Reproductive Characteristics, Host Fish Determination and Culture Techniques of Southern Elktoe, *Alasmidonta triangulata* (Lea, 1858) from Flint River, Decatur County, Georgia

Final Project Summary to the U.S. Fish and Wildlife Service

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Introduction

Alasmidonta triangulata, Southern Elktoe (Lea, 1858) was petitioned for listing in 2010 for listing under the federal Endangered Species Act, and it's formal status is currently under review by USFWS (76 FR 59836 59862). It is an Apalachicola Basin endemic, historically known to occur in the Apalachicola, Chattahoochee, Chipola and Flint River systems in Alabama, Georgia and Florida (Williams et al. 2008, 2014). Though it was formerly widespread throughout the Apalachicola River basin, it is now restricted to a few remaining occurrences, mostly in the Flint River Basin (Wisniewski 2008). In Alabama, less than five specimens have been reported from Uchee Creek in Russell County since the early 1970's and was last collected in 2006. In Florida, it is persisting but very rare in the Chipola and Apalachicola Rivers. In Georgia, *A. triangulata* is currently known only from Chickasawhatchee Creek in Baker County, Potato Creek in Upson County, Patsiliga Creek in Taylor County and Flint River in Decatur County (Wisniewski 2008).

Little is known about *A. triangulata* reproduction, female brooding period, or glochidial hosts. Female *A. triangulata* are presumed long term-brooders, and gravid from late summer or autumn to the following summer (Williams et al 2008, 2014). Glochidial hosts are currently unknown although other *Alasmidonta* species utilize Catastomidae (Suckers) fishes for successful metamorphosis (Bloodsworth et al, 2013; Howard and Anson, 1922). The focus of this study was to determine reproductive periodicity, fish host relationship and initiate propagation methods for juvenile mussels.

Methods

Brood stock collection was completed in the lower Flint River, Decatur County Georgia by visual search with mask/snorkel and hand grubbing by Georgia Department of Natural Resources (GA-DNR) and Alabama Department of Conservation and Natural Resources (ADCNR) biologists (Table 1). Mussel brooders were acquired during one collection attempt in November 2017 (Table 1). All mussels encountered during searches were identified and shell lengths (SL) recorded. A total of six gravid females were transported in a chilled insulated cooler with towels soaked in river water to the AABC (Alabama Aquatic Biodiversity Center Marion, AL Perry County, ADCNR). These mussels were placed in aerated 2 L brood stock holding trays inside a 14° C incubator. Trays were filled with water collected from the brood stock site and 50% of the volume was replaced each day. This effort was a combination of 2 separate host trials initiated between January 11, 2018 and March 9, 2018. Glochidia of *A. triangulata* were liberated by extraction of both outer demibranchs for each trial. Post infection brood stock were tagged with small plastic numbered bee tags adhered with super glue gel on the left valve and returned to site of capture.

We assessed host suitability of 27 fish species across 9 families in laboratory trials. These species were selected based on previous published host studies (Bloodsworth et al. 2013; Howard and Anson 1922) and, likelihood of co-occurrence in typical mussel habitats. Fish were collected from three sites (Table 2). Fishes were collected by backpack and boat electro-shocking, seining and transported to the AABC within hours of their collection. Live fishes were captively held in flow-through systems supplied with well water and wild-fishes were subjected to a daily kanamycin treatment for three days prior to infection. Host trials used 1-46 individuals of each potential host

species depending on availability. Following the infections, fishes used in host trials were euthanized, preserved in ethanol and identified utilizing Boschung and Mayden (2004).

We conducted three separate host trials for each of our primary fish collection study sites. The glochidia of two to four adult mussels were used for each trial depending on the amount required for the numbers and size of host fish tested (Table 3). We took care to avoid using immature glochidia and did not utilize glochidia liberated during transport or the long holding period. *Alasmidonta* spp. are known to cast small numbers of glochidia (Williams et al. 2008; R. Hoch 2017 pers. comm.), but discharged larvae are usually viable for < 48 hours. Fish collected from Little Uchee Creek, Russell County and Cahaba River, Perry County, Alabama were utilized in the initial host trial infection initiated on January 11, 2018 (Table 2). Viable glochidia (n=57,500 infection 1 and n=50,000 infection 2 from two females) were placed into a water bath of 14.3L and 50L respectively (4,020 glochidia/L and 1,000 glochidia/L). Fish (n=115 infection 1 and n=25 infection 2) were placed into the glochidia water bath for 15 min. with heavy aeration. Fish collected from Perry Lakes Park, Perry County and Chattahoochee River, Chambers County, Alabama were used for infection 3 on 3/9/18. Viable glochidia (n=25,100 from four females) were placed into a water bath of 21L (1,194 glochidia/L). Fish (n=25 infection 3) were placed into the glochidia water bath for 15 min. with heavy aeration. Following inoculation, unattached glochidia were rinsed away by placing fish into clean water. Fish were then transferred to size appropriate containers and held individually. Host suitability was monitored in modified recirculating aquarium systems (Pentair Aquatic Eco-Systems AHAB®) for smaller fishes and flow through 50 gal conical tanks for larger individuals. The recirculating systems consisted

of an array of 1 L, 3 L and 9 L tanks. Water temperature was maintained at 18º C ± 2º with Aqualogic Inc. Cyclone chillers. Recirculated water was filtered, and UV sterilized before returning to the tanks. AHAB tanks received approximately 0.5 L water/min and the outflow entered a filter cup with a 153 µm nylon screen to collect glochidia and juveniles. Large conical tanks were equipped with a double stand pipe to draw water from the bottom of the tanks. Tank effluent was filtered through a 153 µm nylon sock. Filters were examined 1 d after inoculation and every 2 d to 6 d thereafter until no glochidia or juveniles were recovered for at least 4 d. Before examining the filters, flow was increased to approximately 2 L/min in the AHAB aquaria and 15 L/min in the conical tanks for 15 min to ensure that all particles were removed. Each filter was rinsed into a glass dish and the number of glochidia and juveniles from each fish were counted under a Nikon SMZ745T stereomicroscope. Juveniles mussels were distinguished from untransformed larvae by the presence of an active foot.

After no juveniles or glochidia had been recovered for 7 d, fishes were euthanized and were examined for remaining encapsulated glochidia. Standard length was recorded, and the fish was preserved in ethanol or frozen. The number of glochidia that initially attached to each fish was calculated as the sum of the number of glochidia and juveniles recovered from the fish throughout the trial. Successful metamorphosis was calculated as the percentage live juveniles from the total attached glochidia. Hosts defined as “primary” if ≥ 50% of attached glochidia transformed to the juvenile stage, “marginal” if 0.1–50% transformed, and “unsuitable” if no transformation occurred (Haag and Warren 1997, 2003). Timing of juvenile metamorphosis was determined by minimum and maximum range and modal time to peak metamorphosis (DPI, days post infection) for juveniles recovered from individual fish.

Juvenile mussels were placed into laboratory HruŜka culture boxes (HruŜka 1992, reviewed in Gum et al. 2011). HruŜka boxes are static flow plastic trays containing 500 mL pond water, cultured algae, and highly organic sediment screened to < 250 µm (Figure 1). Sediment and water were changed weekly for ≈ 4.5 months. On days 56 post metamorphosis light aeration with small air stone was added to boxes to improve water quality. Juveniles were transferred from HruŜka boxes into suspended upwelling system (SUPSYS) buckets at ≈132 days post metamorphosis. Each SUPSYS is connected, through a manifold, to high pressure regenerative blower and draws water in through a screened base and out through a screened air uplift tube (Figure 2). The SUPSYS buckets were submerged in a 1.58-acre (≈ 6,400 m^2) pond. Juvenile mussels from infection three were placed directly into Sediment SUPSYS post metamorphosis on March 26, 2018. Sediment SUPSYS are similarly powered to SUPSYS but this design draws water from the screened sides of the bucket suspended in the water column and out through an air uplift tube (Figure 2). This design provides a solid base for sediment or sand placement along with the juvenile mussels. A thin layer of sieved sand (<250 µm) was used in the base of the Sediment SUPSYS in this trial. Each bucket was rinsed and cleaned weekly. Screen sizes were initially 500 µm, and survivors were then changed to 1500 µm mesh as the animals grew. Both screen sizes were sufficient for survival and growth of animals. Each HruŜka box and SUPSYS was monitored weekly for survivorship and mean shell length measured, for a 10-20 animal subsample (Photos, ImageJ).

Results

Brood Stock Collection

Brood stock was collected on 28 November 2017 at two sites in the Flint River just upstream of Lake Seminole in Decatur County. Water temperature was 15.4 º C. A total of 14 *A. triangulata* ranging in size from 31 – 61 mm shell length were collected including six (31, 32, 32, 45, 57 and 61 mm) that were brooding glochidia. Three were previously encountered mussels tagged by GA-DNR in 2012. These individuals increased in length from 41 to 58 mm, 43 to 58 mm, and 48 to 56 mm respectively over the past 5 years. Nineteen *A. triangulata* were captured, tagged and released by GA-DNR in 2012 (Figure 3).

Reproductive Life History

Demibranch observations of non-gravid animals were thin and slightly transparent, in contrast to gravid demibranchs appeared thicker and cream white in color. Outer demibranchs of all gravid females were slightly inflated and one individual was only partially filled with glochidia or eggs (Figure 4).

Female *A. triangulata* released a clear mucus matrix with white glochidia suspended throughout (Figure 4). Prior to volumetrically counting glochidia, released material was mechanically disrupted with a turkey baster and subsampled to estimate viability. Viable glochidia (n=57,500 infection 1 and n=50,000 infection 2 from two females) completed on 11 January 2018 were placed into a water bath of 14.3L and 50L respectively (4,020 glochidia/L and 1,000 glochidia/L). Nearly continuous casting of glochidia until the 2nd trial initiated on 9 March 2018 extracted only 25,100 glochidia from four females for an infection bath of 1,194 glochidia/L. Glochidia were hooked and had a mean (± SE) length 224 ± 6 µm and height 258 ± 11 µm (Figure 5). Tagged brood stock were returned to site of capture on 5 April 2018 by GA-DNR biologists.

Juveniles were recovered from host fishes 11–34 days after inoculation. The mean time to peak juvenile recovery among 149 individual host tests was 17.5 ± SD 3 days post-inoculation at 18º C ± 2º C (Figure 6). Successful metamorphosis of glochidia was observed on 10 of 27 species tested and 2 of 9 families (Table 5). Five catastomid species were primary hosts (Appalachicola Redhorse, *Moxostoma* sp. cf. *poecilurum*; Creek Chubsucker, *Erimyzon oblongus*; Blacktail Redhorse, *Moxostoma poecilurum*; Lake Chubsucker, *Erimyzon sucetta* and Greater Jumprock, *Moxostoma lachneri*) and four were marginal hosts (Spotted Sucker, *Minytrema melanops*; Highfin Carpsucker, *Carpiodes velifer*; Quillback, *Carpiodes cyprinus* and River Redhorse *Moxostoma carinatum*) (Table 5). Marginal hosts also included one species of cyprinid, Dixie Chub, *Semotilus thoreauianus* with 0.9% metamorphosis (Table 5).

Propagation Culture Techniques

Production of metamorphosed juvenile *A. triangulata* at the AABC in 2018 was 6,143. Shell length growth in the HruŜka culture boxes was consistent at 10 µm/day for 132 days. Juvenile mortality was highest during first two months with 50% survival on day 56, then 23% on day 97, and 15% on day 132 (Figure 7). Mussels were transitioned from HruŜka boxes into SUPSYS buckets at 132 days post metamorphosis. The subset of juvenile mussels placed into Sediment SUPSYS from infection 3 had 100% mortality within a month of the trial.

Mean growth in SUPSYS was 156 µm/day up to day 277 post metamorphosis with 17% survivorship (Figure 8 and 9). Growth was negligible during winter and early spring while mean pond temperatures were below 25° C. The highest growth rates (156 µm/day between days 132 - 277) were observed in SYPSYS. Two notable mortality

events occurred during grow out trials that decreased overall survivorship. One occurred near day 56 in the HruŜka culture boxes, when over 50% juvenile mortality was observed and coincided with an algae bloom in the containers. The addition of aeration with a small air stone eliminated the algae and decreased the mortality rate. The second mortality event occurred in the SUPSYS buckets near day 186. One SUPSYS had 90% mortality in one week, while adjacent SUPSYS buckets had 100% survival and no changes in growth. The cause for this mortality event is still unknown although smaller animals appeared susceptible condition. Overall survivorship in boxes and pond was ~2% to day 277 (Figure 7).

Discussion

Gravid females of multiple sizes and recaptured animals at the two brood stock collection sites is a positive sign for this species, indicating growth, varied age class structure and site habitat stability. *Alasmidonta triangulata* females contain mature glochidia in the winter and appear to depend on suckers as primary glochidia hosts although they remain to be documented in the field (Table 5). Bloodsworth et al (2013) examined *Alasmidonta marginata* fish hosts and reported all catostomid species tested facilitated metamorphosis, and overall, Catostomidae produced more juvenile mussels per fish. In addition, natural infestations of *A. marginata* have been observed in three catostomid species (Howard and Anson 1922). Our results closely mimic *A. marginata* host fish observations indicating *A. triangulata* has a host fish affinity for the Catostomidae. Of the five primary hosts, four are sympatric with *A. triangulata* while Blacktail Redhorse is allopatric (Boschung and Mayden 2004, Williams et al 2008, 2014). Of the four marginal Catastomidae species tested only Spotted Sucker is sympatric with *A. triangulata* throughout its range. Quillback co-occurs in the

Chattahoochee, Chipola, and Apalachicola rivers but not in the Flint River. Highfin Carpsucker and River Redhorse are allopatric but could serve as hosts in future propagation efforts (Boschung and Mayden 2004, Williams et al 2008, 2014).

Several variables affect glochidia metamorphosis and potentially affect the reliability of tests to determine host compatibility. Metamorphosis success varied widely among individual fish (e.g., success on individual *M. poecilurum* ranged from 48-82%). With such variability, marginal hosts such as *M. melanops*, *C. velifer*, *C. cyprinus* and *M. carinatum* may prove to be primary hosts with increased sample sizes. In this study, fish were collected from streams with native freshwater mussel populations present. As a result, we were unable to account for any natural acquired immunity effects. Therefore, estimates for species tested with low sample sizes are imprecise and may require further testing.

Juvenile mortality in HruŜka culture boxes was highest during first two months of grow out but continued throughout the culture process. Improved water quality and algae reduction was accomplished by adding small air stones to the static culture boxes midway through the culture trial. Higher growth rates were observed in SUPSYS buckets compared to culture boxes. Although the unidentifiable cause of a mortality event in the SUPSYS near day 186 indicates additional thermal and nutritional requirement data are needed. Juvenile mussels placed directly into Sediment SUPSYS post metamorphosis had poor survivorship in late March and early April. It is important to note that pond temperatures during this trial were below 20° C, and prior to observed growth from other mussel species being cultured in the pond at the time. This indicates temperature may have impacted early growth and survivorship instead of SUPSYS design. Additional research is also needed to determine the protocols for transitioning

juveniles into SUPSYS buckets for culture and to improve early culture methods to maximize survivorship and growth.

Acknowledgements

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Table 1. *Alasmidonta triangulata* Flint River brood stock collection localities utilized for this study. Date of collection was

Stream	Date	Locality	Lat/Long
Flint River site 1	November 28, 2017	Near Bainbridge, upstream of Lake Seminole boat ramp Decatur County, GA.	31.005315° -84.522451°
Flint River site 2	November 28, 2017	Near Bainbridge, upstream of Lake Seminole boat ramp Decatur County, GA.	30.995332° -84.537882°

Table 2. Potential host fish collection localities used to support *Alasmidonta triangulata* host trials.

Stream	Date	Locality	Lat/Long
Little Uchee Creek	November 16, 2017	AL CR 28, Near Crawford, Russell County	32.410826° -85.106891°
Cahaba River	January 10, 2018	AL Hwy 14, Near Sprott, Perry County	32.669473° -87.241709°
Perry County Lake - Cahaba River oxbow	March 7, 2018	Perry Lakes Park, North of Sprott, Perry County	32.696474° -87.242741°
Chattahoochee River	March 6, 2018	Near Valley, Chambers County	32.793669° -85.140988°

Table 3. *Alasmidonta triangulata* brood stock numbered red bee tags identification numbers, glochidia extraction date, estimated total glochidia, infective glochidia and shell length measurements (mm).

Tag Number	Date	Total Glochidia	Infective Glochidia	Length (mm)	Width (mm)	Height (mm)
1	1/11/18	77,000	76,000	57	28	32
2	1/11/18	32,200	31,500	44	22	28
3	3/9/18	6,600	6,600	33	19	22
4	3/9/18	17,000	15,500	60	33	36
5	3/9/18	800	800	32	18	21
6	3/9/18	2,200	2,200	31	16	21

Table 4. *Alasmidonta triangulata* glochidial infection densities baths utilized in host infection trials.

	Infection #1 Uchee	Infection #1 Cahaba	Infection #2 Cahaba/Chattahoochee
N Fish	115	25	25
N Infective Glochidia	57,500	50,000	25,100
Bath Volume (L)	14.3	50.0	21.0
Glochidia per L	4,020	1,000	1,195
Bath Volume per fish (L)	0.12	2.00	0.84
Glochidia per fish	500	2,000	1,004

Table 5. Summary of metamorphosis in 2018 pairings of *A. triagulata* and potential host fish. Numbers are means± standard deviation (range)

Fish Location/Species	Metamorphosis Success (%)	N Fish	N Attached Glochidia	N Juveniles	N Attached Glochidia/Fish	N Juveniles/Fish
Little Uchee Infection 1						
Dixie Chub (*Semotilus thoreauianus*)	0.9 ±1.4	5	245	4	49.0 ± 35.0	0.8 ±1.3
Blacktail Shiner (*Cyprinella venusta*)	0	46	2,072	0	59.2 ± 61.0	0
Blackbanded Darter (*Percina nigrofasciata*)	0	25	490	0	27.2 ± 21.4	0
Bluegill (*Lepomis macrochirus*)	0	12	1,109	0	100.8 ± 100.3	0
Weed Shiner (*Notropis texanus*)	0	6	299	0	52.8 ± 39.2	0
Bluefin Stoneroller (*Campostoma pauciradii*)	0	5	101	0	20.2 ± 9.8	0
Blackspotted Topminnow (*Fundulus olivaceus*)	0	4	60	0	15 ± 10.5	0
Speckled Madtom (*Noturus leptacanthus*)	0	4	253	0	63.3 ± 42.3	0
Longnose Shiner (*Notropis longirostris*)	0	2	35	0	17.5 ± 12.0	0
Gulf Darter (*Etheostoma swaini*)	0	2	12	0	12	0
Eastern Mosquito (*Gambusia holbrooki*)	0	1	5	0	5	0
Longear Sunfish (*Lepomis megalotis*)	0	1	103	0	103	0

Fish Location/Species	Metamorphosis Success (%)	N Fish	N Attached Glochidia	N Juveniles	N Attached/Fish	N Juveniles/Fish
Redbreast Sunfish (*Lepomis auritus*)	0	1	121	0	121	0
Redear Sunfish (*Lepomis microlophus*)	0	1	33	0	33	0
Silverjaw Minnow (*Ericymba buccata*)	0	1	27	0	27	0
Warmouth (*Lepomis gulosus*)	0	1	30	0	30	0
Cahaba River Infection 2						
Blacktail Redhorse (*Moxostoma poecilurum*)	65.4 ± 15.0	5	1,426	903	285 ± 96.6	180.6 ± 48.6
Highfin Carpsucker (*Carpiodes velifer*)	15.6 ± 7.4	2	184	25	92 ± 28.3	12.5 ± 3.5
Quillback (*Carpiodes cyprinus*)	12.6	1	414	52	414	52
River Redhorse (*Moxostoma carinatum*)	9.0	1	156	14	156	14
Cahaba River Perry Lakes Infection 3						
Creek Chubsucker (*Erimyzon oblongus*)	66	1	53	35	53	35
Lake Chubsucker (*Erimyzon sucetta*)	58.2 ± 1.5	2	48	28	24.0 ± 4.2	14.0 ± 2.8
Pirate Perch (*Aphredoderus sayanus*)	0	3	135	0	135	0
Warmouth (Lepomis gulosus)	0	1	3	0	3	0

Fish Location/Species	Metamorphosis Success (%)	N Fish	N Attached Glochidia	N Juveniles	N Attached/Fish	N Juveniles/Fish
Chattahoochee River Infection 3						
Apalachicola Redhorse (*Moxostoma* sp. cf. *poecilurum*)	85.6	1	526	450	526	450
Greater Jumprock (*Moxostoma lachneri*)	52.8 ± 34.2	8	3,719	2,086	464.9 ± 124.7	260.7 ± 195.1
Spotted Sucker (*Minytrema melanops*)	33.7 ± 12.0	2	93	41	46.5 ± 40.3	20.5 ± 24.7
Yellow Perch (*Perca flavescens*)	0	5	268	0	268	0

Figure 1. HruŜka culture boxes used for culture of newly transformed *Alasmidonta triangulata*. The box is a simple static flow plastic tray, containing 500mL pond water, cultured algae and <250µm sieved sediment. A small air stone for low water movement and aeration were added to the trays on day 56 post metamorphosis.

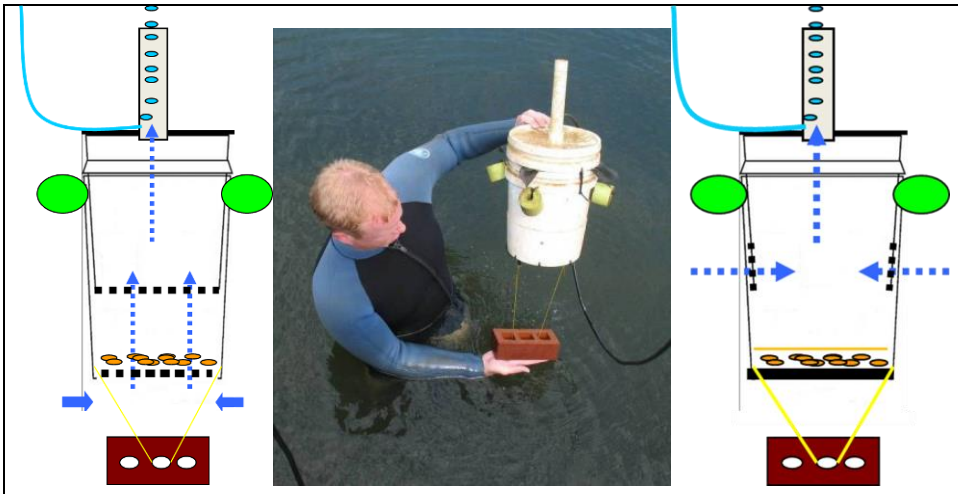

Figure 2. SUPSYS bucket system used in culture of juvenile *Alasmidonta triangulata*. The SUPSYS was powered by a high pressure regenerative air blower. Air lift tube creates flow through the mesh chambered bucket cage from the bottom screen suspended in water column (left) or from the side screen in Sediment SUPSYS (right)

Figure 3. A 2012 tagged *Alasmidonta triangulata* recaptured in the Flint River above Lake Seminole (site 2), November 28, 2017.

Figure 4. *Alasmidonta triangulata* glochidia in a mucus matrix (left); *Alasmidonta triangulata* gravid female with inflated gills

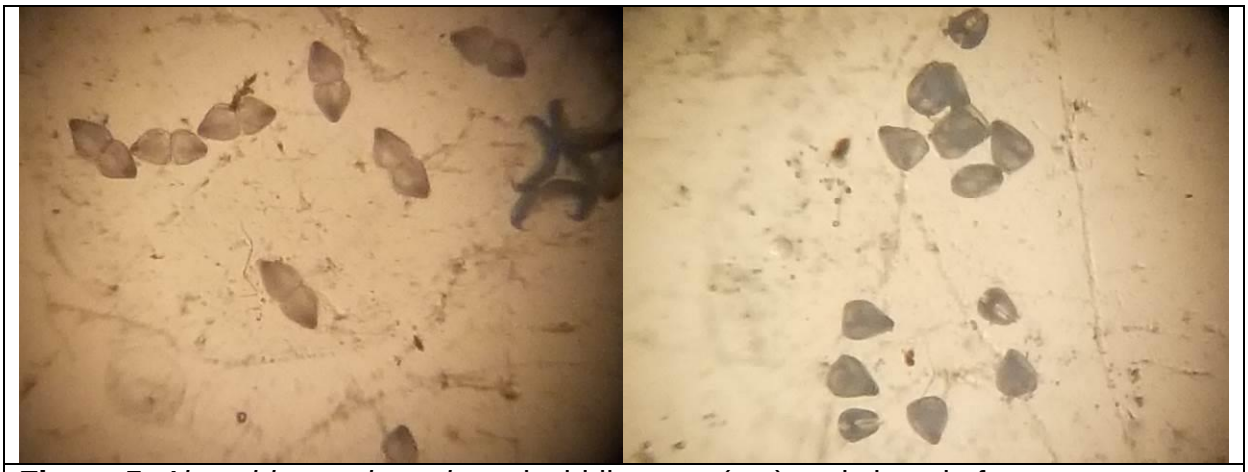

Figure 5. *Alasmidonta triangulata* glochidia open (top) and closed after exposure to NaCl solution (bottom). Estimated dimensions of glochidia are mean (± SE) length 224 ± 6 µm and height 258 ± 11 µm in size.

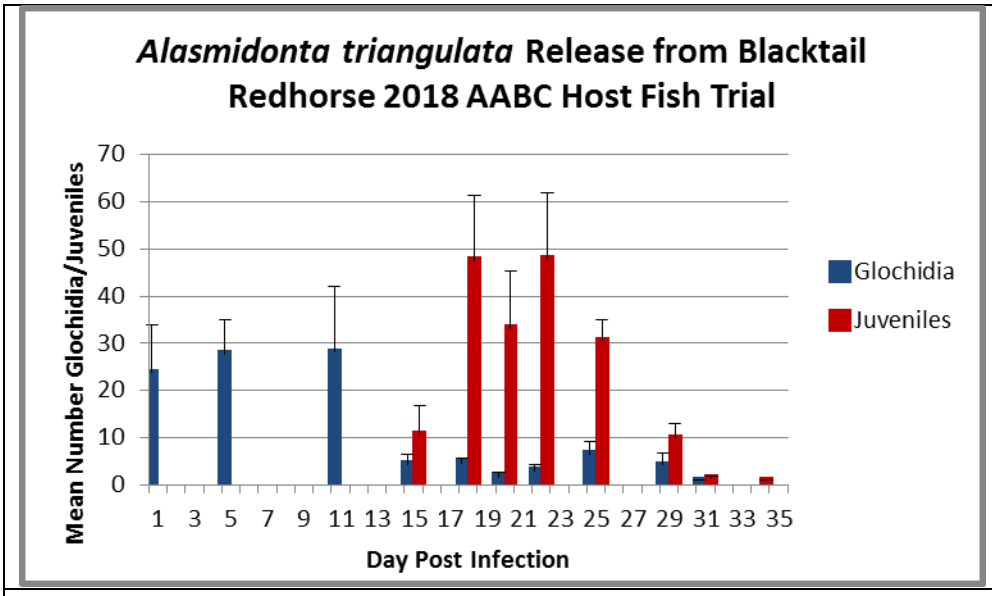

Figure 6. Mean combined results of *Alasmidonta triangulata* glochidia and juvenile release from n=5 *Moxostoma poecilurum* (Blacktail Redhorse) collected from the Cahaba and Applachicola rivers. Bars indicate mean ± standard error.

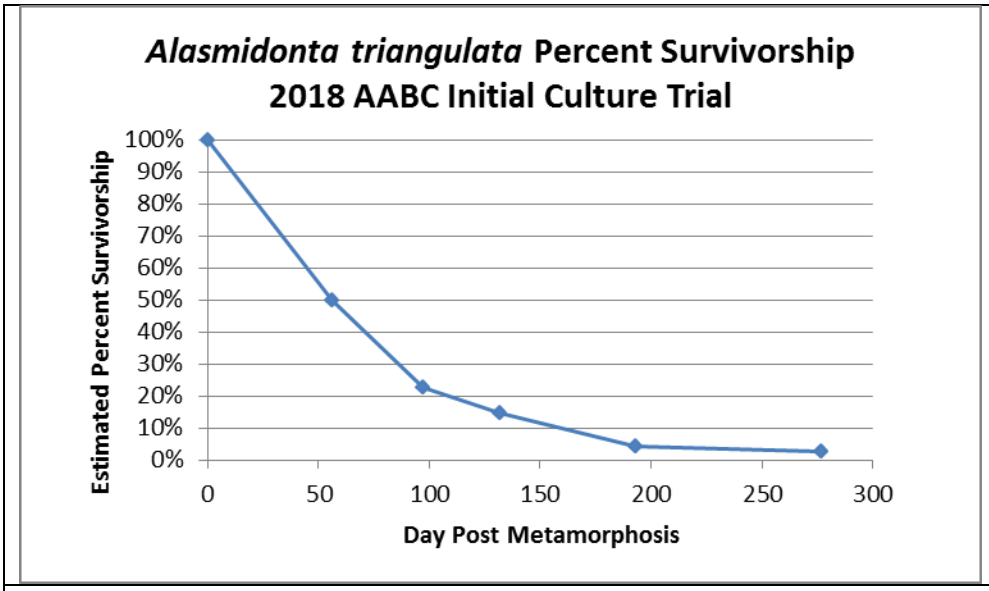

Figure 7. *Alasmidonta triangulata* juvenile mussel survivorship percentage approximately 277 days post metamorphasis.

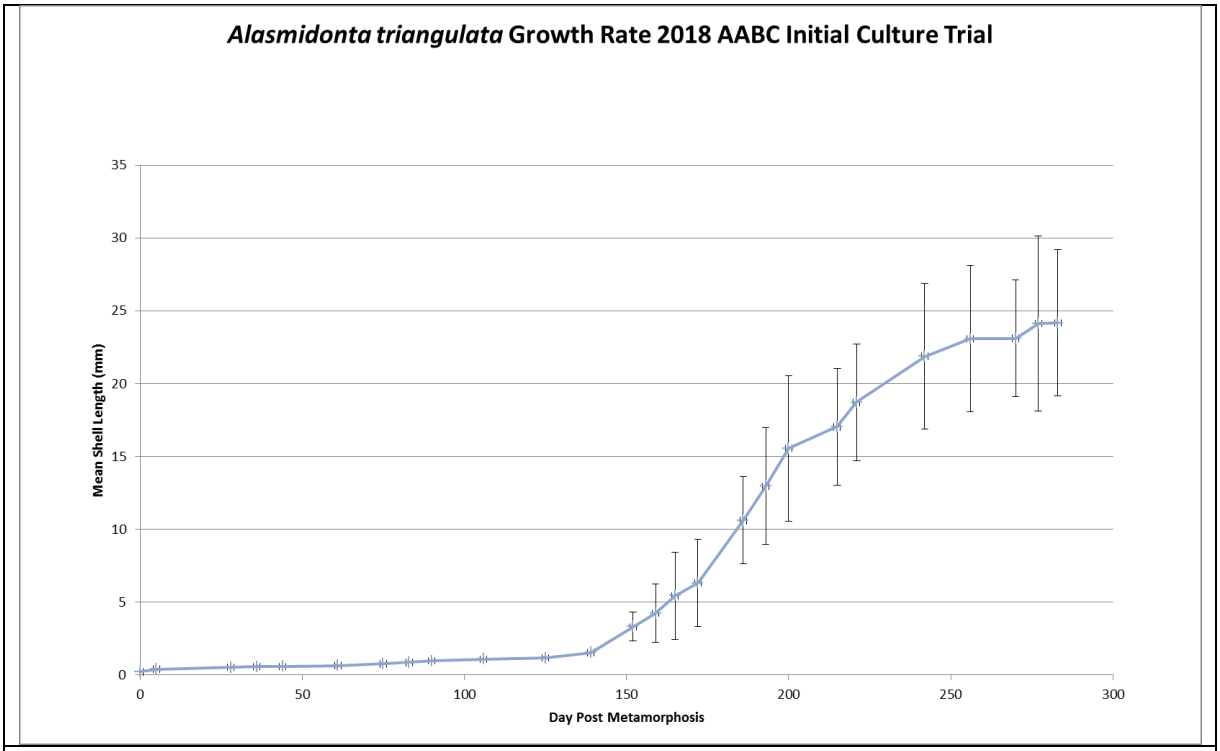

Figure 8. *Alasmidonta triangulata* mean shell length growth (mm) for approximately 277 days post metamorphosis. Bars indicate mean ± standard error.

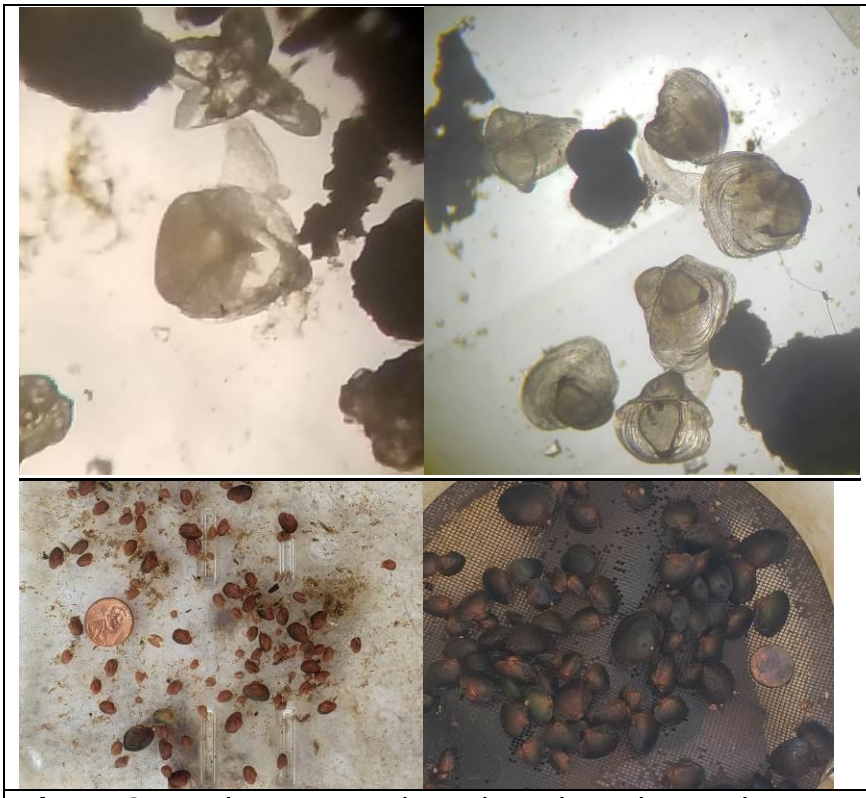

Figure 9. Newly metamorphosed *A. triangulata*, 5 days post metamorphosis (left top). *A. triangulata* juveniles 125 days post metamorphosis (right top) 186 days post metamorphosis (left bottom) 277 days post metamorphosis (right bottom)