

COMMERCIAL PRODUCTION OF SELECTED NATIVE FRESHWATER ORNAMENTAL SPECIES

Reporting Period

September 1, 2017 - August 31, 2018

FUNDING LEVEL:

Year 1	\$74,426
Year 2	\$75,000
Total	\$149,426

INSTITUTIONS AND PRINCIPAL INVESTIGATORS:

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Virginia Tech University.....	Dr. Donald Orth Dr. Eric Hallerman Dr. Michael Schwarz

PROJECT OBJECTIVES

The objectives of this project are to:

- 1) Develop commercial production techniques and protocols for high-value native fishes:
 - a) Blackbanded Sunfish *Enneacanthus chaetodon* (UF)
 - b) Gulf Coast Pygmy Sunfish *Elassoma gilberti* (UF)
 - c) Sailfin Shiner *Pteronotropis hypselopterus* (UF)
 - d) Flagfin Shiner *Pteronotropis signipinnis* (UF)
 - e) Bluenose Shiner *Pteronotropis welaka* (LSU)
 - f) Bluehead Shiner *Pteronotropis hubbsi* (LSU)
 - g) Golden Topminnow *Fundulus chrysotus* (LSU)
 - h) Rainbow Darter *Etheostoma caeruleum* (VT)
 - i) Mountain Redbelly Dace *Chrosomus oreas* (VT)
- 2) Extend this information to commercial clientele and assist with marketing strategies (UF, LSU, VT).

ANTICIPATED BENEFITS

Freshwater fishes native to the U.S. have been largely overlooked by domestic ornamental producers and cultivation of these species for sale within the country as well as to export markets would help to further increase the resiliency of the U.S. ornamental aquaculture industry through diversification. Furthermore, culture of these endemic species may prove advantageous for U.S. farmers as these species are generally more tolerant of cooler temperatures than their tropical counterparts, allowing them to be extensively cultured across a wider geographic area. Commercial production of North American freshwater species for ornamental markets has generally occurred on a small scale. While a significant

number of native species have been cultured in captivity, empirical evidence to support management decisions and production goals is limited. Fundamental information regarding reproduction, larval culture, and production techniques is critical when evaluating a species for commercial propagation and these bottlenecks will ultimately dictate the success of domestication and cultivation efforts. Accordingly, the objectives of the proposed research aim to define effective culture protocols for nine species of freshwater fishes endemic to the U.S. Species specific protocols will be immediately transferred to stakeholders within the southern region to aid in commercialization of research species.

PROGRESS AND PRINCIPAL ACCOMPLISHMENTS

Objective 1 - Develop commercial production techniques and protocols for high-value native fishes
*Sub-objective 1a, 1b, 1c, and 1d. Develop commercial production techniques and protocols for Blackbanded Sunfish *Enneacanthus chaetodon*, Gulf Coast Pygmy Sunfish *Elassoma gilberti*, Sailfin Shiner *Pteronotropis hypselopterus*, and Flagfin Shiner *Pteronotropis signipinnis* (University of Florida).*

The overall goal of the current grant is to develop culture protocols to facilitate commercial production of high-value native fishes. Thus, prior to development of culture protocols wild broodstock must be acquired. Significant effort was made during year one of the project to acquire broodstock of the Blackbanded Sunfish, Flagfin Shiner, Sailfin Shiner, and Gulf Coast Pygmy Sunfish. Following collection, all four species were held at the University of Florida Tropical Aquaculture Laboratory for conditioning for subsequent spawning and larval culture trials.

*Blackbanded Sunfish (*Enneacanthus chaetodon*)*

The Blackbanded Sunfish (*Enneacanthus chaetodon* [Baird 1855]) exhibits a disjunct distribution throughout its range, from New Jersey to the central highlands of Florida. While specimens are captured sporadically from locations in and around the Ocala National Forest, just one site, Rachel Lake, exhibits consistent captures in Florida. Multiple collection trips were taken to gather specimens from Rachel Lake, a low pH heavily vegetated lake in north Florida. Approximately 200 Blackbanded Sunfish were captured during these sampling efforts representing a mixed population of both sexually mature and immature individuals. All specimens were transferred to recirculating aquaculture systems with a hardness and alkalinity of 17.1 mg/L and a pH of 7.0 in an attempt to mimic natural environmental conditions from Rachel Lake and promote gonadal development. The initial population was further distributed among numerous 37 – 110 L aquaria with 2 – 15 individuals per tank. Artificial spawning substrate in the form of simulated aquatic vegetation, bottle brushes, and a mix of sand and gravel were provided. A single spawning event from a pair of Blackbanded Sunfish resulted in viable fry, which were reared in a separate 37 L aquarium on the broodfish recirculating system. Larvae were initially fed a diet of *Artemia franciscana* nauplii and gradually transitioned to commercially available microparticulate diets (Golden Pearls, protein, 55%; lipids, 15%; ash, 12%; moisture, 8%). This preliminary trial suggests the species to be amenable to common larviculture protocols used in production of freshwater ornamental species. Experimental objectives involving embryo handling and larvae have yet to be completed with this species. However, broodstock are currently conditioned and we anticipate successful spawning in the coming months.

Gulf Coast Pygmy Sunfish (Elassoma gilberti)

The Gulf Coast Pygmy Sunfish (*Elassoma gilberti*) can be found from southwestern Georgia to northern Florida. Gulf Coast Pygmy Sunfish were collected from McBride Slough near Wakulla Springs. Roughly 100 individuals were captured here and at Rachel Lake. All of the fish from McBride Slough were lost to parasitic infestation. Rachel Lake broodstock began spawning consistently in June 2018 and experiments evaluating spawning substrate preference, incubation strategy, embryo disinfection, larval dietetics, and characterization of larval development were completed.

Broodstock were housed in four 37 L aquaria on a recirculating system containing 1600 L of spawning water with the following water quality parameters: hardness = 35 mg/L, CaCO₃ alkalinity = 35 mg/L, pH = 7.5, and temperature = 24.4°C. The photoperiod regime was set at 11 hours daylight and 13 hours night. Spawning media in each aquarium consisted of eight spring-green, eyelash-yarn mops (ICE yarns, GSC Tekstill Dis Tic. Ltd, Istanbul, Turkey), each approximately 18 cm long, attached to a styrofoam float on the surface of the water. Each aquarium was stocked with 10 female and 5 male *E. gilberti*. Brood fish were fed live *Artemia franciscana* nauplii and adult *Moina* sp. twice per day *ad libitum*. The mops were removed from each aquarium and agitated manually to remove the slightly adhesive eggs and the bottoms of the aquariums were then siphoned to remove any eggs that had been dislodged in transit. For all comparative egg and larvae trials, eggs collected during this process were enumerated and stocked into subsequent experiments. Due to the limited number of eggs collected each day, egg incubation, egg disinfection, and larval feeding experiments were temporally replicated.

Spawning substrate preference experiments were completed in six 37 L aquaria contained within a recirculating system. Each tank was provided with three types of spawning media: 1) a single floating eyelash yarn mop in spring green, 2) a 670 ml square plastic container with a single black bottle brush and 3) a 670 ml square container that contained a single layer of smooth river rock gravel (10 – 50 mm). Each aquarium was stocked with two mature males and five mature females and the media were checked for eggs and larvae late morning every Monday, Wednesday, and Friday over a six-week period. Harvested contents were examined under a dissecting microscope to enumerate the fertilized eggs, unfertilized eggs, and hatched larvae in each media type.

Egg incubation studies were carried out using twenty embryos stocked into either a 150 ml plastic sample cup with 40 µm screened bottom (static incubator, SI), or an upwelling incubation apparatus fashioned from a 50 ml conical tube (upwelling incubator, UI). Both incubation containers were transferred to a single 3 L polycarbonate tank, where incoming flow was directed to the bottom of the UI with a plastic tube and allowed to exit through 40 µm mesh surrounding the top of the UI. The SI was placed in a foam ring, which allowed it to float within the 3 L tank. After 3 days of incubation, hatching success was recorded by counting larvae in each incubation container. This process was repeated four times as spawning permitted to achieve adequate replication. Egg disinfection experiments were conducted using varying doses of 1) formalin (Parasite-S [37 % formaldehyde], Syndel, Ferndale, WA, USA), 2) iodine (Ovadine [10 % povidone-iodine buffered solution,] Syndel, Ferndale, WA, USA), and 3) hydrogen peroxide (3% hydrogen peroxide, Equate, Bentonville AR, USA). Four groups of 10 eggs were counted into individual SIs, and transferred to subsequent 150 ml sample cups containing serially diluted disinfectants of the following concentrations: 1) formalin: 2,000 mg/L, 1,000 mg/L, 500 mg/L, 0 mg/L; 2) iodine: 200 mg/L, 100 mg/L, 50 mg/L, 0 mg/L; 3) hydrogen peroxide: 1,000 mg/L, 500 mg/L, 250 mg/L, 0 mg/L. Eggs remained in disinfectant solutions for 15 minutes for formalin and hydrogen

peroxide, and 10 minutes for iodine. Following a 3-day incubation, the number of eggs hatched for each SI was recorded. This process was repeated four times for each chemical, as broodstock spawning permitted, to allow for temporal replication.

Larval dietetics experiments were stocked with 10 larvae into each of four 3 L tanks at 2 days post hatch (dph) as spawning permitted. At the onset of exogenous feeding (4 dph), fish from each tank were fed twice per day with either newly hatched *Artemia* nauplii (instar 1-2), or one of three commercially available microparticulate diets: MD1 (Larval AP-100, Ziegler Bros Inc., Gardners, PA), MD2 (Golden Pearls 50-100 μm , Brine Shrimp Direct, Ogden, UT, USA), and MD3 (Otohime A1, Marubeni Nisshin Feed Co. LTD, Tokyo, Japan). *Artemia* were fed at a rate of 100 nauplii/L, and each microparticulate diet at a rate of 20 mg for each feeding time. On 18 dph, fish were harvested from each tank and placed on a Sedgewick Rafter cell and photographed for subsequent morphometric analysis. Survival and standard length were determined and the experiment was replicated temporally ($n = 6$) as spawning permitted.

A descriptive growth and development trial was stocked with 40 eggs into four SI in a single 10 L tank with flow through well water. Beginning at 4 dph, larvae were fed newly hatched *Artemia* nauplii (1-2 instar) *ad libitum* twice per day. Five of the resulting larvae were randomly sampled from among the four SIs at 1, 3, 5, 9, 12, 15, 22, 26, and 30 dph. The larvae were placed in water on a Sedgewick Rafter slide and photographed under a trinocular dissecting microscope with digital camera attachment. After photographs were taken, the larvae were promptly returned to their original SI within the 10 L rearing tank. Larval development milestones, including eye pigmentation, swim bladder inflation, flexion, and hypural plate formation were documented and larvae were digitally measured.

Adult *E. gilberti* preferentially spawned in yarn mops, with 77.5% of the total eggs collected from this media type. Eggs from bottle brush and gravel media comprised 15.3% and 7.1% of the total, respectively. Average calculated fecundity assuming equal contribution by each female per tank on days where spawning was observed was 1.1 ± 1.7 eggs/female. However, considering not all eggs were captured in spawning media and not all females likely contributed to a given spawning event, fecundity estimates may be underrepresented. Eggs incubated in static incubators exhibited a mean hatching success of $71.25 \pm 22.5\%$, which was significantly higher than those incubated in upwelling incubators ($13.75 \pm 7.5\%$). There were no significant differences in hatch rates relative to a control following immersion in formalin, iodine or hydrogen peroxide at three different concentrations per chemical. Average hatch rate was generally high in each trial, with mean values across all concentrations of 71.85% for formalin, 61.33% for iodine, and 85.63% for hydrogen peroxide (Table 1). Average survival to 18 dph across all treatment groups in the larval dietetics trial was $50.0 \pm 23.2\%$ and was not significantly different among treatments (Figure 1). However, fish fed *Artemia* exhibited statistically higher standard length ($4.92 \text{ mm} \pm 0.53$) than MD1 ($4.16 \text{ mm} \pm 0.21$), MD2 ($4.32 \text{ mm} \pm 0.34$), and MD3 ($4.40 \text{ mm} \pm 0.51$) (Figure 1). Results from the developmental trial showed average length increased from $3.41 \text{ mm} \pm 0.14$ at 1 dph to $5.06 \text{ mm} \pm 0.31$ at 30 dph (Figure 2). Larvae exhibited eye pigmentation at 1 day post hatch (dph), swim bladder inflation at 5 dph, flexion at 12 dph, and hypural plate formation at 22 dph. The results of these trials provide necessary information for the successful culture of *E. gilberti*, as well as valuable baseline data for captive rearing of other members of the genus. While the rearing protocols described here are intended to facilitate commercial production of *E. gilberti* as an ornamental, additional motivation to culture this species or congeners may come from conservation or restoration initiatives.

Table 1. Percentage of *E. gilberti* embryos that hatched following immersion in three disinfectants. Doses are assigned as follows: Formalin: Control = 0 mg/L, a = 500 mg/L, b = 1,000 mg/L, c = 2,000 mg/L; Iodine: Control = 0 mg/L, a = 50 mg/L, b = 100 mg/L, c = 200 mg/L; Hydrogen Peroxide: Control = 0 mg/L, a = 250 mg/L, b = 500 mg/L, c = 1,000 mg/L. Percentage hatch did not vary significantly within each disinfectant among concentrations ($P \geq 0.164$).

Disinfectant	Dose			
	Control	a	b	c
Formalin	67.5 ± 12.6	82.5 ± 5.0	70.0 ± 14.1	67.5 ± 5.0
Iodine	66.7 ± 25.2	53.3 ± 5.8	56.7 ± 23.1	50.0 ± 36.1
Hydrogen Peroxide	90.0 ± 14.1	80.0 ± 14.1	90.0 ± 14.1	82.5 ± 17.1

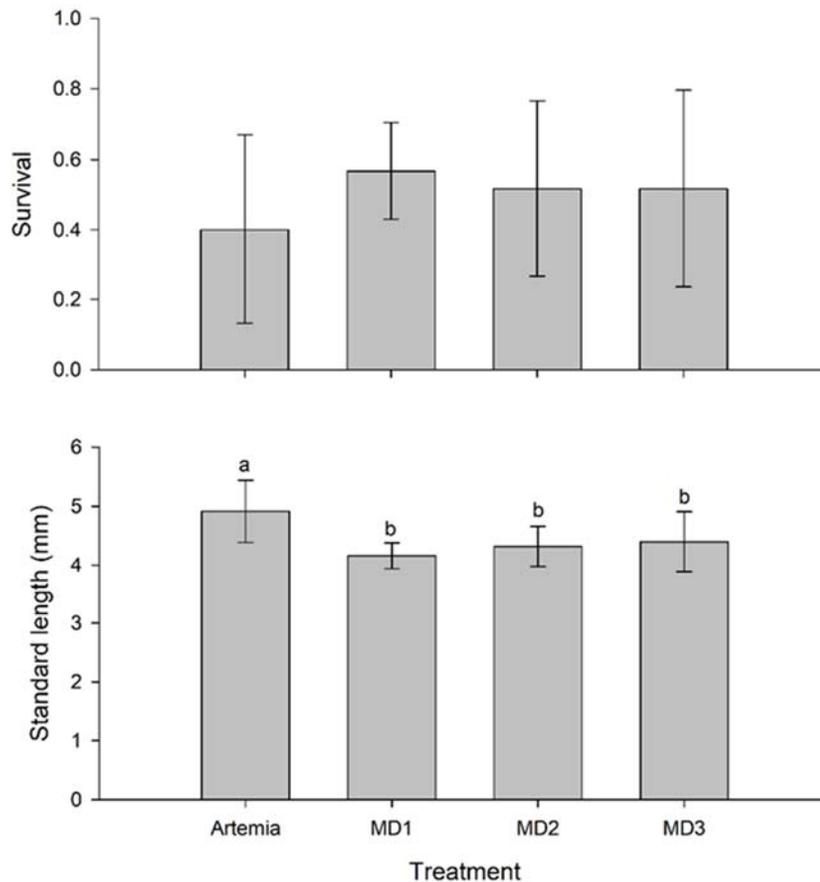
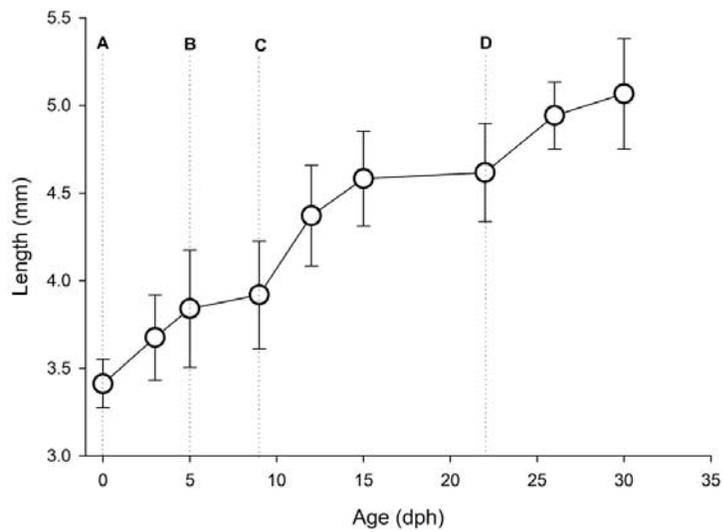


Figure 1. Survival ($P = 0.370$) and standard length ($P = 0.004$) of *Elassoma gilberti* following initial 14 days of exogenous feeding, being fed exclusively either *Artemia* nauplii, Ziegler Larval AP-100, Golden Pearls, or Otohime A1. Different letters among treatments denotes significant differences ($P \leq 0.05$). Flagfin Shiner (*Pteronotropis signipinnis*) and Sailfin Shiner (*Pteronotropis hypselopterus*)

a.



b.

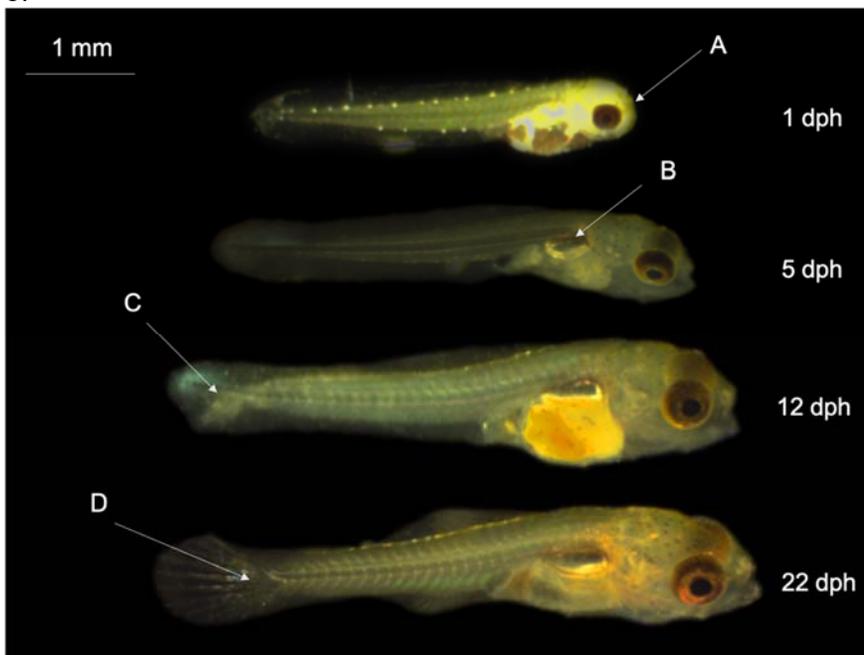


Figure 2. a.) Growth of *Ellassoma gilberti* between 0 and 30 days post hatch (dph). A: eye pigmentation (1 dph), B: swimbladder inflation (5 dph), C: initiation of notochord flexion (9 dph), D: hypural plate formation (22 dph). b.) Development of *E. gilberti* between 1 and 22 dph.

The Flagfin Shiner (*Pteronotropis signipinnis*) is distributed throughout Gulf Slope drainages from Mississippi to Florida. In Florida, it can be found in large numbers in panhandle drainages. Approximately 200 Flagfin Shiners were collected from a tannic stream near Ebro, Florida and from McBride Slough near Wakulla Springs. All specimens were transferred to a recirculating aquaculture system with a hardness and alkalinity of 17.1 mg/L and a pH of 7.0 in an attempt to mimic natural environmental conditions and promote gonadal development. A diet of frozen blood worms

(Chironomidae), frozen *Artemia*, live hatched *Artemia* nauplii and flake food was fed *ad libitum* to broodstock daily. Sand and river gravel substrates were provided for spawning. This initial population was subsequently diagnosed with microsporidian and myxozoan parasites and were removed from forthcoming experiments. Additional specimens were collected during year two in Louisiana (~100) and Florida (~100) due to disease related losses.

The Sailfin Shiner (*Pteronotropis hypselopterus*) is distributed in Gulf and Atlantic drainages from South Carolina to Florida and can be found as far south as Tampa in peninsular Florida. Two collecting trips were taken around the Tampa Bay area, one to Bell Creek and another to a small stream near Picnic, Florida. No specimens were captured at Bell Creek and ~24 were captured near Picnic. Additional collecting trips will be taken to Picnic to increase the number of broodstock. The captured individuals are being conditioned on varied diet of live, frozen, and prepared feeds in static water systems with periodic water changes.

Flagfin and Sailfin shiner spawning has been inconsistent irrespective of manipulations in environment and spawning substrates. Difficulties with these species has served as an impetus to pursue similar lines of investigation with a closely related and phenotypically similar species, the Metallic Shiner (*Pteronotropis metallicus*) during year two. Approximately 200 Metallic shiners were collected from a small tributary of the Alafia River near Picnic, FL. These broodstock were quarantined for 30 days and conditioned for spawning with a diet of frozen blood worms and frozen *Artemia*. Broodstock were held in nine 27 L aquariums on a recirculating system and began spawning in August of 2018. A suite of experiments, similar to those described for the Gulf Coast Pygmy Sunfish, were carried out. These experiments included evaluations of spawning substrate preference, incubation strategy, embryo disinfection, and larval dietetics. Methods for these experiments mirrored those of the Pygmy Sunfish with the sole exception of a 4 male: 4 female stocking density for substrate preference experiments.

Experimental results indicated Metallic shiners preferred floating yarn mops as a spawning substrate, followed by the bottle brush and then the gravel. The shiners exhibited a relatively high batch fecundity (12 eggs/female per spawn), continually spawning for more than four months. Static and upwelling incubation techniques resulted in similar hatch rates. Submersion in three different concentrations of iodine and hydrogen peroxide resulted in similar hatch rates (>60%) with respect to the control. However, the highest dose of formalin evaluated (2,000 ppm) resulted in significantly lower hatch than the lower doses and the control, indicating potential toxicity (Figure 3). Survival and growth were similar for fish fed one of three manufactured diets relative to *Artemia*. Survival and growth ranged from 16 – 44% and 5.8 – 6.1 mm respectively, across dietary treatments. These results indicate that commercial microparticulate diets are a viable replacement for *Artemia* during larviculture of the Metallic Shiner.

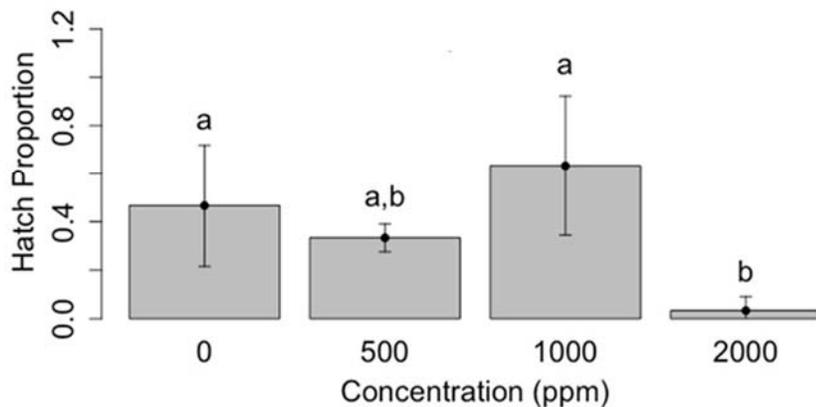


Figure 3. Proportion of *P. metallicus* embryos that hatched following immersion in Formalin: Control = 0 mg/L, a = 500 mg/L, b = 1,000 mg/L, c = 2,000 mg/L. Different letters among treatments denotes significant differences ($P \leq 0.05$).

Sub-objective 1e and 1f. Develop commercial production techniques and protocols for Bluenose Shiner Pteronotropis welaka and Bluehead Shiner Pteronotropis hubbsi (Louisiana State University).

Prior to the start of the project, our lab identified bluenose shiner habitat and made an initial collection of ~55 individuals. Flagfin shiner were also collected at the same site for Sub-objective 1d. Trips to possible sites for the bluehead shiner made during 2017 and 2018 were unsuccessful at locating this species.

Museum loans of historic bluenose shiner collections from the Tulane University Biodiversity Research Institute's ichthyology collection were accessed to assess external morphometrics and collect gonads for gonadosomatic index (GSI) and histology. These previous collections were conducted within April through June and represent a critical period in gonad development. Increases in GSI were observed for both males and females from April to late May, with decreasing GSI observed in mid to late June (Figure 4). Male and female bluenose shiner gonads were processed for histology and we are currently scoring oocyte development on these historical samples.

Bluenose shiner collected in the spring of 2017 were stocked in 114-L tanks within the same recirculation system. Each tank was stocked with five males and 8 – 10 females and was maintained at a temperature of 12°C and reduced photoperiod (10 L: 14 D). On April 3rd the temperature of system was increased by 2°C every week while day length (light) was increased by one hour every 1.5 weeks. After 4 weeks the fish reached a temperature of 23°C and photoperiod of 13 L: 11D and were maintained at those parameters for spawning trials. A small spawn mat (4 X 6 cm) was placed in the bottom of each tank and checked twice weekly for eggs. We collected no eggs after four weeks at the target temperature and photoperiod.

Due to reports of this species spawning within close proximity to sunfish nests we created artificial nests with larger rocks placed in a petri dish, which could be inspected for eggs by siphoning. After the

placement of these nests in each of the three tanks we introduced a 2-ml suspension of green sunfish sperm over the nests as a potential olfaction cue for spawning. No eggs were collected from the nests, however, we did collect a single unfertilized egg from a spawntex mat within 48 hours of milt introduction.

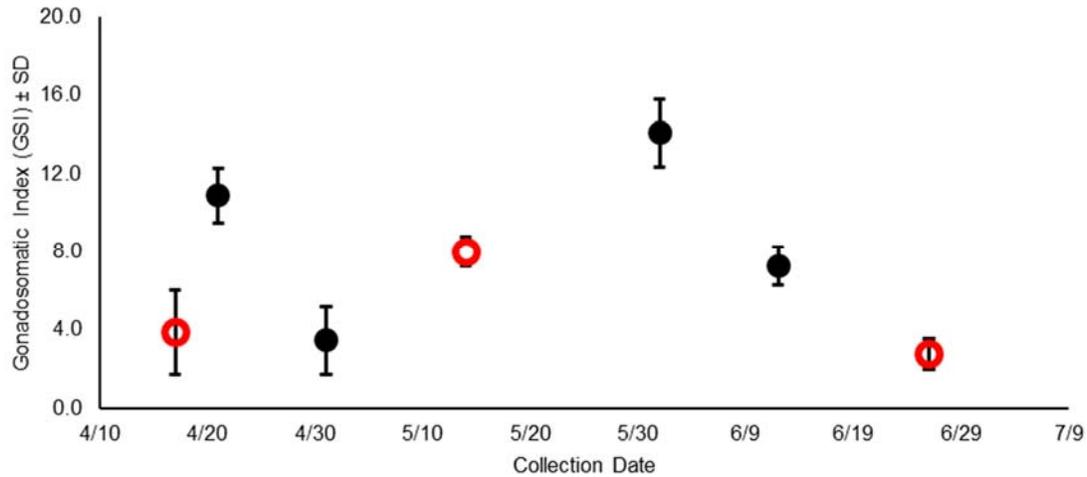


Figure 4. Female Bluenose shiner GSI (+ SD) from historical collections. Closed circles represent different collection locations while open circles represent the same location sampled multiple times between April and June.

Two weeks after the introduction of artificial sunfish nests and sunfish milt a sub sample of fish were selected for Ovaprim injections. Three males and 6 females were weighed and given an intraperitoneal injection (IP) with Ovaprim at a rate of 50 $\mu\text{l/g}$ and placed in three floating mesh baskets (16 x 12 x 14 cm) each with one male and two females. Each floating mesh basket was siphoned for eggs daily for a total of 5 days after Ovaprim injections and no eggs were observed from these collections. The females were anesthetized with clove oil and palpated to investigate oocyte maturation. Three of the six females released small, opaque oocytes. One of the six females released clear oocytes containing relatively clear yolk and we combined those eggs (47) with newly collected milt in an attempt to produce in vitro fertilization. Embryos (33) hatched at approximately 50 hours after fertilization at $\sim 24^{\circ}\text{C}$ (Figure 5). As post hatch development proceeded, the larvae used yolk reserves and began to develop cranial and pericardial deformations which eventually resulted in 100% mortality approximately 7-9 days after hatch.



Figure 5. Bluenose shiner larvae at approximately 24 hours post hatch.

Substrate (sand and rocks), water, and submerged vegetation were collected from the stream sites and placed in three identical 114-L tanks. Each tank contained two artificially constructed sunfish (*Lepomis* spp.) nests and a sunfish decoy floating above one of these two nests. Males (one per tank) and females (4-5 per tank) were stocked in each tank and allowed one week to acclimate prior to hormone injections. Each tank was assigned a hormone treatment and females received an IP injection of one of the following hormones: (Table 2).

Table 2. Hormone treatments and dose administered to female bluenose shiners.

Treatment	Dose	N
Ovaprim	75 µg/g	4
cGnRH-IIa	100 µg/g	5
mGnRH-Ia	100µg/g	4

Nests were checked for the presence of eggs every 24 hours for a total of 72 hours following hormone injections by siphoning the sunfish nests. Throughout this period there was no evidence of volitional spawning as no freely released eggs were collected for any hormone treatment. One female from each hormone treatment was gently palpated to observe the extent of oocyte maturation at 72 hours post injection. Resulting eggs were observed for maturational status and compared to oocyte stages described in Harrell et. al. (1990). Only one female from each hormone treatment group was palpated as we have experienced mortality from palpations in the past. When palpated, the female treated with Ovaprim released eggs that appeared to be undergoing atresia and breaking down. Oocytes from a cGnRH-IIa treated female were relatively clear and contained yolk that was not yet coalesced into a single oil droplet (Figure 6). The female treated with mGnRH-Ia produced immature eggs that were small in diameter and opaque.

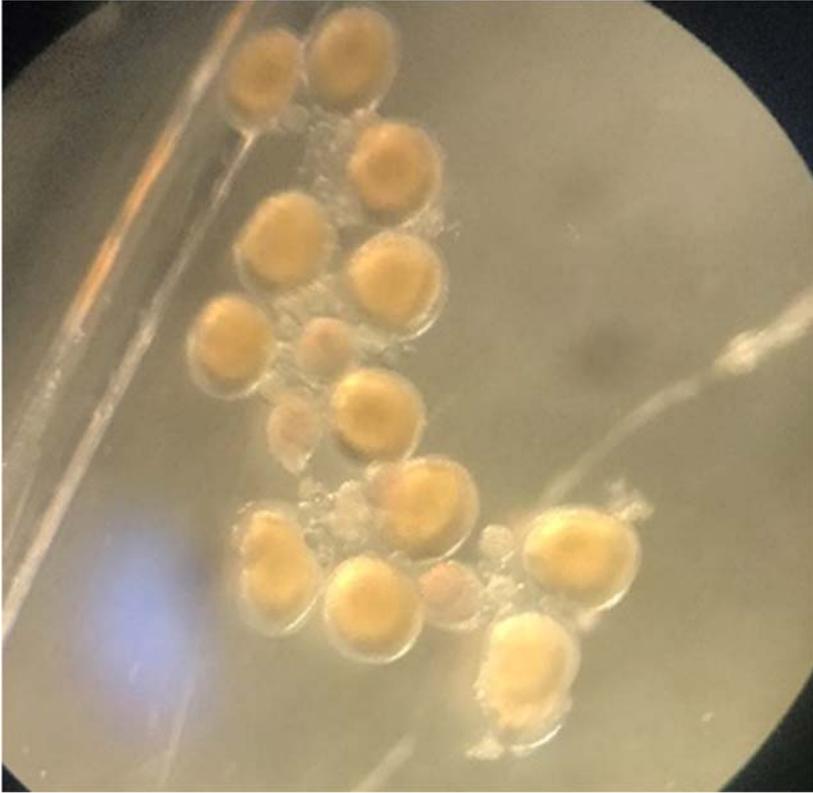


Figure 6. Oocytes palpated from a bluenose shiner treated with cGnRH-IIa. Yolk deposition and egg clarity indicate that these females are investing in maturing eggs within our current temperature and photoperiod regimes.

Previous simulations of natural conditions (vegetation, substrate, artificial sunfish nests) within recirculation systems used manipulated temperature and photoperiod regimes to induce volitional spawning without success (Figure 7), with indoor manipulations previously described. An overview of each of the aforementioned experimental setups is presented in Table 3.

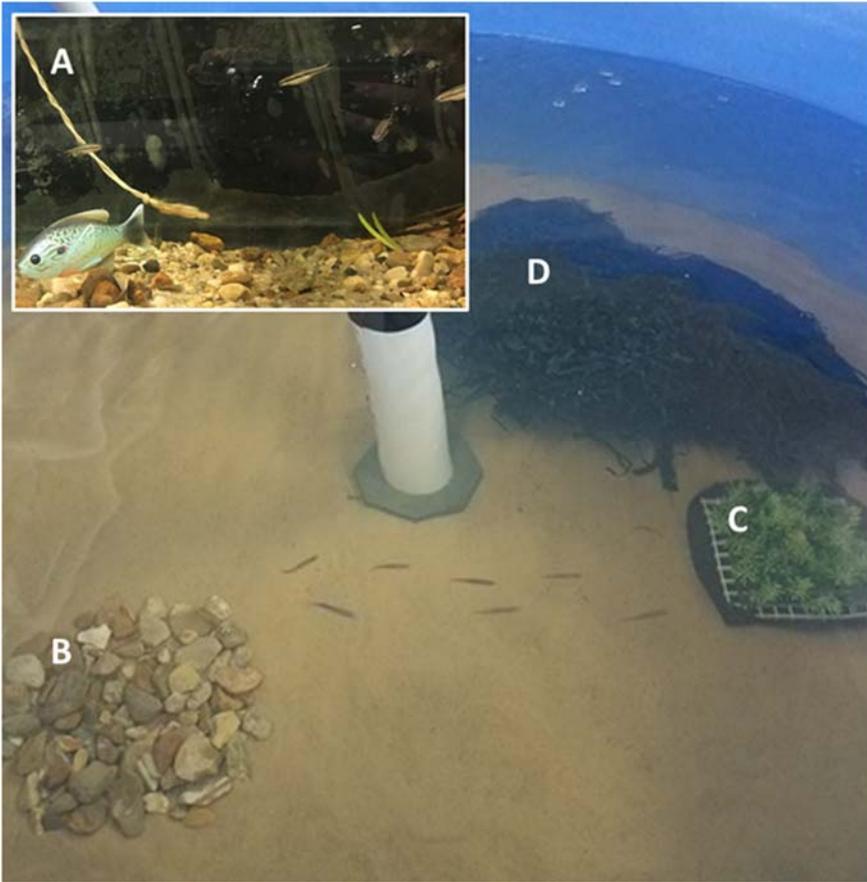


Figure 7. Indoor simulations of potential spawning substrates to induce volitional spawning of Bluenose Shiners (*Pteronotropis welaka*) under increasing photoperiod and temperatures. Decoy sunfish (A) were suspended over artificially constructed sunfish nests within 100-L tanks. Larger 2000-L tanks were utilized to provide (B) artificially constructed sunfish nests, (C) short, and (D) long artificial vegetation.

Table 3. Various methods and experimental setups previously employed to induce volitional spawning of Bluenose Shiners. Indoor experiments did not result in volitional spawning for this species.

Tanks Used	Hormone Injections	Temperature Manipulation	Photoperiod Manipulation	Conductivity Adjustment	Substrates	Sunfish Cues
100 L Indoors	Yes	None	None	None	Natural stream vegetation and substrate	Artificial sunfish lures
2000 L Indoors	No	Yes	Yes	No	Sand and pom-poms	Constructed nest
100 L Indoors	No	Yes	No	Yes	None	One instance of sunfish milt
2000 L Indoors	Yes	No	No	No	Sand and pom-poms	Live bluegill over constructed nest
2800 L Outdoors	No	Natural	Natural	Natural	River silt, natural vegetation	Live Longear Sunfish, constructed nest

Three outdoor 1,400-L mesocosms were stocked with 3 Longear Sunfish and Bluenose Shiner (3 males: 9 females) in late Spring of 2018. Tubing and air stones were outfitted to each tank to provide oxygen for the fish. Netting was secured over the tanks to prevent predation. Shade cloth covered 60% of the total tank surface area to reduce thermal stress that could result from full exposure to direct sunlight (Figure 8). Pavestone pond pebbles were poured into a concentrated area of the tank on the margin of the shade cloth to simulate sunfish nests and allow for visual observations of the nest. Water temperature was recorded hourly with a temperature data logger beginning on July 9, 2018. Natural vegetation (Southern Water nymph *Najas guadalupensis* and Upright Burhead *Echinodorus berteroi*) was maintained through periodic mechanical removal when growth began to overtake the tanks, specifically when the simulated nests were crowded over by vegetation.

On July 19, larval fish were observed in a mesocosm. Eight of these larvae were caught and identified using a larval fish key in the lab to be Longear Sunfish. Another batch of larval Longear Sunfish was observed in the same mesocosm on August 20. Although no larval minnows were identified, the presence of one or more juvenile Bluenose Shiner were observed within the month of July that could indicate volitional spawning. Complete harvest of the mesocosms was completed within January 2019 and we found one young of the year Bluenose Shiner. Based on the size of this juvenile, spawning possibly occurred relatively soon after initial stocking.

The use of outdoor tanks stocked with sunfish as a potential spawning cue for Bluenose Shiners resulted in Bluenose Shiner spawning within the early summer. Predation of larval and juvenile shiner by sunfish spawned at the same time was anticipated to limit the production of larvae from these initial outdoor trials.



Figure 8. Outdoor 2000-L tanks with partial shade cover and aeration were stocked with Longear Sunfish and Bluenose Shiners and provided several vegetation types. Sunfish were provided larger substrate for nest construction (white circle) and reproduced throughout the late-spring to early-summer months.

*Sub-objective 1g. Develop commercial production techniques and protocols for Golden Topminnow *Fundulus chrysotus* (Louisiana State University).*

A local population of golden topminnows was identified and collected for this project prior to the performance period. These original 180 fish were stocked into outdoor pools containing submerged vegetation in the Spring of 2017. A regenerative blower supplied continuous aeration to all tanks via airstones. In May 2017 we stocked 30 fish each into four 2.5-m diameter outdoor static tanks containing approximately 2.0 m³ of water and 0.3 m³ of river silt. Two of these tanks were stocked with a 3:1, Female to Male ratio while the remaining two tanks were stocked at a rate of 2:1. Spawning mats consisted of 33 x 43 cm, 4-cm thick, sections of spawning substrate (Spawntex) on a wire frame, suspended horizontally approximately 8 cm below the water surface from two floats made of sealed PVC tubing. These mats were collected twice weekly and checked for newly fertilized eggs.

Submerged vegetation within these four treatment tanks precluded our ability to quantify egg production because it appears the fish were not depositing eggs on our artificial substrate. Rather than terminate the study we allowed the fish to produce embryos throughout the summer in order to build up an F1 generation for spawning studies within the Spring/Summer of 2018.

In September 2017 adult broodfish were moved to an indoor recirculation system to examine growth parameters over cooler winter months (Temperatures between 12 – 20°C). We stocked 30 sexually mature adults (6.4 ± 0.15 g) into four circular 200-L tanks within a recirculation system. Fish were fed a 48% protein/ 14% lipid diet daily for a total of approximately 10% body weight. All fish were weighed

every four weeks and average weight reported in grams (Figure 9). These fish were used in subsequent spawning studies to create recommendations for stocking density and optimum broodfish size.

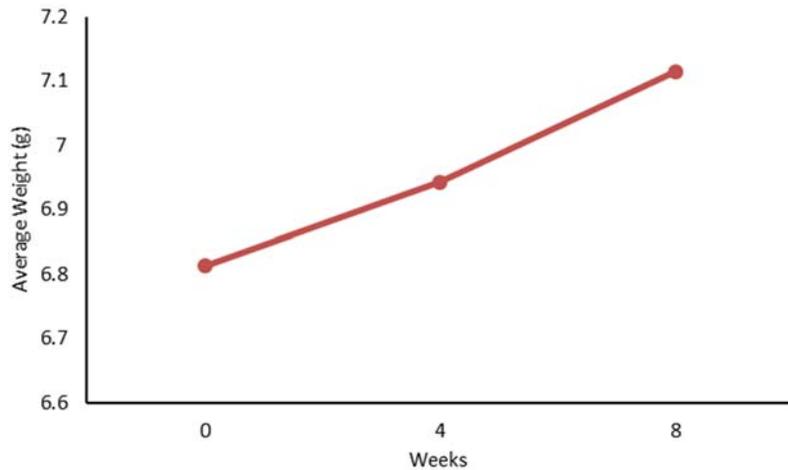


Figure 9. Average weight of golden topminnow broodfish reared within indoor tanks within November and December 2017.

During year two of the project a study was conducted to determine size related fecundity and egg production metrics to facilitate development of recommendations for female broodfish size (Small: $5.7 \pm 0.19\text{g}$; Medium: $8.53 \pm 0.16\text{g}$; Large: $12.2 \pm 0.47\text{g}$). Each 100-L aquarium contained an air stone, and 53 L of recirculating filtered water maintained at $28.1 \pm 0.84^\circ\text{C}$ and 14 h light:10 h dark. Six females and three males from each size class were stocked in triplicate. Males were stocked within approximately the same size range of their associated female size class (Small: $5.7 \pm 0.19\text{g}$; Medium: $8.53 \pm 0.16\text{g}$; Large: $12.2 \pm 0.47\text{g}$).

Females were anaesthetized and palpated every 3-4 days, for 2.5 weeks. Each fish was weighed before palpation to provide an exact egg/gram female fecundity measurement for each fish. Palpations were performed with consistency on each date (by the same person) through application of light pressure to the coelom, extracting only ripe eggs. Extracted eggs were counted and recorded in association with each individual female's weight.

Golden Topminnow embryos and newly hatched larvae were measured for length at hatch and yolk volume morphometrics. Twice weekly egg collections were conducted from broodfish stocked within 2000-L outdoor tanks. Viable embryos were cleaned, transferred to a new petri dish labeled with the collection date and tank, held for image capture, and stored in a non-light controlled, laboratory oven acting as an incubator (Temperature: $25.7 \pm 0.65^\circ\text{C}$ [mean \pm SD]) to maintain all embryos at a consistent temperature throughout the study. Mortality and hatched larvae were recorded daily. Newly hatched larvae were anaesthetized and recorded to measure total length and yolk volume.

The number of eggs female⁻¹ day⁻¹ (including all females each day) produced by Golden Topminnows were 1.1 ± 0.70 , 2.0 ± 0.69 , and 5.2 ± 1.40 for small, medium, and large female broodfish, respectively (Figure 10). This results in potential daily egg production from the two larger sized females groups at approximately 230 and 415 eggs per day for every 1000 g of female broodfish stocked. Egg area was

significantly greater for the largest size class, however, yolk volume was not different among female size classes (Table 4). These results indicate that although fecundity differs across female sizes it is possible that yolk reserves are conserved across female sizes.

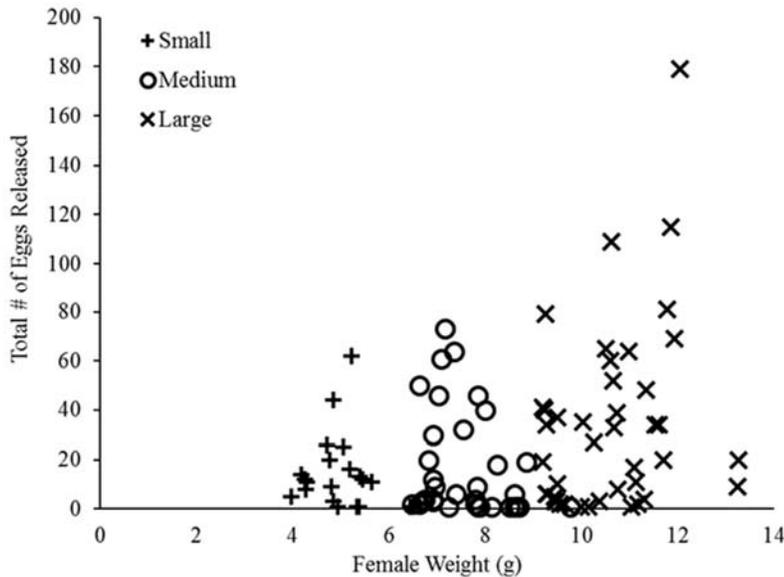


Figure 10. The relationship between Golden Topminnow female weight and the number of eggs released for small, medium, and large size classes from palpations every 3-4 days for 2.5 weeks.

Table 4. Mean \pm SEM egg area (mm^2) and yolk volume (mm^3) for newly collected Golden Topminnows. Within columns, different letters denote significant differences among female size classes for each embryo parameter ($P \leq 0.05$).

Female Size Class	Embryo Morphometrics	
	Egg Area (mm^2)	Yolk Volume (mm^3)
Small 3.6 \pm 0.1 g <i>n</i>	3.37 \pm 0.02 _y 151	2.29 \pm 0.02 151
Medium 5.9 \pm 0.1 g <i>n</i>	3.40 \pm 0.02 _y 156	2.33 \pm 0.02 156
Large	3.47 \pm 0.01 _z	2.35 \pm 0.02

*Sub-objective 1h and 1i. Develop commercial production techniques and protocols for Rainbow Darter *Etheostoma caeruleum* and Mountain Redbelly Dace *Chrosomus oreas* (Virginia Tech).*

The year one grant award was received late in the breeding season, which interfered with field work needed to collect reproductively ready broodstock candidates and limited our success. We had no access to Rainbow Darter, but worked as planned with Mountain Redbelly Dace. We worked, as noted below, with Fantail Darter *Etheostoma flabellare* and Creek Chub *Semotilus atromaculatus*. We have Redline Darter *Etheostoma rufilineatus* and Logperch *Percina caprodes* broodstock candidates on hand for next year's spawning season. At the Hampton lab, the broodstock environmental optimization (enhancement), broodstock conditioning/spawning tanks, and larval protocol development systems were constructed and 50 adult rainbow darters were received through a commercial supplier in year one.

In Blacksburg, Virginia project-related fish were held in the 9000-sq. ft. aquaculture center in recirculating aquarium or tank systems adapted to rearing the focal species for this project. Much of the spawning work was carried out in two recirculating aquarium systems, each with twelve 20-gallon tanks; each tank has fine gravel, and some have pebbles for chub nest construction. Redbelly dace are held in two 75-gallon recirculating tanks. Logperch broodstock are held in one 75-gallon recirculating tank, and larvae in four 10-gallon tanks or in an AHAB system (Pentair Aquatic Eco-systems). We find that the latter provides small, easy-to-clean and monitor space which is ideal for larval fish. In Hampton, the AQUA-AHAB, a turnkey recirculating aquaculture system manufactured by Aquaneering, has been procured and installed. This system has 18, 10L tanks to be used for optimizing of broodstock environment (enhancement) and for larviculture. The main broodstock conditioning and spawning system was brought online, with three 100-L tanks connected to a robust RAS and a digitally controlled chiller. This system is ready to receive fish as soon as sufficient numbers are available from the supplier for shipping.

Mountain Redbelly Dace spawn from mid-spring to early summer on the gravel nests of other minnows, especially chubs. We stocked approximately 30 Mountain Redbelly Dace (at a ~1:1 sex ratio) in a 75-gallon recirculating tank with an artificial chub mound made of large gravel set over a wire mesh providing a place for incubating eggs and rearing larval dace (Figure 11). We added fresh, macerated chub testis to induce spawning of the dace. While we never saw eggs or larvae, in 2017, two small juvenile dace were found, which proved easy to raise on brine shrimp and microworms (nematode *Panagrellus redivivus*). In 2018, a dozen larval dace were collected and raised.

We collected Fantail Darter egg masses from the wild. Untreated eggs did not survive. Iodine-treated eggs showed low survival. Eggs treated daily with 100 ml of 3% over-the-counter strength hydrogen peroxide per 3 L of water exhibited high survival. The eggs hatched in May 2017, and high survival was observed for those not used for another purpose.

Swim-up fry of all species were fed newly hatched Brine Shrimp *Artemia* sp. nauplii and microworms produced onsite. Adult Logperch also took redworms, blackworms, and frozen *Tubifex* worms. Attempts were made to wean juveniles of all species to dried *Tubifex* worms, frozen bloodworms, flaked tropical fish food, and ground trout chow. All species successfully made transition of non-live foods/feeds at about 6-8 weeks old.



Figure 11. The photograph on the left shows the gravel breeding mound with the meshing completely covered. The photograph on the right is of the meshing placed underneath gravel breeding mound.

In planned breeding for spring 2018, following recommendations from Conservation Fisheries Incorporated (Knoxville, TN), single male and 2-3 female Redline Darters will be held in aquaria with gravel and tiles under which the females can deposit eggs. Logperch will be held in small and larger groups in aquaria with sand to determine whether we can obtain spawn in that manner. Mountain Redbelly Dace will be held with spawning chubs or in tanks to which chub ovarian fluid is added to induce spawning.

During year two at the Virginia Tech Aquaculture Center in Blacksburg, adult Rainbow Darters at a sex ratio of two males to four females were stocked into 17 20-gallon tanks with pebble substrate and submersible pumps for flow. The tanks had lights set to mimic the natural diurnal cycle. Eight of the tanks were chilled (15°C in April and October, 10°C March and November, 5°C December and January), and nine were at the ambient temperature of the laboratory (~15-20°C). Adult fish were fed a mixture of live blackworms, frozen brine shrimp, and frozen bloodworms. This spawning setup in the chilled tanks was successful; we collected over 700 eggs and 23 larval rainbow darters. We observed good hatching success without egg treatment. The young ate newly hatched brine shrimp (Figure 12 and Figure 13).



Figure 12. Approximately two-week-old rainbow darters after feeding on brine shrimp.



Figure 13. Approximately five-week old rainbow darters.

Objective 2 - Extend this information to commercial clientele and assist with marketing strategies (UF, LSU, VT).

Results of completed experiments have been disseminated through various presentations at professional meetings. Farm visits and tours of research facilities have also allowed for education of stakeholder groups. Interested commercial clientele are periodically informed of pertinent research developments.

IMPACTS

Culture methods for the Gulf Coast Pygmy Sunfish and Metallic Shiner developed during this project are a solid foundation for the commercial production of select native North American ornamental fishes, which may be transferable to close congeners. Recommendations on Golden Topminnow broodfish size and expected egg output will help producers with production goals for this species as fecundity is low compared to many other ornamental species. Bluenose Shiner appear to require a nest mate associate such as a sunfish to initiate spawning as they potentially use the sunfishes nest as spawning substrate.

PUBLICATIONS, MANUSCRIPTS OR PAPERS PRESENTED

1. Presentations:

a. Oral

DiMaggio, M., T. Lipscomb, Q. Tuckett, A. Wood, S. Ramee, J. Patterson, and C. Watson. 2019. Evaluation of Culture Protocols for two Florida Native Ornamental Species: *Elassoma Gilberti* and *Pteronotropis metallicus*. 39th Annual Meeting of the Florida Chapter of the American Fisheries Society. Haynes City, Florida.

Fetterman, J. A., Murr, C. E., and C. C. Green. 2018. Morphology, gonadosomatic index, and gonad histology assessment of the Bluenose Shiner (*Pteronotropis welaka*). Louisiana Chapter of the American Fisheries Society, Annual meeting, May 24-25, 2018, Baton Rouge, LA.

Fetterman, J. A., Murr, C. E., and C. C. Green. 2019. Bluenose Shiner (*Pteronotropis welaka*) Spawning Trials and Suggested Culture Methods. Louisiana Chapter of the American Fisheries Society, Annual meeting, May 23-24, 2019, Thibodaux, LA.

Fetterman, J. A., Murr, C. E., and C. C. Green. 2019. Weight dependent intraspecific variation in female Golden Topminnow *Fundulus chrysotus* reproduction. World Aquaculture 2019, New Orleans.

Fetterman, J. A. and C. C. Green. 2019. Use of historical collections to aid in reproductive assessments of the Bluenose Shiner. North American Native Fishes Association Annual Meeting. May 31, 2019, Jackson. MS.

Lipscomb, T., Q. Tuckett, A. Wood, S. Ramee, J. Patterson, C. Watson, and M. DiMaggio. 2019. Development of aquaculture techniques for two Florida native ornamental fishes: *Elassoma gilberti* and *Pteronotropis metallicus*. Aquaculture 2019. New Orleans, Louisiana.

b. Posters

Murr, C. E., Fetterman, J. A., and C. C. Green. 2018. Application of hormones as a potential spawning aid for a species of conservation concern. Louisiana State University Discover Day, April 10, 2018, Baton Rouge, LA.

Murr, C. E., Fetterman, J. A., and C. C. Green. 2018. Application of hormones as a potential spawning aid for a species of conservation concern. Louisiana Chapter of the American Fisheries Society, Annual meeting, May 24-25, 2018, Baton Rouge, LA.

RESULTS AT A GLANCE

- Bluenose Shiner appear to require a nest mate associate such as a sunfish to initiate spawning as they potentially use the sunfishes nest as spawning substrate.
- Results indicate that although fecundity differs across female sizes for the Golden Topminnow, it is possible that yolk reserves are conserved across female sizes.
- Commercial microparticulate diets are a viable replacement for *Artemia* during larviculture of the Gulf Coast Pygmy Sunfish and Metallic Shiner.