilization, %-fertilization was 31.94 ± 7.19%. The second female (wt = 245 g, TL = 263 mm) to spawn in the 6-wk treatment produced 1ml of eggs, presumably a partial spawn, although %-fertilization was 74.50 ± 29.92%.

Gonad Samples

There was no difference between male GSI between treatments (3-wk GSI = 0.01 ± 0.00; 6-wk GSI = 0.01 ± 0.00; winter GSI = 0.01 ± 0.00; spring GSI = 0.00 ± 0.00; Kruskal-Wallis, $p < 0.05$)(Figure 4.3). There was a difference between GSI of immature

(< 250 mm TL with yellow-opaque gonads; GIS < 0.008) and mature fish, where mean immature GSI was 20.04% smaller than 3-wk males, 39.74% smaller than 6-wk males, 70.50% smaller than 3-wk females and 82.44% smaller than 6-wk females. For females, there was a difference between immature fish and all other treatments, however no other treatment GSI was different (immature GSI = 0.01; winter GSI = 0.02 ± 0.00; 3-wk GSI = 0.03 ± 0.02; 6-wk GSI = 0.03 ± 0.02; spring GSI = 0.04 ± 0.01; Kruskal-Wallis, $p < 0.05$)(Figure 4.4A).

Immature crappie oocytes were smaller than all other treatments ($p < 0.001$)(Figure 4.4B). However, no difference in egg size was found between 3-wk and 6-wk treatments, which were larger than winter adults but smaller than spring adults. Similarly, crappie female gonad development shows a relationship of increasing egg diameter from immature stages to spring spawning stages, where 3-wk and 6-wk ED are larger than winter ED, but smaller than Spring ED (Figure 4.5).

Discussion

This is the first study to demonstrate white crappie can be induced to spawn out-of-season. Further, this is the first study to describe techniques for long-term (> 60 d) holding of wild adult crappie in recirculating tank systems. Experiments utilized wild-captured adults, which were acclimated to recirculating tank systems using manipulation of temperature and salinity to minimize disease following tank transfer (Culpepper and Allen unpublished), and established feeding techniques resulting in high survival (> 90%) across the entire out-of-season experiment.

Developing methods for tank acclimation, health maintenance and feeding of adult white crappie over a long time were needed any in-tank spawning experiments.

Crappie are easily stressed in tank systems and are prone to systemic bacterial disease without proper precautions, especially in the first few weeks of acclimation following wild capture (Busack and Baldwin 1988; Smeltzer and Flickinger 1991). Little information is currently available on the indoor, tank culture requirements of adult crappie. A short-term adult crappie tank acclimation study by Culpepper and Allen (unpublished) identified that decreasing temperature (< 16^0 C) and increasing salinity (3 – 5 ppt) will minimize mortality during this sensitive time period. In this study, these acclimation methods were used during the first 2 wk following wild capture. Low salinity was maintained throughout the acclimation phase of the out-of-season spawning experiment, and resulted in low mortality rates (< 5%). Two wk before induced spawning all tanks were transitioned to freshwater and mortality remained low.

Several experiments on feed-training larval and juvenile crappie in ponds (Baldwin et al. 1990; Arslan and Phelps 2004; Cuevas-Uribe et al. 2009), raceways (Thomas 1995) and tanks (Dudenhoeffer et al. 2014) have demonstrated young crappie can be trained to accept artificial feeds. Dudenhoeffer et al. (2014) raised juvenile white and black crappie in a RAS with 73-96% mean survival over an 84 d experiment. Feed training juvenile white crappie (4.3g mean weight; 10-15 fish/L) was accomplished over 2-3 wk using a transitionary diet; beginning with live brine shrimp until active feeding occurred, then moving to brine shrimp mixed with starter feed (1.2 or 2 mm, slow-sinking, extruded feed formulated for steelhead (anadromous Rainbow trout) and ground krill (Dudenhoeffer et al. 2014). No literature is available on feed training adult white crappie in tank systems, although public aquariums have reported success using fresh and/or frozen chopped fish, squid or shrimp (Culpepper and Allen, unpublished (Ch1)).

In this study, feed consumption was variable, although there was an overall trend of increasing consumption in the last 6 wk of the experiment. This increase is likely due to crappie acclimating to feed type and tank conditions and increased metabolic requirements with a steadily increasing temperature. Based on visual observation, the high variability in %BW/d observed between tanks within the same treatment was due to individual differences, presumably how well each crappie adapts to tank conditions. A few crappie readily adapted to feeding in tanks, with active feeding occurring within minutes of feeder fish being added to the tanks, while others did not feed over the entire experiment. Several larger crappie exhibited agonistic behavior (territoriality and chasing) during the experiment, possibly affecting food consumption of other crappie in the tank. Similar behavior has been observed with largemouth bass (Petit et al. 2001). Most crappie fed in the absence of human presence and during the night (based on feed consumption from evening to morning), further indicating crappie are sensitive to tank environments. A study examining the effect of white crappie nutritional status before spawning found 92% of female crappie held in experimental ponds exhibited gonad growth and initiated vitellogenesis regardless of feeding rate, including starvation (Bunnell et at.2007), suggesting that the low feed consumption rate in this study (3-wk: 0.33 %BW/day; 6-wk: 0.41 %BW/day) was not a significant factor contributing to gonadal development. Beyond the tank culture and out-of-season spawning methods provided in these experiments, adult artificial feed training poses the largest hindrance towards year-round production and the development of crappie as a food fish. Additional tank culture experiments are needed to determine if adult crappie can be trained to accept artificial feeds. Possible feed training solutions could come from holding crappie over

longer time periods (multiple years), beginning feed training at an earlier age (larvae or juveniles) and manipulating tank density to improve feeding rates. Increasing tank density may reduce agonistic behavior and stressors, improving feeding rates and spawning performance (Baker and Ayles 1990).

Overall the experiment resulted in poor spawning success (one 3-wk and two 6-wk) and low volumes of viable eggs produced. In comparison, other fish species spawned out-of-season have had 50-100% spawning success (Lang and Tiersch 2007; Müller-Belecke and Zienert 2008; Targonska et al. 2014), however in these species tank culture and induced spawning methods were well developed before out-of-season spawning experiments. In this study, although spawning success was low, gonadal development was occurring in females based on increasing GSI and ED. GSI and ED comparisons with wild crappie during winter and spring indicate both the 3-wk and 6-wk treatment groups were in intermediate stages of gonadal development. The 6-wk treatment, while not different than the 3-wk, did have a greater standard error indicating greater variation in maturation stages between females, with a trend towards increased egg diameter with time. Results from gonad histology corroborate the trend observed in female GSI, where mean 3-wk and 6-wk EDs were between winter and spring reproductive stages (ED (mm): winter = 0.53; 3-wk = 0.71; 6-wk = 0.72; spring = 0.90). This is also supported by the finding of Thomas and Kilambi (1981), who defined vitellogenic (Stage III) egg sizes between 0.34 - 0.64 mm and mature (Stage IV) egg sizes between 0.58 - 1.02 mm. Similar sizes of mature black crappie eggs have been reported (1.27 ± 0.02 mm)(Cook et al. 2006). Both white and black crappie have smaller mature ED than other centrarchids (bluegill ED = 1.47 ± 0.02 mm; largemouth bass ED = 2.09 ± 0.03mm; pumpkinseed

Lepomis gibbosus, ED = 1.50 ± 0.02 mm; rock bass *Ambloplites rupestris,* ED = 3.07 ± 0.04 mm; smallmouth bass *Micropterus dolomieu*, ED = 3.11 ± 0.03 mm (Cook et al. 2006). While the 3-wk and 6-wk female white crappie did have viable oocytes, ED was smaller than in females during the natural spawning season, likely accounting for the poor %-fertilization and egg volumes observed. It is also possible differences in reproductive data (GSI and ED) among comparisons with winter fish may be due to population or environmental differences between collection sites.

Few studies have focused on spawning centrarchids out-of-season (Parsons 1992; Mischke and Morris 1997; Matthews and Stout 2013). A preliminary study on black crappie was unsuccessful, with one female exhibiting gonadal development although it died before spawning (Parsons 1992). When comparing the results of this experiment to similar centrarchid out-of-season spawning experiments, it is important to consider both bluegill and largemouth bass are easily acclimated to tank conditions, formulated feeds and spawn readily in tanks without the need for hormone injections (Mischke and Morris 1997; Morris and Clayton 2009; Matthews and Stout 2013). Previous induced spawning experiments (Culpepper and Allen unpublished) using wild white crappie collected during the natural spawning season found crappie would not spawn volitionally in tanks with or without hormone induction, however other studies reported success with volitional spawning in black crappie in aquaria following hormone injections (Al-ablani and Phelps 1997; Arslan and Phelps 2004; Gomelsky et al. 2005; Cuevas-Uribe et al. 2009). Therefore, final reproductive maturation in tanks, regardless of spawning method, requires using hormone induction. Thus, egg maturation assessment is a critical component of determining optimal timing for hormone administration. Unfortunately,

currently utilized maturation assessment techniques, such as dissection or egg histology require fatal sampling methods (Thomas and Kilambi 1981). Ultrasound, a technique commonly used for sex determination and maturation assessments in other fish species (Martin-Robichaud and Rommens 2001), may have useful applications for crappie broodstock management in the future.

Future experiments on white crappie out-of-season spawning may benefit from extending the seasonal shift time to 9-12 wk to allow more time for complete gonad maturation, and also extend the duration of the final spring temperature (21° C) until GSI and ED reach Stage IV maturity. During this experiment, GnRHa was administered to all crappie 5 d after reaching spring photoperiod and temperature conditions based on visual observations of enlarged, gravid females. Maturation assessments from terminal sampling were not conducted due to limited numbers of fish. Post-experiment GSI and ED analyses supported the finding of Thomas and Kilambi (1981) that report egg maturation occurs at a female GSI of > 0.35 and an ED of > 0.7 mm. Bi-weekly sampling of females for GSI and ED during the later stages of future out-of-season spawning experiments (temperature > 18° C) may allow for more accurate reproductive staging and improved hormone administration timing.

Adult white crappie reproductive maturation can be induced in RAS through photoperiod and temperature manipulation and hormone (GnRHa) administration to induce spawning. This study has also shown white crappie can be maintained for long time periods and acclimated to a live-feed diet in closed, tank systems through maintaining cool water temperatures (~15° C) and low salinity (3-5 ppt). While overall spawning success rates were poor for both seasonal shift treatments, this experiment has

demonstrated adult crappie can be spawned out-of-season. Greater spawning success may be achieved using a longer seasonal shift, which should facilitate reproductive maturation.

Tables

Table 4.1 Comparison of white crappie *Pomoxis annularis* out-of-season spawning post-experimental data for 3-wk and 6-wk seasonal shift treatments

3-Wk Seasonal Shift										
		TL (mm)			Wt (g)			Gonad Wt (g)		
Sex	*n*	Mean	±	SE	Mean	±	SE	Mean	±	SE
Immature	6	227.3	±	14.9	161.7	±	28.9	1.08	±	0.50
Females	7	284.9	±	38.8	367.9	±	131.2	6.46	±	2.61
Males	8	282.5	±	49.9	347.5	±	195.2	3.15	±	2.52
6-Wk Seasonal Shift										
Immature	2	220.0	±	11.3	147.5	±	10.6	0.78	±	0.05
Females	7	315.7	±	61.0	415.0	±	234.6	11.36	±	8.42
Males	12	268.1	±	36.8	292.6	±	91.5	2.65	±	1.25

Table 4.2 Daily water quality summary for out-of-season spawning white crappie *Pomoxis annularis* recirculating systems

	3-Wk				6-Wk			
	Mean	±	SE	*n*	Mean	±	SE	*n*
D.O.[a] (mg/L)	12.22	±	0.19	66	10.15	±	2.11	84
pH	7.68	±	0.83	66	7.67	±	0.2	84
Salinity (ppt)[b]	2.6	±	0.1	66	2.8	±	0.7	84
TAN[c](ppm)	0.13	±	0.03	15	0.08	±	0.12	23
NO_2 (ppm)	0.05	±	0.04	15	0.04	±	0.04	23

[a]Total ammonia nitrogen (TAN); [b]salinity ≤ 0.5 ppt for the last 2 weeks of the experiment
[c]Dissolved oxygen (D.O.)

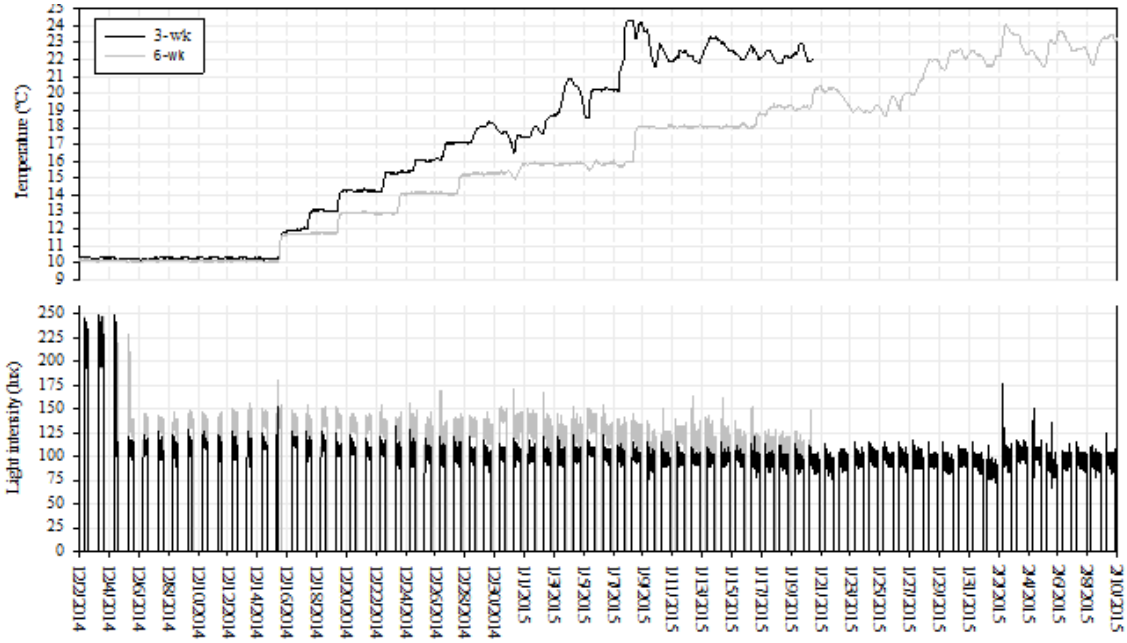

Figure 4.1 White crappie *Pomoxis annularis* out-of-season spawning temperature (°C) and light intensity (lux) data from 3-wk and 6-wk seasonal shift treatments

Note the decline in light intensity at 5 December 2014 is due to a reduction in the number of florescent bulbs per side to create a more even lighting intensity between treatments. Also, the sharp increase in temperature from 7 January 2015 – 8 January 2015 was caused by overcompensation of additional water heaters which were turned on due to a cold front at the facility that day. Errors bar were not presented, as the standard error of mean temperatures and light intensities were not different ($p < 0.05$) at each time point between the replicate dataloggers ($n = 4$) in each treatment.

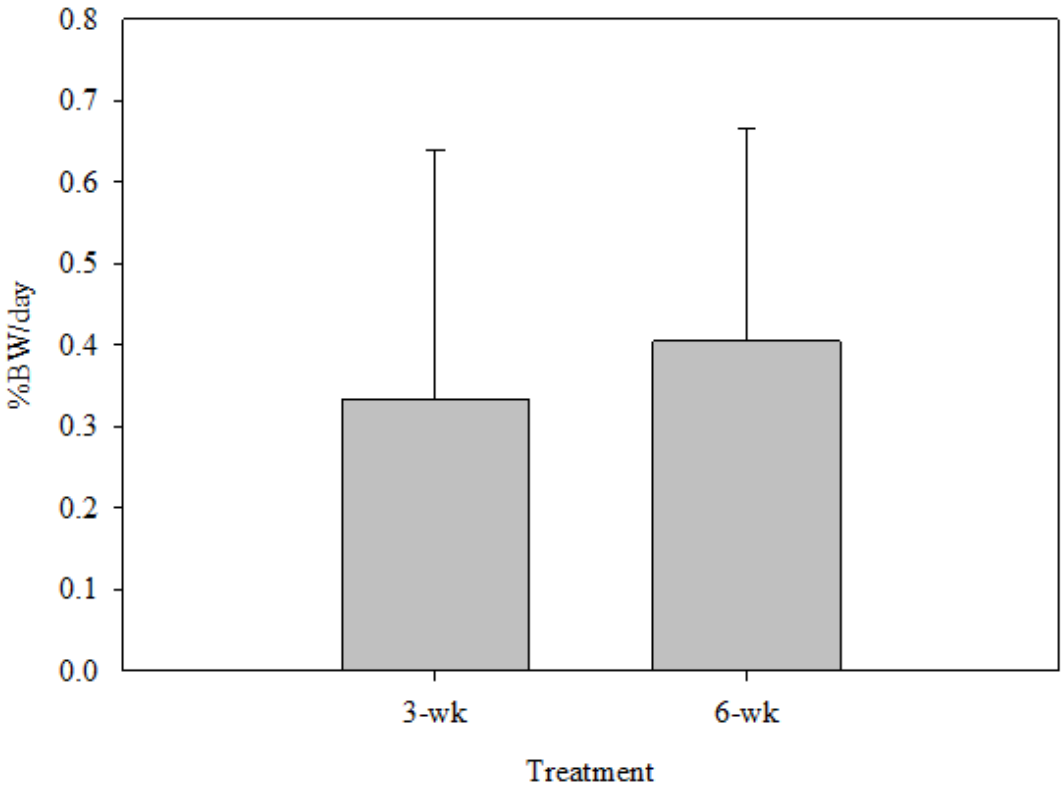

Figure 4.2 White crappie *Pomoxis annularis* feed consumption, in terms of mean %-body weight consumed per day (%BW/d)

The duration of the out-of-season spawning experiment was 49 d for 3-wk (n = 9 tanks) and 64 d for 6-wk (n = 10 tanks) treatments. No significant difference was found using one-way ANOVA ($p < 0.05$).

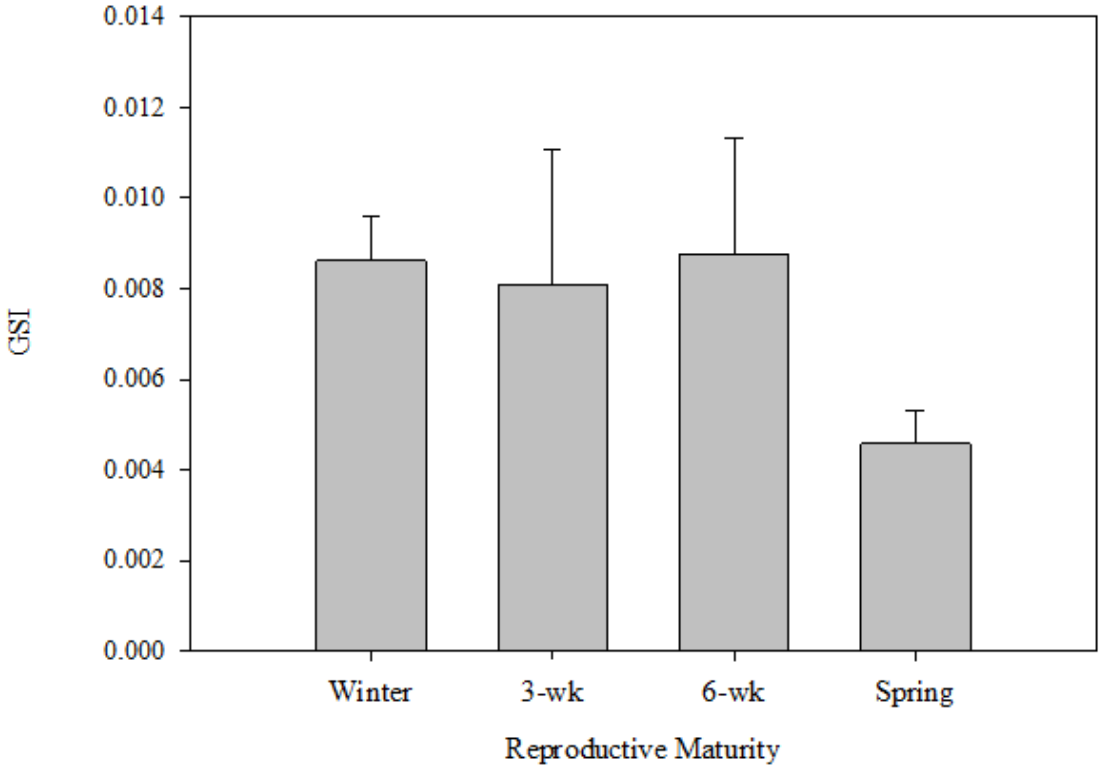

Figure 4.3 Comparison of male white crappie *Pomoxis annularis* gonadosomatic index (GSI)(mean ± S.E.)

between out-of-season spawning 3-wk (n = 8) and 6-wk (n = 12) seasonal shift treatments and wild caught males from Eagle Lake, Vicksburg, MS (17 December 2014)(Winter; n = 4) and Enid Reservoir, Enid, MS (21 April 2014)(Spring; n = 8). No significant difference was found using Kruskal-Wallis rank-sum test ($p < 0.05$).

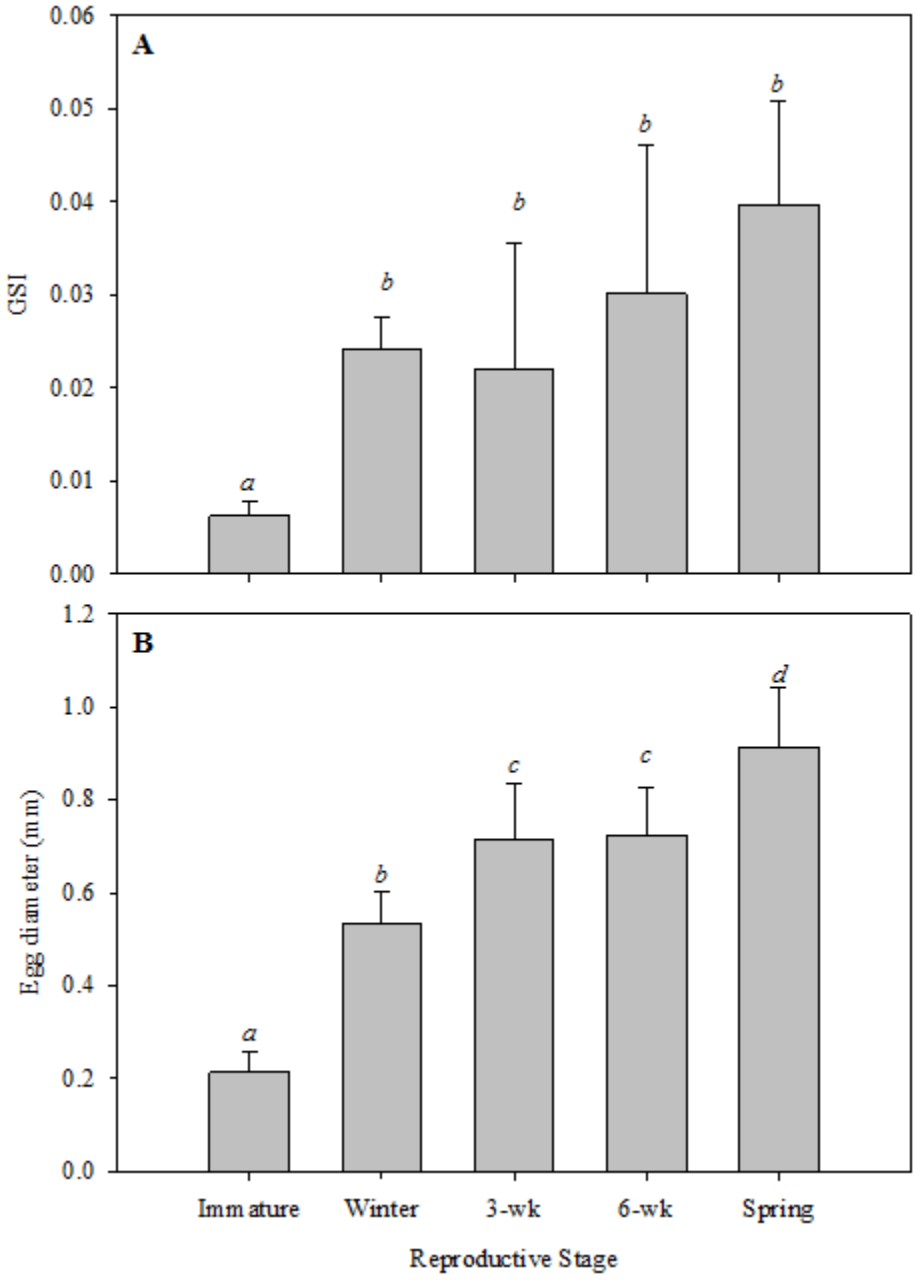

Figure 4.4 White crappie *Pomoxis annularis* gonadosomatic index (GSI)(A) and egg diameter (B)

in immature (Immature; n = 3) females, wild caught females from Eagle Lake, Vicksburg, MS (17 December 2014)(Winter; n = 6), out-of-season spawning 3-wk (n = 5) and 6-wk (n = 7) seasonal shift treatment females and spawning season females from Enid Reservoir, Enid, MS (21 April 2015)(Spring; n = 4). The letters beside each reproductive stage (A) represent differences ($p < 0.05$) using Kruskal-Wallis test and Nemenyi post-hoc comparison. The letters beside each reproductive stage (B) represent a difference ($p < 0.05$) using a one-way ANOVA and Tukey's honest-significant-difference test.

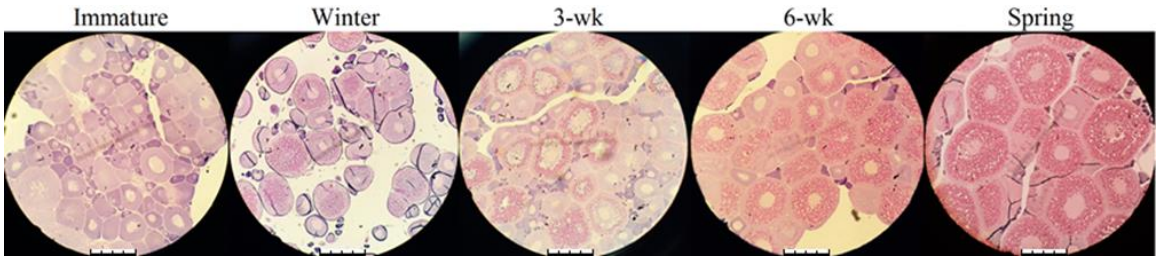

Figure 4.5 Female white crappie *Pomoxis annularis* egg development with different reproductive stages

. Immature fish from 3-wk and 6-wk out-of-season spawning (OSS) treatments (immature, wild caught females from Eagle Lake, Vicksburg, MS (17 December 2014) (Winter, OSS 3-wk and 6-wk seasonal shift treatments and spawning season females from Enid Reservoir, Enid, MS (21 April 2014)(Spring). Slides were stained with hematoxylin and eosin, all images are at 10x magnification and the scale bar represents 1 mm.

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