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Development of aquaculture protocols and gonadal differentiation of green sunfish (*Lepomis cyanellus*)

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ABSTRACT

We provide detailed rearing methods and describe green sunfish (*Lepomis cyanellus*) gonadal development and histological differentiation for both sexes. Developing in-depth aquaculture protocols and describing the gonadal differentiation of green sunfish could facilitate strategies to control nuisance populations, enhance stocking programs, and provide information for this species' use in bioassay trials or toxicology studies. Our methods resulted in consistent year-round production of green sunfish and allowed us to identify the timing of their gonadal differentiation through histological assessment. Our spawning methods provided year-round volitional spawns from green sunfish broodstock. Our rearing methods involved weaning larval green sunfish off live nauplii and onto only artificial diets by 37 days post-hatch (dph). Most of the offspring generation reached sexual maturity by 213 dph. Green sunfish are gonochoristic, with testes and ovaries differentiating directly from undifferentiated gonads. Ovaries begin to differentiate by 39 dph and testes begin to differentiate by 69 dph. This information can provide biologists consistent means to produce this Centrachid and understand their gonadal development.

1. Introduction

Green sunfish (*L. cyanellus*) is a North American Centrarchid species native to the Great Lakes, Hudson Bay and the Mississippi River basins (Fuller et al., 2021). Green sunfish are considered invasive in other areas of the world (Yun-Chang et al., 2008) and have been found in most U.S. states outside of their native range (Lemly, 1985; Dudley and Matter, 2000; Fuller et al., 2021). Their widespread distribution can be attributed to aquaculture practices, stocking as game fish, and releases as forage fish for largemouth bass (*Micropterus salmoides*) (Welcomme, 1988; Halos et al., 2004). Green sunfish have been implicated in the decline of many native aquatic species, including: Gila chub (*Gila intermedia*) and Chiricahua leopard frog (*Rana chiricahuensis*) in Arizona (Rosen et al., 1995; Dudley and Matter, 2000), California roach (*Hesperoleucus symmetricus*) and Ranid frogs in California (Moyle, 1976; Hayes and Jennings, 1986), and multiple native fish populations in North Carolina (Lee et al., 1980). Their aggressive behavior also negatively impacts sport fisheries (McKechnie and Tharratt, 1966). For example, their presence has been shown to cause stunting and competition with more desirable game fish, such as bluegill *Lepomis macrochirus* (Werner and Hall, 1977). Conversely, green sunfish are a desirable bait for catfish (Brunson and Morris, 2000) and Green Sunfish x Bluegill hybrid fisheries have been developed because this hybrid is a fast growing and aggressive sportfish (Lewis and Heidinger, 1971; Brunson and Robinnette, 1985).

Controlling invasive green sunfish populations is challenging and

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successful removals have required extensive efforts (Reinthal et al., 2020), sometimes coupled with indiscriminate methods such as piscicide treatments (Blasius, 2002; Ward et al., 2015). Genetic biocontrol methods, such as the Trojan sex chromosome strategy (TSC), are species-specific population control strategies that were modeled to effectively suppress or extirpate nuisance populations of various fishes (Gutierrez and Teem, 2006; Senior et al., 2013; Schill et al., 2017; Teem et al., 2020; McCormick et al., 2021). The TSC eradication strategy works through the continuous release of YY males (males with two Y chromosomes instead of an XY chromosome complement) or ZZ females (sex reversed males in a ZZ-male/ZW-female sex determined species) into a nuisance population. When these TSC carrying fish spawn with wild-type females the resulting progeny are 100% male. With significant introductions of TSC carriers, the sex-ratio of the population can theoretically be skewed towards all male resulting in a population decline and, ultimately, extirpation (Gutierrez and Teem, 2006; Senior et al., 2013; Schill et al., 2017; Teem et al., 2020).

As with any eradication strategy, understanding the basic biology of the invasive species is critical for success (Kolar et al., 2010). An initial assessment of the feasibility of using the TSC strategy for green sunfish requires identifying this species' sex determination system. Male heterogametic sex determination systems (XY-male/XX-female) and female heterogametic sex determination systems (ZZ-male/ZW-female) have been discovered in fishes by spawning sex-reversed individuals with wild-type individuals and then assessing the sex ratios of the progeny (Desprez et al., 1995; Gomelsky et al., 2002). These methods require effective sex-reversal treatments and aquaculture practices.

Fish are most susceptible to sex reversal by the administration of exogenous steroids if steroidal treatments are applied prior to gonadal differentiation and finish when gonadal differentiation is first observable through histology (Hackmann and Reinboth, 1974; Piferrer, 2001). A prior study investigated gonadal differentiation of green sunfish, sampling gonads on a monthly time-scale and consolidating 2–3 month old green sunfish into one group for analysis (Yun-Chang et al., 2008). Although Yun-Chang et al. (2008) research generally described gonadal development in green sunfish, it did so with relatively coarse temporal resolution and lacked a report of the percentage of the cohort that had undergone gonadal differentiation in each age sampled. Knowing the percentage of a cohort that is undifferentiated at each age, coupled with more frequent sampling of gonads and length-at-age data, is essential for designing a steroid treatment regimen that could result in 100% sex reversal (Malison et al., 1986; Gao et al., 2009).

Sex-reversal treatments and overall aquaculture operations can benefit from having aquaculture protocols that transition fish onto artificial diets early in their development. Weaning fish onto artificial diets can facilitate the administration of exogenous steroids (Yamamoto, 1963). In addition, use of artificial diets is less labor intensive than live-feeds and can reduce transmission of parasites and disease derived from live-feed sources (Støttrup, 2003; Gugliandolo et al., 2008; Dhont et al., 2013). Although methods are available for weaning the closely related Bluegill onto artificial feeds for sex-reversal treatments (Wang et al., 2008), green sunfish and bluegill prefer different foods (Werner and Hall, 1977). Our pilot studies showed that bluegill rearing methods proved ineffective for weaning green sunfish onto artificial diets without resultant high mortality rates.

Green sunfish aquaculture protocols and detailing the gonadal differentiation of this species are not just necessary for examining its potential use in a TSC strategy, but can also facilitate their use in toxicology studies (Uguz, 2008) and bioassay trials (Hunn et al., 1968; Carr, 1976; Adams and Johnsen, 1986). This information can also aid managers interested in producing and stocking sunfish hybrids (Mischke et al., 2007) or green sunfish mono-sex cultures (Al-Ablani, 1997). The objectives of this study were to 1) develop and report aquaculture protocols for green sunfish that resulted in consistent spawns and featured early weaning onto artificial diets, and 2) identify the timing and pattern of green sunfish gonadal differentiation. This research will facilitate the initial consideration of a TSC eradication strategy for green sunfish and help with laboratory and hatchery operations using green sunfish for bioassay trials, toxicology studies, or stocking programs.

2. Methods

2.1. Broodstock collection and stocking

On May 22, 2018 and May 21, 2019, we collected 25 and 37 adult green sunfish, respectively, by boat electrofishing from Parker Canyon Lake, Arizona, USA (GPS coordinates 31°25′37.0“ N, 110°27’25.0” W). During transit to the University of Arizona Fisheries Propagation Laboratory, fish were given a one-hour prophylactic treatment with a mixture of 0.05 ppm malachite green and 15 ppm formalin (ProForm C, Koi Care Kennel, Utah, USA). Water used at the UA Fisheries Propagation Laboratory was chlorinated well water that we dechlorinated with 25 ppm of sodium thiosulfate before use in the laboratory's aquaculture systems. Fish were acclimated to quarantine tanks by removing half of the water in the transport coolers and then slowly filling the coolers with quarantine tank water via 4.8-mm diameter airline tubing until the coolers were full. For quarantining, fish were divided randomly into three roughly even groups and stocked in two 437 L aquariums equipped with a Fluval FX6 filter (Rolf C. Hagen Inc., Baie-D'Urfe, Canada) and one 1135.62 L Rubbermaid tank (Rubbermaid, Georgia, U.S.A.) with an AquaClear 110 Power Filter (Hagen, Massachusetts, U.S.A.). The quarantine tanks were bare-bottom and contained large plastic plants for cover. Fish were kept in these tanks for two weeks before being sexed and stocked in broodstock tanks. During those two weeks, fish were fed thawed, previously frozen, mysis shrimp (~6 shrimp/fish) once a day.

After two weeks in quarantine, fish were sexed by applying pressure to the ventral surface of the abdomen to express gametes. Fish that did not express gametes were sedated for 3–5 min using 50 ppm of MS 222 buffered with 150 ppm of sodium bicarbonate. Once sedated, a plastic catheter of 0.97 mm ID × 1.27 mm OD was inserted into the urogenital opening to sample gametes (Brunson and Morris, 2000). Fish of known sex were selected as broodstock and were stocked in three 473 L glass aquariums each containing two 300-W Jager EHEIM drop-in heaters (EHEIM GmbH & Co, Deizisau, Germany) and two AquaClear 110 filters. These broodstock tanks had bare glass bottoms. Each tank was divided into three sections with removable screen partitions. Each section of the tank contained four plastic plants for cover and an artificial nest constructed from a terra cotta drain plate that was 30.5 cm in diameter x 3.8 cm deep filled with pea gravel (particle size 5–15 mm diameter). Each section of the tank contained one male and one female for a total of three males and three females in each tank. Water quality in broodstock tanks was maintained within these parameters: water temperature 23–27 °C, ammonia <0.5 ppm, nitrite <1.0 ppm, and pH 8.0–8.4. For the first two weeks in the broodstock tanks, fish were fed thawed shrimp once a day. Broodstock were transitioned onto Skretting (Nutreco, Amersfoort, Netherlands) Classic Brood 5-mm pellet (46% crude protein, 12% crude fat) over a two-week period. The broodstock's transition from shrimp onto the pellet diet was accomplished by reducing the number of shrimp fed to each tank by half over the course of a week and supplementing with pellets ad libitum. By the end of the second week all tanks were fed only pellets ad libitum. The appropriate rate to feed ad libitum was based on how much the fish could eat in two minutes before the diet settled on the bottom of the tank. Broodstock were fed once a day. The green sunfish broodstock mean total length (TL) was 153.6 mm (SD 47.2 mm).

2.2. Broodstock spawning

Changes in photoperiod and temperature were conducted to induce spawning in the broodstock tanks (Kaya and Hasler, 1972; Smith, 1975; Dupree and Hunter, 1984; Bryan et al., 1994). Over a 2-week period, fish were transitioned from a 12-h photoperiod to an 18-h photoperiod by

increasing the light-on time by 25 min each day and temperature was increased from 23 °C to 29 °C by increasing the temperature ~ 1 °C every two days. After the broodstock were transitioned to an increased photoperiod and temperature, artificial nests were checked for eggs and larvae once a day for four weeks. After four weeks with no spawns, different sex ratios were attempted in each tank section (1:2 male to female, 2:2 male to female, 2:3 male to female) to induce spawns (Smith, 1975; Bryan et al., 1994) and reduce the number of injuries and mortalities occurring with females. The three sex ratios were attempted for four weeks unless patterns of injury or mortalities were observed before the trial period ended. No changes were made to the temperature and photoperiod while the three different sex ratios were attempted. Finally, all screen partitions were removed from tanks and lower stocking densities of one male and two females, two males and two females, and two males and three females per tank were attempted to provide more room for males to perform courtship behavior (Bryan et al., 1994). These lower stocking densities with the different sex ratios were attempted for four weeks.

After four months of unsuccessful spawning trials in the 473 L aquariums, two recirculating aquaculture systems were constructed to facilitate the male's circular courtship behaviors and reduce stress on females. Each system was composed of two 360 L round polyethylene tanks (99.06 cm inside diameter x 50.80 cm depth) connected by 4.83-cm (outside diameter) PVC pipe to a 189 L sump filled with biomedia and a split return for each tank using two submersible magnetic drive pumps (Danner Model 7, Danner Manufacturing Inc., New York, U.S.A.), a 40 W Lifegard UV sterilizer (Lifegard Aquatics, California, U.S.A.) and a ¼ hp. Coralife chiller (Central Aquatics, Wisconsin, U.S.A.). Each of the four broodstock tanks contained two artificial nests with a different design than previously used. These artificial nests were 19 L buckets (27.94 cm inside diameter) that were cut to a 7–10 cm depth and filled with pea gravel (particle size 5–15 mm diameter) (Mischke and Morris, 1997). Two males and three females were transferred to each of the four broodstock tanks. Using methods of Mischke and Morris (1997) except utilizing only ambient light, the broodstock tanks' water temperatures and photoperiods were then manipulated to mimic an artificial winter (~15 °C water temperature with 8 h of light) and returned to an artificial summer (27 °C water temperature and 16 h of light) over six-weeks to induce spawning. Despite courtship behavior and nest guarding by the males, no spawns occurred during the next five months, and traumatic injuries were noted on the females. Even though there already were three large plastic aquatic plants in each tank for cover, a circular laundry basket (50.80 cm inside diameter x 38.10 cm depth) with two ~12.70 cm square holes cut in the sides were placed upside-down in each tank to provide the females more cover and to reduce conspecific aggression. The artificial nests were on the outside of the upside-down laundry baskets. One month after the addition of the laundry baskets, the fish began to spawn regularly, and the artificial nests were checked for eggs once a day for a year.

2.3. Hatching, larval rearing, and growout of offspring generation

Nests with eggs were placed in a 37.9 L plastic tub and given a 30 min 100-ppm formalin bath. Nests were then placed in one of four larval rearing tanks consisting of 37.9 L plastic tubs each outfitted with a 50 W Jager EHEIM drop-in heater, air stone, and QANVEE Bio Sponge filter (Taian Qanvee Aquarium Equipment Co., Ltd., Shandong, China). Each larval rearing tank was randomly assigned one artificial nest with eggs from each broodstock tank resulting in a total of four replicates undergoing the following larval rearing treatment. For the first 30 days of rearing, tank temperatures were maintained between 25 and 28 °C. After 30 days post-hatch (dph), heaters were removed from tanks and water quality parameters were maintained at: temperature 15–24 °C, ammonia <0.25 ppm, nitrite <1.0 ppm, and pH 8.0–8.4. Initially, methods used to wean young bluegill onto artificial diets (Gao et al., 2009) were followed closely. Briefly, the feeding of AP-100 microfeed (Zeigler Bros., INC., Pennsylvania, U.S.A.) starting at 10 dph resulted in nearly 100% mortality and those that survived to 30 dph were stunted. We attribute this to the green sunfish's reluctance to feed on this diet and the concomitant reduction in water quality in the recirculating systems we used.

The failed attempts at rearing green sunfish using artificial diets following the Gao et al. (2009) methods required us to experiment with and develop the following feeding regimen (Table 1). The first feeding was given once yolk sacs were absorbed and larvae started their swim up stage (3–4 dph). Larvae were fed <24-h old brine shrimp nauplii four times per day at a rate of ~125 nauplii/L (estimate based on weight of unhatched cysts and ~ 90% hatching rate). At 25 dph we fed the green sunfish, now post-larvae, nauplii four times a day and began feeding Otohime B1 diet (B1: 200–360 μm, 51% crude protein, 11% crude fat) (Pentair Aquatic Eco-Systems, North Carolina, U.S.A.) twice a day. When juveniles were 30 dph, we fed them nauplii once a day and started feeding B1 diet six times a day using an EHEIM automated fish feeder. At 37 dph, we stopped feeding nauplii and only fed B1 feed 6 times a day. Juvenile green sunfish were transitioned onto Otohime B2 diet (B2: 360–620 μm, 51% crude protein, 11% crude fat) at 100 dph by feeding them half B1 and half B2 for two weeks. This and subsequent transitions in diet were accomplished by mixing the fish's current feed with the next stage of feed. At 122 dph fish were transitioned onto Otohime C1 diet (C1: 580–840 μm, 51% crude protein, 11% crude fat). At 175 dph, fish were transitioned onto Otohime C2 diet (C2: 840–1410 μm, 51% crude protein, 11% crude fat) and were fed six times a day with the automated feeder in addition to being fed ad libitum twice a day. At 285 dph, fish were transitioned onto Skretting 2-mm pellets (46% crude protein, 12% crude fat) and fed this diet ad libitum twice a day for the duration of the rearing trials. The automated feeder administered 66.8 mg (SD = 14.24 mg, $n = 10$ measurements of diet dispersed), 224.0 mg (SD = 79.89 mg), 390.2 mg (SD = 133.05 mg), 552.2 mg (SD = 245.97 mg) during each feeding for B1, B2, C1, and C2 diets respectively. The estimated feed rates in percent body weight per day were 13%, 6%, and finally 3% for fish 0–30 dph, 31–90 dph, and > 90 dph, respectively.

At 30 dph, all but 50 randomly selected fish were removed from the four larval rearing tanks to reduce crowding during growout. At 285 dph, all surviving fish from each larval rearing tank were transferred to four 757 L round fiberglass tanks that comprised a recirculating aquaculture system (RAS). The RAS was composed of thirty 757 L round fiberglass tanks connected to a filtration system featuring a Lifegard ¾ hp. in-line pump, an Emperor 750 W UV sterilizer (Pentair Aquatic Eco-Systems), a DF-6 Polygeyser bead filter (Aquaculture Systems Technologies, Louisiana, U.S.A.), and a Dayton ½ hp. in-line pump (Dayton Electric Mfg. Co., Illinois, U.S.A.). Aeration was provided to each tank by a blower (WW80 Whitewater, Pentair Aquatic Eco-Systems).

The four tanks were siphoned once a day to remove uneaten food and waste. A 10% water change was performed weekly. Fish mortalities were fixed in 10% neutral buffered formalin and occasionally submitted to the University of Arizona's Aquaculture Pathology Laboratory for investigation. Larval rearing tanks where *Aeromonas* infections persisted

Table 1
Diet and feeding regimen for green sunfish during their first year of growth.

DPH	Diet	Quantity
3 (swim up stage) – 25	< 24-h old brine shrimp nauplii	4×/day at ~125 nauplii/L
25–30	Otohime B1 Diet	2×/day at ~66.8 mg
30–37	< 24-h old brine shrimp nauplii	1×/day at ~125 nauplii/L
30–100	Otohime B1 Diet	6×/day at ~66.8 mg
100–122	Otohime B2 Diet	6×/day at ~224.0 mg
122–175	Otohime C1 Diet	6×/day at ~390.2 mg
175–285	Otohime C2 Diet	6×/day at ~552.2 mg + 2×/day ad libitum
285–355	Skretting 2 mm pellet	2×/day ad libitum

after a week of 10–30% daily water changes were given oxytetracycline treated feed (1.12 g oxytetracycline/454 g of diet) for 10 days.

Study fish were periodically measured for TL (mm) and weighed (g) during the rearing period. Between 9 and 22 randomly selected fish from each of the four larval rearing tanks were measured and weighed at 30 dph. At 91 dph, 285 dph, and 355 dph all fish from each of the four larval rearing tanks were measured for TL. Absolute growth rates (AGR) were calculated using the formula $AGR = (TL_2 - TL_1)/T \times 100$. Where TL_1 and TL_2 are the mean fish total lengths at the start and end of the growth period for each of the four larval rearing tanks, and T is the time between measurements. This absolute growth rate formula was adapted from Wang et al. (2008), but we used total lengths instead of weights because weights of fish <30 dph were suspected to be inaccurate due to unstable scale readings. A one-way ANOVA was conducted on the grand mean AGR for each growth period to determine if they differed substantially. A Von Bertalanffy growth curve for fish age 1–155 dph was constructed using FSA package (Ogle, 2016) in R studio. A simple linear regression was used to describe the function of age on the TL in fish aged 1–355 dph.

2.4. Gonadal development and histology

Swim-up larvae were stocked in two tanks with identical configurations as the four 37.9 L larval rearing tanks described above. Stocking densities were determined by counts of swim-up larvae from a sample of 500 mL of water when fish were 5 dph. One tank was stocked at a relatively high density (~20 larvae/L) compared to the other tank (~8 larvae/L). Five fish were collected upon hatching, euthanized by immersion for 10 min in 100 ppm of MS 222 buffered with 150 ppm sodium bicarbonate, and fixed in buffered formalin. Subsequently, five fish were collected and euthanized this way from the high-density tank and the low-density tank every 7 days for the first 30 dph and then every 10 days from 30 dph to 159 dph. In addition, fish were collected at 322 dph from one of the four rearing tanks to ensure gonads from sexually mature fish were sampled. Only abdominal sections or gonads were fixed for histology in fish that were over 18-mm TL. Tissue was fixed at a ratio of 1:10 tissue to 10% neutral buffered formalin. A multiple linear regression with TL as a function of age (dph) and tank density (high vs low) was used to evaluate differences in growth rates between the high-density and low-density larval rearing tanks.

Fixed samples 1 dph – 109 dph were submitted to a commercial laboratory (Animal Reference Pathology, Utah, U.S.A.) for histology processing and hematoxylin and eosin staining. Samples 119–322 dph were submitted to Fishhead Labs (Florida, U.S.A.) for histology processing and hematoxylin and eosin staining. Samples were cut along the sagittal plane to bisect the gonad. Histological sections in fish 109 dph and younger were scanned for digital review by Animal Reference Pathology. In samples between 119 dph and 322 dph histological sections were photomicrographed using an AmScope 40×-2000 × 3 W LED Seidentopf trinocular compound microscope and AmScope 14MP camera (United Scope, LLC, California, U.S.A.).

Gonadal tissue in histological sections were sexed and described using various references (Nakamura et al., 1998; Arezo et al., 2007; Uguz, 2008; Gao et al., 2009; Lowerre-Barbieri et al., 2011; Mazzoni and Quagio-Grassiotto, 2017; van der Ven and Wester, 2021). Sex differentiation of gonadal tissue was described by comparing our histological sections with descriptions and histological images from the references listed above. Cytological comparisons were made with previously described green sunfish gonad differentiation (Yun-Chang et al., 2008) and with bluegill gonad differentiation (Gao et al., 2009). The timing of anatomical differentiation of the gonads could not be compared to bluegill anatomical differentiation because our examination of green sunfish gonads along the sagittal plane, as opposed to the transverse plane, made it difficult to observe certain anatomical changes that may have preceded cytological differentiation (Jensen and Shelton, 1983; Sacobie and Benfey, 2005; Gao et al., 2009).

3. Results

3.1. Broodstock spawning and hatching of offspring generation

After adding the laundry baskets to the broodstock tanks, we observed 27 spawns over a one-year duration. Broodstock spawned throughout the year under the lab's ambient photoperiod and tank temperatures (Table 2). Fish were able to spawn four times in a month, but mean number of days between spawns for each tank was 19 ($n = 4$, SD = 14.15 days, 95% CI = 0 days – 42 days). We observed two spawns within the same week in one broodstock tank.

Eggs hatched 24–36 h post spawn. Larvae were benthic and fed off their yolk-sacs for the first 3–4 days. Swim-up larvae readily consumed <24 h old nauplii.

3.2. Growth rates of offspring generation

Growth rates began to decrease in green sunfish at about 91 dph and a Von Bertalanffy growth curve model suited TL data for fish measured 1–155 dph (Fig. 1). However, mean growth extended to 355 dph (Fig. 2A) exhibited a linear function with age (simple linear regression, $F_{1,18} = 1490$, P value <0.0001). Mean TL (mm) increased by 0.26 (95% CI = 0.244–0.272 mm) with each day (Fig. 2B). Green sunfish mean total lengths were 4.08 mm (SD = 0.04 mm, $n = 4$), 12.11 mm (SD = 1.91 mm, $n = 4$), 26.46 mm (SD = 2.17 mm, $n = 4$), 76.76 mm (SD = 7.51, $n = 4$) and 95.75 mm (SD = 6.38 mm, $n = 4$) at 1, 30, 91, 285 and 355 dph, respectively. Changes in mean AGR during growout were small and insignificant (One-Way ANOVA, $F_{3,12} = 0.43$, P value = 0.735). The AGRs for ages 1–30 dph dph, 30–91 dph, 91–285 dph, and 285–355 dph were 27.69 (95% CI = 17.19–38.19, $n = 4$), 23.45 (95% CI = 15.47–31.44, $n = 4$), 25.91 (95% CI = 21.13–30.69, $n = 4$), and 27.12 (95% CI = 16.24–38.00, $n = 4$). Fish density showed no effect on growth rates between the high-density and low-density larval rearing tanks used for histology (multiple linear regression, $t_{2,126}$ *low density* $\beta = -0.10$, $P = 0.92$). Mature gametes were expressed from most offspring individuals at 213 dph. Volitional spawns of the offspring generation occurred within the first year, but spawns were only detected as larvae and juveniles in the growout system, so no exact date for the onset of spawning can be reported.

3.3. Survival of offspring generation

Cannibalism was observed in the first 30 days of growout. Cannibalistic individuals typically exhibited faster growth rates and were culled once their TL became an outlier among the other larvae (roughly double in TL). *Aeromonas hydrophila* infections were common in larval rearing tanks, especially during the transition from live nauplii onto an

Table 2
Conditions present in green sunfish broodstock tanks when spawning began and continued for a year.

Condition	Description
Broodstock tank	360 L, 99.06 cm inside diameter x 50.80 cm depth
Water depth	40.46 cm
Water temperature	23–27 °C
Photoperiod	18 h light, 6 h dark
Filtration	Baffled 189 L sump filled with bioballs, UV sterilizer
Pump and flow rate	Danner Model 7, ~473 L/h
Diet and feeding	Skretting Classic Brood 5-mm pellet ad libitum once a day
In-tank cover	three large plastic aquatic plants (~ 60 cm in length), 1 upside-down laundry basket (50.80 cm inside diameter x 38.10 cm depth) with two ~12.70 cm square holes in the sides
Artificial nests	Two 19 L buckets cut down to 7–10 cm depth filled with pea gravel
Sex ratio	2 males: 3 females

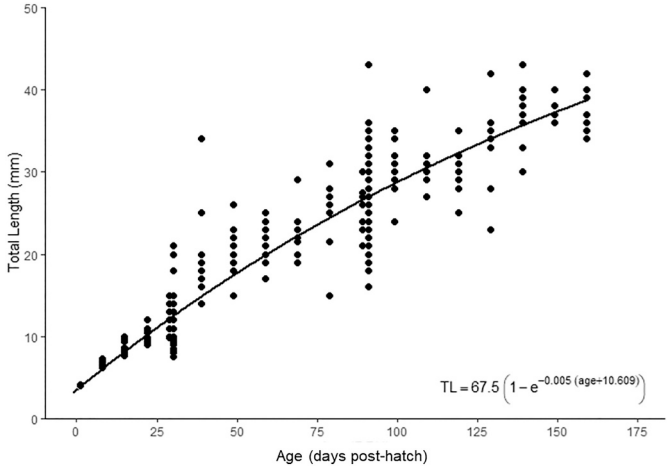

Fig. 1. Von Bertalanffy growth curve for 1–155 dph green sunfish. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

artificial diet. The infection presented as internal hemorrhaging that could be seen through the skin. This bacterial strain was detected via PCR in the larval and juvenile carcasses submitted to the University of Arizona's Aquaculture Pathology Lab. Oxytetracycline treated diet cured infections and reduced mortalities.

Survival was lowest during the first 30 dph. Since exact counts were not taken of fish younger than 30 dph, precise survival rates for fish less than 30 dph are unknown. By using volume-based tank densities from the high-density and low-density histology tanks, the survival rate from 1 to 30 dph is roughly estimated to range between <10% and up to 69.31%. Mortality rate decreased with age. Mean survival was 49.50% (SD = 23.17%, $n = 4$), 68.25% (SD = 18.81%, $n = 4$), 100% (SD = 0%, $n = 4$) between 30 and 91 dph, 91–285 dph, and 285–355 dph, respectively.

3.4. Gonadal development and histology

Gonads were observable in fish as young as 8 dph (TL = 6.2–7.3 mm). The undifferentiated gonads were observed ventral to the posterior end of the swim bladder and dorsal to the posterior section of the intestine (Fig. 3A). All gonads in fish 8–29 dph (TL = 6.2–15.0 mm) were undifferentiated. Undifferentiated gonads were comprised of primordial germ cells supported by a scant fibrovascular stroma (Fig. 3B). Presumptive ovarian differentiation was first observed at 39 dph (TL = 16–34 mm). The onset of ovarian differentiation (Fig. 4) was subtly characterized by the clustering of oogonia and germ cell meiosis. Oogonia were usually observed alongside various maturation phases of chromatin-nucleolus oocytes (Fig. 4A). Basophilic perinucleolus stage oocytes were observed in females at 49 dph. By 59 dph (TL = 19–23 mm) most females had ovaries predominated by previtellogenic oocytes (Fig. 4B) and the ovarian cavity could be identified. Between 59 and 159 dph (TL = 19–37 mm) ovaries enlarged and there was an increase in the number of previtellogenic oocytes. Various stages of vitellogenesis was observed in females sampled at 322 dph (TL = 62–100 mm) (Fig. 4C).

Although stromal aggregations and subtle lobule formations were observed in two fish at 59 dph (TL = 17 and 20 mm), definitive testicular differentiation was not observed until 69 dph (TL = 20–24 mm). In young testes, spermatogonia were cytologically indistinguishable from undifferentiated primordial germ cells, but the proliferation of the germ cells and their organization in clusters of four or more were suggestive of spermatogonia differentiation (van der Ven and Wester, 2021). Presumptive spermatogonia were also slightly smaller ($\bar{x}$ = 9.51 μm, 95% CI = 8.55–10.47 μm, $n = 12$) than undifferentiated primordial germ cells ($\bar{x}$ = 12.92 μm, 95% CI = 11.07–14.76 μm, $n = 12$). By 69 dph some males exhibited spermatogonia and primary spermatocytes forming loose tubular formations lined by seminiferous epithelium (Fig. 5A). Spermatogonia predominated the tissue in the testi at 89 dph (TL = 21–30 mm) and their organization into tubules became more conspicuous. Meiotic activity in testes was not observed in males until they were 99 dph (TL = 30–35 mm). Haploid spermatids resulted from meiotic divisions of spermatocytes. The clustered spermatids were small (~1–2 μm), darkly basophilic stained, and were roughly round in shape (Fig. 5B). Adult males (322 dph) had testes comprised of germ cells organized in tight tubules that were lined by thickened seminiferous epithelium (Fig. 5C). Adult males had spermatocytes at varying levels of maturity and lumina filled with mature spermatozoa (Fig. 5C).

By 99 dph all gonads had differentiated and were easily discernible as testis or ovary via histological examination (Fig. 6). We observed a heavily male-skewed sex ratio in fish sampled between 79 and 322 dph (71.67% male). We did not observe any evidence of protogynous sex differentiation (Shapiro and Rasotto, 1993). Timing of gonadal differentiation in the high-density and low-density tanks was synchronous and the sex ratios in fish older than 79 dph were male skewed from both the high-density (84.62% male) and low-density tanks (76.19% male).

4. Discussion

4.1. Aquaculture of green sunfish

Detailed aquaculture and rearing methods are essential for any

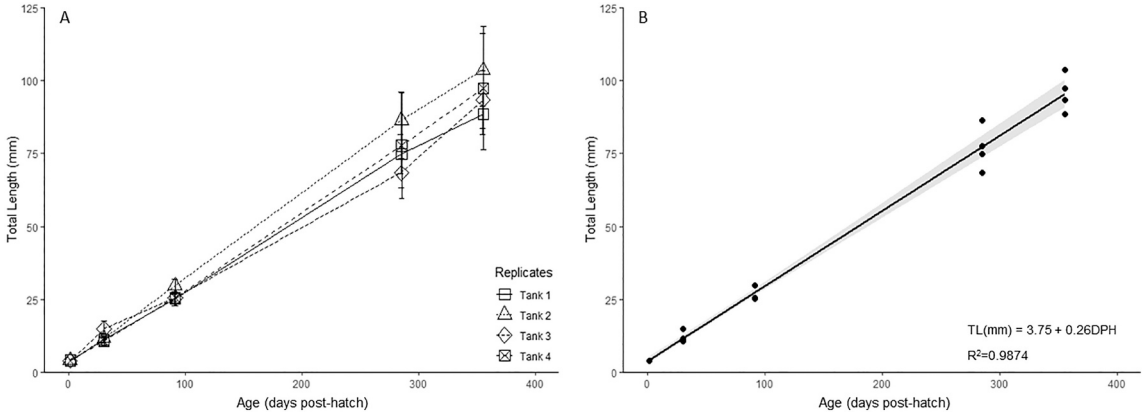

Fig. 2. (A) Growth of green sunfish as depicted by total length (TL mm) versus age (days post-hatch) for each larval rearing tank. (B) Growth of green sunfish from 1 to 355 dph as estimated by linear regression of TL on age with 95% confidence bands for the line of best fit. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

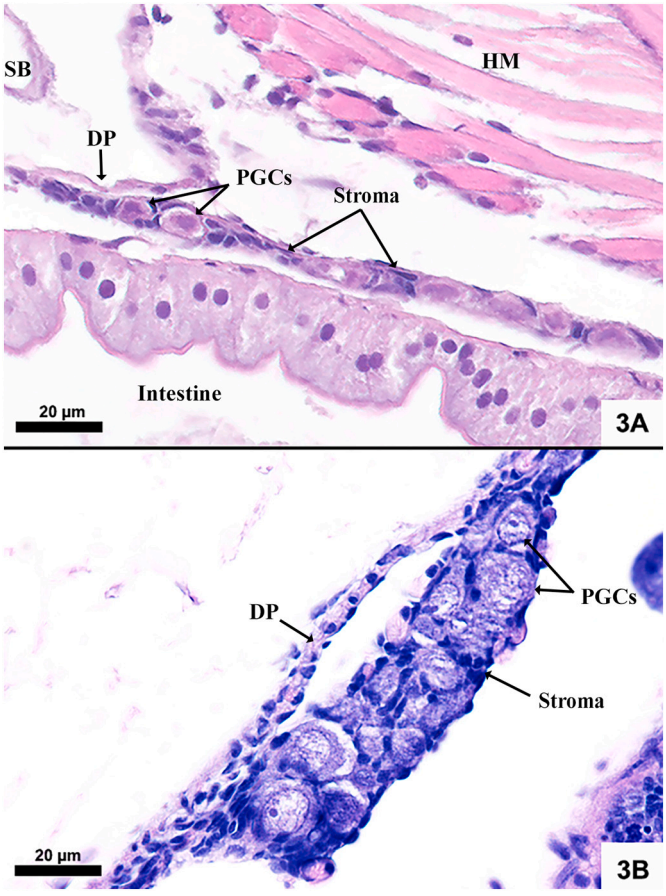

Fig. 3. Photomicrographs of undifferentiated gonad in 8 dph green sunfish (3A). Primordial germ cells (PGCs) were supported by mesenchymal stroma dorsal. The gonad is positioned dorsal to the posterior end of the intestine (DP = dorsal peritoneum, SB = swim bladder, HM = hypaxial muscle). 3B) As evident in this 22 dph green sunfish, gonads grew larger and PGCs were abundant before any histological differentiation was observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stocking program. In the case of green sunfish, such knowledge could prove useful for enhancing a native stock (Cotton and Wedekind, 2007; Lorenzen, 2008) or to develop and maintain a TSC broodstock for population eradication efforts where the species is invasive (Schill et al., 2016). Protocols are also essential for the maintenance and propagation of fishes for research in a laboratory setting (Lawrence, 2011). Using our feeding regimen (Table 1), we were able to wean green sunfish completely onto artificial diets by 37 dph. Transitioning fish onto artificial diets can help laboratory and hatchery operations by reducing cost and labor (Jones et al., 1993). This species' ability to continuously spawn and ability to be reared in simple, indoor systems, makes them an appealing species for a variety of studies. In addition, this protocol can be scaled up from the laboratory to the hatchery level in order to increase production.

The current protocol allowed us to obtain year-round volitional spawns from green sunfish broodstock. Despite collecting wild-caught broodstock in May, at the beginning of their spawning season (Hunter, 1963; Etnier and Starnes, 2001), the first attempts to spawn green sunfish using 473 L rectangular glass aquaria with various male to female ratios and stocking densities were unsuccessful. The glass aquaria we used were double the volume of those used by Smith (1975) and were stocked at the same sex ratio of 2 males to 3 females, yet we noticed male-on-male and male-on-female aggression that resulted in stress and occasional injury. Moving the broodstock to 360 L round tanks enabled

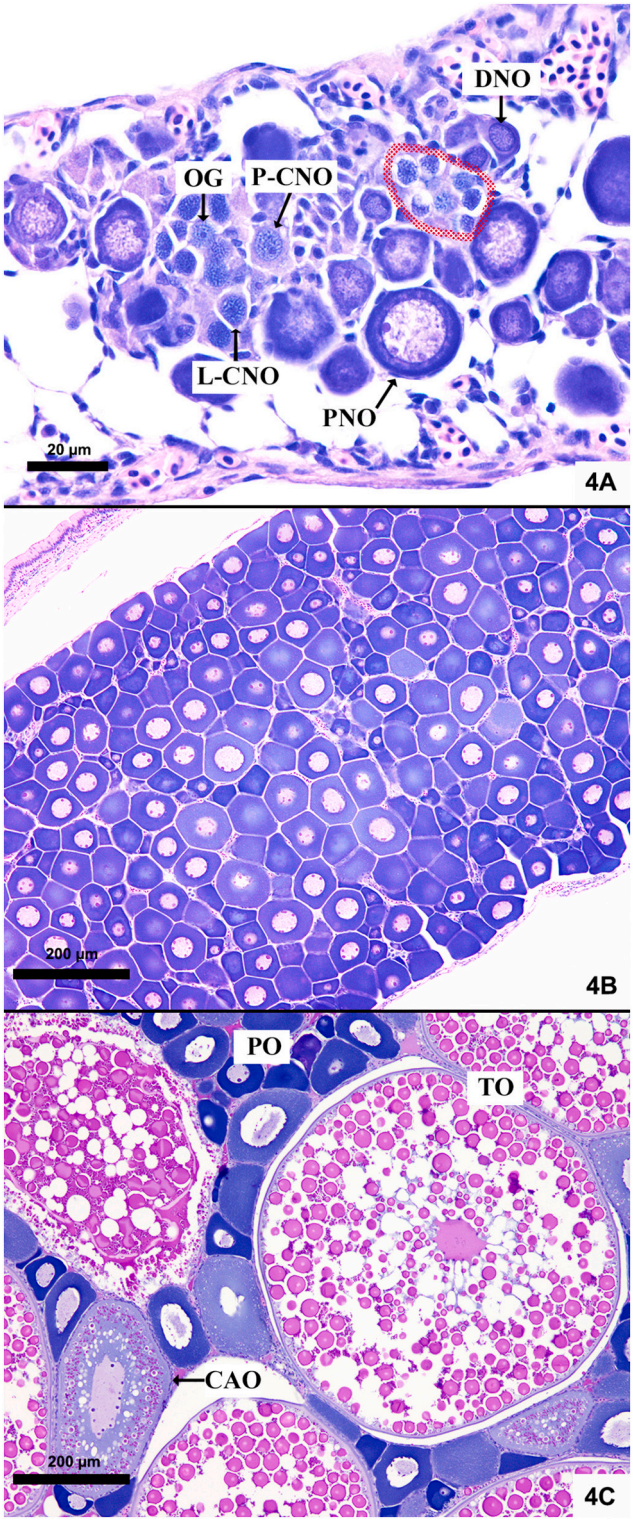

Fig. 4. Photomicrograph 4A shows an oogonium (OG), a cluster of proliferating oogonia (within red circle), a leptotene stage chromatin nucleolus-oocyte (L-CNO), a pachytene stage chromatin nucleolus-oocyte (P-CNO), a diplotene stage perinucleolus ooctye (DNO), and a previtellogenic stage perinucloelus oocyte (PNO) in 49 dph green sunfish. 4B) Ovaries in green sunfish between 59 and 159 dph were predominated by previtellogenic oocytes, as seen in this 99 dph female. 4C) Ovaries of a 322 dph green sunfish contained mature tertiary vitellogenic oocytes (TO), primary vitellogenic oocytes (PO) and intermediate cortical alveolus stage ("oil droplet stage") oocytes (CAO). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

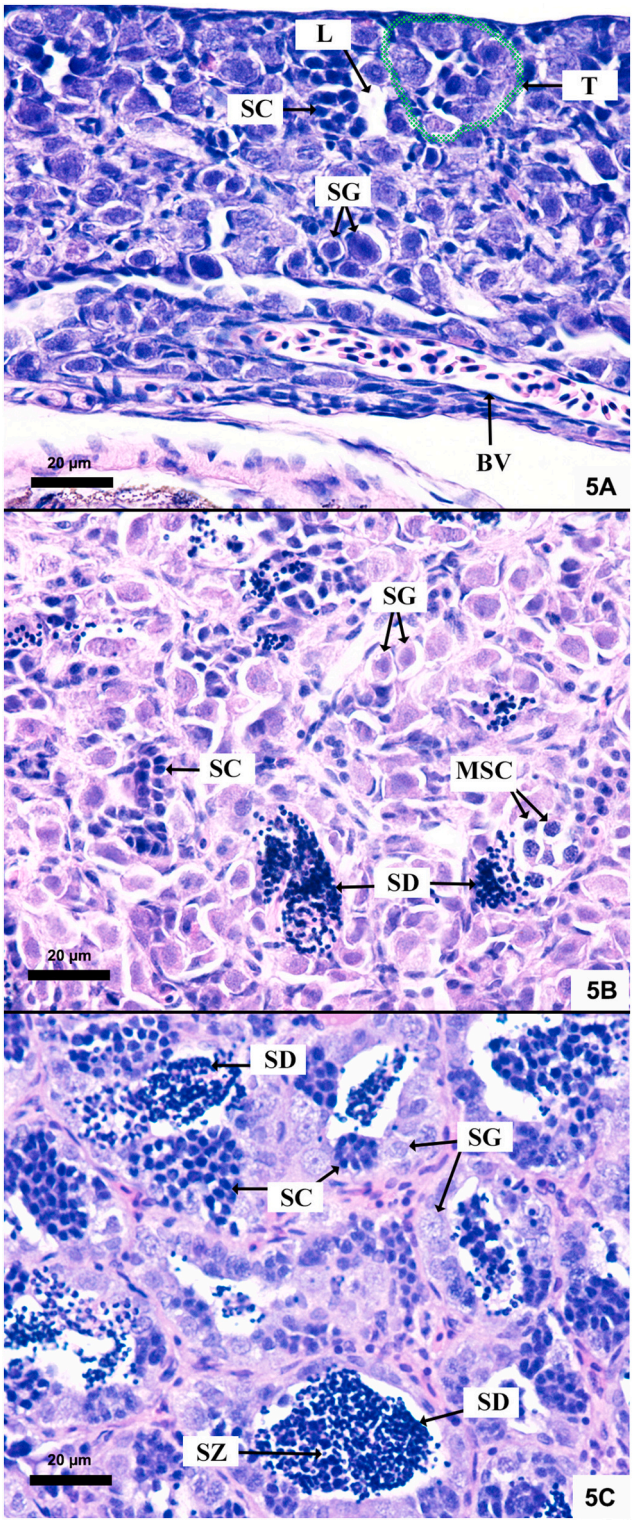

Fig. 5. 5A) Photomicrograph of testis in 69 dph male green sunfish. Clusters of spermatogonia (SG) and primary spermatocytes (SC) are present. Primary spermatocytes and spermatogonia border presumptive lumina (L). Seminiferous epithelium can be seen bordering early formations of tubules (T – border of tubule outlined in green). Blood vessels (BV) increased in size in developing testes. 5B) shows spermatocytes undergoing meiosis (MSC) and haploid spermatids (SD) first seen in 99 dph males. 5C) By 322 dph the tubules are well defined and separated by seminiferous epithelium and the lumina are filled with mature spermatozoa (SZ). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

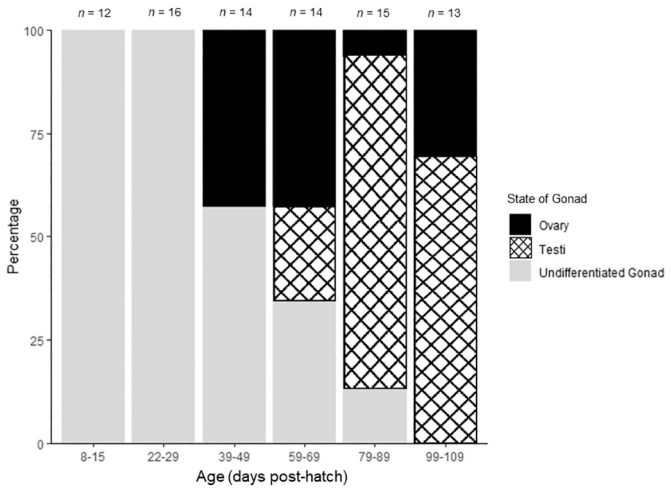

Fig. 6. Gonadal differentiation in green sunfish given rearing methods and growth rates in the present study. Ovaries begin to differentiate by 39 dph and testicular differentiation was detected after 49 dph. By 99 dph all sampled gonads were clearly differentiated as either ovaries or testes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the females and smaller males to feed more freely and decreased the incidence of enclosure wall trauma. Nevertheless, it was not until we added upside down laundry baskets with holes cut out of the sides that we observed consistent spawns. These laundry baskets were often utilized by the females while the males were more often observed guarding an artificial nest. The importance of in-tank cover had not been discussed in any of the *Lepomis* aquaculture literature we reviewed (Smith, 1975; Dupree and Hunter, 1984; Bryan et al., 1994; Mischke and Morris, 1997; Brunson and Morris, 2000). Cover and tank features, such as substrate, have been shown to increase aquaculture performance in other species (Barnes et al., 2005; Krebs et al., 2017). Aquaculturists have used submerged boxes with one side open to permit more spawns to occur in largemouth bass *M. salmoides* broodstock tanks (Breder Jr., 1936). Providing structure and in-tank cover for green sunfish broodstock appears essential due to the male's aggressive courtship behavior. Our observations also suggest a stocking ratio of 2:3 male to female can help alleviate conspecific aggression and reduce stress when attempting to induce spawning.

Despite unsuccessful spawning attempts for the first ten months, we chose not to try the use of hormone injections to cue spawning (Cuevas-Uribe et al., 2009). Since other researchers had reported consistent volitional spawns with sunfishes in captivity without the use of hormone injections (Smith, 1975; Bryan et al., 1994) we were confident that under the proper conditions (photoperiod, sex ratio, density, etc) we would obtain similar results. We observed spawns year-round after broodstock were exposed to a 4-week artificial winter and then maintained in an 18-h photoperiod with water temperatures between 23 and 27 °C the remainder of the year. It is unclear if the green sunfish would have consistently spawned without the artificial winter treatment if the upside down laundry baskets were added to the broodstock tanks first. The ability of the green sunfish to spawn continuously under a static photoperiod and temperature mimics findings by Smith (1975) where he was able to obtain volitional spawns from longear sunfish *Lepomis megalotis* maintained in a 16-h photoperiod in 25 °C water. The green sunfish's ability to continuously spawn in the laboratory is a beneficial trait for any aquacultured species (Stieglitz et al., 2017) or research animal (Gale and Buynak, 1982; Segner, 2009).

The growth rates of the lab-reared green sunfish varied widely within and among tanks. These results are validated by existing literature on green sunfish growth rates. By 30 dph we observed a mean TL of 11.9 mm which is slightly larger than the TL reported by Meyer (1970) at the

same age. However, Smith (1975) was able to rear green sunfish to a TL of 30 mm by 30 dph. Outliers in our study did exhibit similar growth rates as those reported by Smith (1975), but these fish were routinely culled due to their cannibalistic behavior. Smith's (1975) observations were likely the result of a relatively low stocking density of 50 embryos/60 L, whereas we cultured entire spawns in 37 L tanks until the fish were 30 dph before adjusting rearing densities. Smith (1975) also reported spawns by 16 weeks when the green sunfish had a mean TL of 100 mm. Based on our regression model, the mean TL of green sunfish in our rearing system was 32.87 mm at 16 weeks. Our observed growth rate and onset of sexual maturity in the offspring generation more closely matched results seen by Yun-Chang et al. (2008), who reported sexual maturity at 7–8 months at a TL of 87.50–94.50 mm. We observed sexually mature individuals by 213 dph when the mean TL was ~60 mm. In addition to starting with a lower stocking density, Smith (1975) fed brine shrimp to post larval green sunfish, whereas we only fed artificial diets after 37 dph. These two factors might have resulted in a faster growth rate than observed in our study. This variability in growth rates is another example of the plasticity of *Lepomis'* development and this genus's tolerance for overcrowding (Mittelbach, 1988; Aday et al., 2006).

As with most cultured species, green sunfish survival rates increased with size and age (Lorenzen, 1996). The survival rate of fish between 1 and 30 dph is likely overestimated considering green sunfish spawns can range from 2000 to 10,000 eggs (Yun-Chang et al., 2008). Green sunfish larvae stay benthic for at least 3 days, and we based stocking density estimates on counts of swim-up larvae from a sample of 500 mL of water when fish were 5 dph. It is possible that the water samples were not an accurate representation of the starting tank densities. Despite a continuous daily feeding regimen that ensured nauplii were always present in the tanks, growth variability likely resulted in significant reductions in survival due to concomitant cannibalism during the first 30 days of growout. Sorting and grading has been shown to reduce cannibalism in warmwater species (Kelly and Heikes, 2013). Routinely grading green sunfish during the first two months post hatch may increase survival rates and alleviate increasing growth disparities during growout.

A. hydrophila infections were another significant cause of mortality, especially during the weaning period from live nauplii to an artificial diet. *A. hydrophila* is a common bacterial infection in freshwater fishes that can cause severe disease and losses in cultured warmwater species (Cipriano et al., 1984; Swann and White, 1991). Uneaten diet would temporarily collect on the bottom of the larval rearing tanks when the juvenile green sunfish were transitioned from live nauplii to the artificial diets. Although there was no change in the tested water quality parameters during this time, the accumulation of uneaten food and waste, coupled with increased tank densities due to fish growth, may have facilitated *A. hydrophila* infections. Oxytetracycline treated diet cured infections and reduced mortalities, but prevention of these infections is obviously preferred. Bottom-draining tanks and increased filtration, especially UV filtration, during diet transitions might have helped prevent any changes to water quality conditions and helped reduce stress that facilitated infections (Swann and White, 1991). Continual feeding of live diets is labor intensive and makes administering treatments and medication via feeding challenging or impossible. With self-cleaning rearing tanks and more intensive filtration systems, the benefits to research and aquaculture operations of transferring the green sunfish onto an artificial diet during their development could outweigh any potential risk of disease outbreaks.

4.2. Gonadal differentiation of green sunfish

In this study we were able to describe the microscopic features of differentiation in green sunfish gonads using histology. We found that green sunfish are gonochoristic, with testes and ovaries differentiating from undifferentiated gonads. Ovarian differentiation preceded testicular differentiation by 30 days. By 99 dph all sampled gonads showed clear morphological sex differentiation (Fig. 6). We noted a heavily male-skewed sex ratio in fish sampled between 79 and 322 dph (71.67% male). We did not detect any transitions from ovaries to testes in the form of intersex gonadal tissue in our sampled individuals. In many fish species, increased temperatures or high stocking densities have been shown to increase the proportion of males in a reared cohort (Baroiller et al., 1995; Roncarati et al., 1997; Ospina-Alvarez and Piferrer, 2008). Temperature effects on sex ratios have also been identified in the closely related bluegill (Shen et al., 2016). It is possible that temperature effects or rearing densities could have a resulted in male-skewed sex ratios in our sampled fish.

The transformation of primordial germ cells to oogonia and the subsequent development of chromatin-nucleolus stage oocytes were the first observed signs of ovarian differentiation. The development of oogonia and chromatin-nucleolus stage oocytes has been described by many researchers with regards to ovarian differentiation (Jensen and Shelton, 1983; van der Ven and Wester, 2021; Uguz, 2008; Gao et al., 2009), yet we found these cytological differences a challenging benchmark to identify and instead, the presence of perinucleolus stage oocytes seemed to be a more reliable and conspicuous indicator of ovarian differentiation. Yun-Chang et al. (2008) also remarked that at one-month of age, distinguishing between male and female gonads is difficult. As similarly expressed by Yun-Chang et al. (2008), the most notable feature in a developing ovary is the highly basophilic cytoplasm in previtellogenic oocytes. This feature was ubiquitous in ovaries older than 49 dph.

Ovaries between 59 and 159 dph grew with little histological change aside from an increase in number of primary oocytes and the development of a thin layer of vacuoles along the periphery of the oocyte nucleus. Yun-Chang et al. (2008) observed larger flat droplets and increased vitellogenesis in green sunfish 4–5 months old. We only observed that level of vitellogenesis in 322 dph ovaries, after fish were moved to larger growout tanks. The relative delay in vitellogenesis observed in our study could be due to the high stocking density the fish were kept in during the first 150 days of growout or due to differences in diet (Schreck et al., 2001). Ovaries of sexually mature females exhibited various levels of vitellogenesis, ranging from primary to fully mature. This variety of oocytes at different stages of development could explain the green sunfish's ability to continuously spawn under the lab's broodstock holding conditions.

The first signs of testicular differentiation were clusters of presumptive spermatogonia that were loosely organized into lobules and supported by stroma. During the first two months of development, the spermatogonia were indistinguishable from undifferentiated primordial germ cells, but as previously described in other papers, germ cell clusters are indicative of testicular differentiation (Yun-Chang et al., 2008; Gao et al., 2009; van der Ven and Wester, 2021). Yun-Chang et al. (2008) reported the formation of seminal lobules in 1-month old testes. We did not observe this until fish were 59 dph. Aside from this earlier detection of differentiation, the overall pattern and monthly timing of testicular development described by Yun-Chang et al. (2008) was similar to our own findings, except they do not report any meiotic activity in testes until fish are four months of age, yet we saw spermatocytes yielding spermatids in fish as young as 99 dph.

Many researchers have discussed the plasticity of the timing of sexual development in fishes (Billard et al., 1981; Eyeson, 1983; Blay, 1985; Gao et al., 2009). The onset of ovarian differentiation and age at first spawn of our green sunfish was similar to that observed by Yun-Chang et al. (2008), yet we did not observe vitellogenesis in ovaries sampled up to 150 dph. Yun-Chang et al. (2008) described advanced stages of vitellogenesis in fish 4–5 months of age and observed earlier testicular differentiation as well, but our green sunfish displayed earlier testicular maturation.

The timing and pattern of testicular differentiation of our green sunfish resemble that of bluegill (Gao et al., 2009), but we observed earlier oocyte development in green sunfish than bluegill. Despite sharing similar growth rates and undifferentiated gonad descriptions as

compared to bluegill, we observed 49 dph green sunfish ovaries that already contained perinucleolus oocytes whereas Gao et al. (2009) did not observe oocytes at this stage of development in bluegill until 90 dph. Gao et al. (2009) demonstrated the plasticity in the timing of sexual development in bluegill by comparing the timing of sexual differentiation between a slow-growing batch that was stocked at a high density and a fast-growing batch stocked at a relatively low density. They also noted that sexual differentiation was a function of length, not age. It is likely that green sunfish exhibit similar plasticity in the timing of their sexual development and that age is an index that can be used to estimate TL under specific rearing conditions (Fig. 2B). Nevertheless, there was no difference in the timing of gonadal differentiation in our low-density and high density larval-rearing tanks. Under our rearing conditions, we first saw cytological differentiation of ovaries by 39 dph. Although cytological testicular differentiation was first detected at 69 dph, some individuals still had undifferentiated gonads at 89 dph. No fish sampled after 99 dph had undifferentiated gonads. Under the concept of the labile period of fish sex determination (Hackmann and Reinboth, 1974; Piferrer, 2001), green sunfish under similar rearing and feeding conditions could display permanently altered sex differentiation if exposed to endocrine disruptors or exogenous sex hormones before 39 dph (TL = 16–34 mm) through 99 dph (30–35 mm). Unsurprisingly, our proposed labile period is similar to the labile period identified in bluegill (Gao et al., 2009). However, since sex differentiation begins with biochemical processes (Haffrey et al., 2009) and anatomical differentiation typically precedes cellular differentiation (Jensen and Shelton, 1983; Sacobie and Benfey, 2005), beginning the treatment period earlier than 39 dph may be necessary for complete sex reversal. Starting to wean juvenile green sunfish onto artificial diets by 25 dph, as we did in this study, could help facilitate the administration of exogenous steroids during a critical period for altering their sex differentiation (Yamamoto, 1963) and assist in the development of TSC carrying fish (Schill et al., 2016; Teem et al., 2020).

5. Conclusions

We were able to obtain year-round spawns of green sunfish, rear juveniles using artificial diets, and produce an F2 generation in captivity. The ability to be reared successfully and to spawn so frequently may lend the species to a wide variety of laboratory studies. A detailed description of gonadal differentiation of green sunfish that identified their labile period in sexual development was also obtained. The acquisition of both detailed aquaculture protocols and an understanding of the gonadal development of green sunfish may contribute to enhanced stocking programs or alternatively, assist with eradication of invasive populations via development of TSC broodstocks.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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