FINAL PERFORMANCE REPORT

Federal Aid Grant No. F11AP00447 (E-75-R-1)

Propagation and Augmentation of the Ouachita Rock Pocketbook

Oklahoma Department of Wildlife Conservation

July 20, 2011 through July 19, 2015

FINAL PERFORMANCE REPORT

STATE: Oklahoma **GRANT NUMBER:** F11AP00447 (E-75-R-1)

GRANT TYPE: Traditional ESA Section 6

GRANT TITLE: Propagation and Augmentation of the Ouachita Rock Pocketbook

GRANT PERIOD: July 20, 2011 – July 19, 2015

REPORTING PERIOD: July 20, 2011 – July 19, 2015

PRINCIPAL INVESTIGATOR: Dr. Chris Barnhart, Department of Biology, Missouri State University, 901 S. National, Springfield, MO 65897; chrisbarnhart@missouristate.edu

Objective:
Augment Ouachita Rock Pocketbook populations in the Little River of Oklahoma and Arkansas with juveniles reared from glochidia collected from the Little River.

Background:

The Ouachita rock pocketbook mussel, *Arkansia wheeleri* (Ortmann and Walker 1912) is a rare and critically endangered species. The live collections of this species reported in the past decade include 3 sites in the Little River in Arkansas and Oklahoma, one site in the Ouachita River, and one site in the Kiamichi River in Oklahoma (USFWS 2004; Vaughn and Pyron 1995; Seagraves 2006; Galbraith et al. 2008; Harris et al. 2010; W. Posey, AGFC personal communication). Only 1-13 live individuals were found per site in these collections and it is likely that site populations are less than a few hundred individuals (Seagraves 2006). Although potential habitats remain to be surveyed, the limited range, rarity, and the loss or decline of the species at historically known sites of occurrence suggests that *Arkansia wheeleri* may be on the brink of extinction (Harris et al. 2010).

Native freshwater mussels use particular fish species as hosts for the metamorphosis of the parasitic glochidia larvae. Barnhart (2010) investigated fish hosts of *A. wheeleri*. The fish species tested and the percent metamorphosis of attached glochidia were golden shiner (*Notemigonus crysoleucas*) (31%), duskystripe shiner (*Luxilus pilsbryi*) (11%), freshwater drum (*Aplodinotus grunniens*) (3%), blue catfish (*Ictalurus furcatus*) (0%), largemouth bass (*Micropterus salmoides*) (0%), hybrid sunfish (*Lepomis macrochirus* x *cyanellus*) (0%), orangespotted sunfish (*Lepomis humilis*) (0%), and walleye (*Sander vitreus*) (0%). Metamorphosis was complete within 10 days at 23ºC. An earlier study reported 11 potential hosts, mainly centrarchids, with metamorphosis success up to 70% (Seagraves 2006), but those results have been questioned (Barnhart 2010).

The present project was initiated by ODWC to develop captive propagation as a tool for conservation of this globally endangered species. Early in the project it was evident that locating *A. wheeleri* females would be limiting, so a decision was made to also investigate the rock pocketbook, *Arcidens confragosus*, as a surrogate for methods development. *Arcidens*

confragosus is the closest relative of *A. wheeleri.* A recent morphometric and genetic analysis (Inoue et al. 2014) concluded that the genetic differences between the two species are relatively small, notwithstanding their distinctive shell morphologies. The authors supported previous suggestions (summarized by Graf and Cummings 2007) that the genus *Arkansia* be synonymized with *Arcidens*.

The main obstacles to captive propagation of *A. wheeleri* are 1) obtaining brooding females with glochidia larvae suitable for culture, 2) low and inconsistent metamorphosis success on known host fish, and 3) identification of suitable methods for grow-out of early juveniles. We used golden shiner as fish hosts and also tested metamorphosis of glochidia in cell culture media (i.e. in vitro) as an alternative to use of fish hosts. We tested grow-out in static and recirculating systems and we are currently testing grow-out of *A. confragosus* in a new pulsed flow-through culture system that is in development.

Approach:

Metamorphosis on host fish:

We used standard methods described previously (Dodd et al. 2005). Briefly, glochidia were recovered by injecting sterile water into the marsupia of the female mussel to flush the contents of the water tubes into a dish. The glochidia were then rinsed through a 400 micron Nitex filter using a spray of water to break up clumps and free the glochidia from egg membranes. Glochidia were then suspended in a known volume of water and quantified by counting the number present in ten 20-microliter subsamples (total 2 ml) and multiplying by the total volume of the suspension. Viability was measured as the percentage of a subsample of glochidia that closed in response to a salt solution.

In each of two trials (one trial per species), glochidia were used to inoculate 106 golden shiners (4-5 inch SL) obtained from Anderson Farms, Lonoke AR. The fish were inoculated by the bath method using 4,000 viable glochidia per liter in 16 liters for a total exposure time of 20 minutes. The water was agitated to maintain suspension of the glochidia (see video at link listed in Figure 1). Following inoculation, the fish were rinsed and placed in recirculating systems designed for the recovery of glochidia and juveniles (Barnhart 2003; Dodd et al. 2005). The time course of drop-off was monitored at 1-2 day intervals from six fish kept in individual tanks, with three kept at 14ºC and the other 3 kept at 22ºC. The remaining 100 fish were kept in a larger recirculating system at 14ºC and were monitored as a group.

In vitro metamorphosis

We used medium similar to that described by Owen (Owen 2009, Owen et al. 2010, Lima et al. 2012, and Christopher Owen, personal communication) with the exception of replacing fish serum with rabbit serum. The medium was titrated to pH 7.65 after equilibration with 1% CO_2. Five ml of medium was distributed to each 6-cm polycarbonate Petri dish using a syringe with 0.6 micron filter for sterilization. Glochidia for in vitro metamorphosis were washed a minimum of 3 times with sterile filtered water and once with sterile filtered medium before distribution to culture dishes. Approximately 100-200 glochidia were placed in each dish.

The dishes were held in covered glass boxes that were flushed with 1% CO_2. The gas mixture

was prepared from CO_2 and air using Matheson gas mixing pumps and monitored ($\pm$ 0.1%) with an infrared CO_2 analyzer. Three ml of the medium was replaced in each dish at 2-day intervals. Medium changes were carried out in a laminar hood to maintain sterility. The condition of the glochidia was monitored by examination of the dishes on a dissecting microscope. The development of the anterior and posterior adductors was visible without opening the dish and was used to judge completion of metamorphosis.

Growout

The juveniles of both mussel species obtained from fish hosts were cultured in static sediment boxes; this approach is similar to that described by Eybe et al (2013). Metamorphosed juveniles were placed in 1-L plastic boxes holding 0.5-L river water and 20 ml of sediment. Sediment was collected biweekly from a terrestrial wetland area (Compton Hollow Conservation Area, Webster County, MO). The sediment was sieved to <150 microns and aerated continuously before use. No stirring or aeration was used in the culture boxes, which were partially covered to allow some evaporation and resulting convection to occur. Water was James River water with microalgae added as food (10 L water plus 120 µL Shellfish Diet and 200 µL of *Nannochloropsis* concentrate, Reed Mariculture, Campbell, CA). Water and sediment were changed weekly. No food was added between water changes. Temperature averaged 19.2ºC.

After approximately 1 month, surviving juveniles were transferred to sediment trays with flowing water delivered from 240-L recirculating systems. These systems were fed at hourly intervals from microprocessor-controlled peristaltic pumps. The food was a mixture of 10 parts Reed Mariculture *Nannochloropsis* concentrate and 27 parts Shellfish Diet (V:V) in 1 liter of water, with delivery rate adjusted to maintain 2-3 ppm of cell volume in the water.

Results and Discussion:

Glochidia of both one female *Arkansia wheeleri* and the closely related *Arcidens confragosus* were obtained and metamorphosed using golden shiners (*Notemigonus crysoleucas*) as host fish; additionally, the in vitro approach was employed, involving the of placement of glochidia into a nutritive liquid medium in lieu of the fish host. Successful gill attachment and subsequent metamorphosis of glochidia to the golden shiners was limited and generally lower than seen from previous propagation efforts; shiners used in this grant originated from Anderson Farms, AR, while shiners used in previous trials originated from Lost Valley Hatchery in Missouri. This result was partly attributed to holding the fish at a lower temperature (14ºC vs 22ºC) during the parasitic period. Recovery of juveniles from fish hosts was higher at 22ºC as compared to 14ºC. In vitro methods were improved during the study, as shown with 100% metamorphosis and 1,320 juveniles (presently in culture) for *A. confragosus*. Such results have increased optimism and the likelihood of success for the survival of *Arkansia* glochidia in the laboratory. We remain hopeful that additional gravid *Arkansia* can be located during winter of 2015 for continued propagation efforts in future segments of this grant.

Mussels:

Two brooding *A. confragosus* collected in November 2014 and originating from Pickwick Lake, AL were used in the surrogate species trial. The broods of both *A. confragosus* females were fully fertilized and viability was nearly 100%. Glochidia were recovered from both females on 11/15/14 for infecting fish hosts; outside of the scope of this grant, additional glochidia were

collected from one of the females on 9/11/15 for in vitro metamorphosis. This female had been stored (with weekly feedings) at 10ºC for 10 months. Glochidia viability was still excellent after 10 months and metamorphosis in vitro was 100% (see below).

A single brooding female *A. wheeleri* was collected by USFWS personnel from the Little River below Millwood Dam, AR on December 10, 2014. The mussel was immediately transported to Tulsa by David Martinez and then to MSU by MSU staff biologist Beth Glidewell. It was held at 10ºC for 6 days before glochidia collection began. Judging from growth lines, the mussel was estimated to be approximately 4 years of age and weighed 80.7 grams. Glochidia were collected on December 16, 2014. The mussel was then shipped overnight to Chris Davidson (USFWS Conway AR) for return to the collection site on December 17, 2014.

The *A. wheeleri* brood was fully fertilized and yielded approximately 66,500 glochidia. The condition of the glochidia appeared excellent, and development appeared to be complete. (Figure 1 and link in the legend). Viability was nearly 100%; this was a clear contrast to the partially fertilized condition of two females examined in previous years (Barnhart 2010).

Metamorphosis on fish hosts.

Attachment of glochidia to fish that were monitored individually was about 250 per fish for *A. confragosus* and about 125 per fish for *A. wheeleri* (Table 1). These numbers per fish were within the range expected from previous experience (Barnhart 2010). However, the apparent attachment in the groups of 100 fish was only about 50 per fish for both *A. confragosus* and *A. wheeleri*, leading us to suspect that the recovery of glochidia and juveniles in the group recirculating systems was compromised.

Metamorphosis success was variable on individual fish (Table 1) ranging from 0-45% for *A. confragosus*, and 0-30% for *A. wheeleri*. Metamorphosis success was lower at 14ºC compared to 22ºC (Tables 1). This result was unexpected; we anticipated a higher return and better quality juveniles from the fish kept at lower temperature, based on previous experience with other species (e.g. Roberts and Barnhart 1999). In the batches of 100 fish at 14ºC, overall apparent metamorphosis success was 5.1% for *Arcidens confragosus*, and 10.4% for *Arkansia wheeleri*. These results were substantially lower than the 31% average metamorphosis of *A. wheeleri* on golden shiner observed at 23ºC in 2010 (Barnhart 2010). The difference is probably partly due to the lower temperature. Another possible explanation is population differences in the host fish; in the 2010 trials, the golden shiners were obtained from Lost Valley Hatchery of Missouri Department of Conservation (MDC) at Warsaw, MO, while the fish used in 2014 originated from Anderson Farms in Lonoke, Arkansas. Though this seems unlikely, population differences among host species do occur (e.g. Douda et al. 2014).

The fish that were monitored individually revealed the time course of metamorphosis and drop-off for each species (Figures 2, 3). Time from infection with glochidia to the drop-off of juveniles was more than 3-fold longer at 14ºC as compared to 22ºC. The peak of drop-off of juveniles for the fish at 22ºC and 14ºC was about 11 and 44 days, respectively, for *Arcidens*, and about 10 and 24 days for *Arkansia*. We plan to use 22ºC for future metamorphosis of *A. wheeleri* on fish hosts.

In vitro metamorphosis

The first in vitro trial was made with *A. wheeleri* in December of 2014. Juveniles produced in that trial were not vigorous and did not survive long in culture.

Culture of juvenile mussels

The juveniles of both mussel species obtained from fish hosts were cultured in static sediment boxes at ~18-20 C using wetland sediment, filtered river water, and *Nannochloropsis* as food. This approach is similar to that described by Eybe et al (2013). The box method was chosen because we have reason to think that *Arcidens* juveniles are mechanically fragile, and we have had good success using it for other fragile species, including *Anodonta californiensis* and *Margaritifera falcata*. *Arcidens confragosus* juveniles placed in sediment boxes survived longer, and some individuals reached 3 months of age and over 1 mm shell length (Figure 4). However, both *A. wheeleri* and *A. confragosus* failed to thrive in these systems. On 3/3/15, 40 *A. confragosus* and 32 *A. wheeleri* juveniles remained alive. The mean shell length on that date was 1.1 mm (*A. confragosus* at 108 days) and 0.55 mm (*A. wheeleri* at 74 days). Larger numbers of juveniles are needed to adequately test culture methods. The number of juveniles available was small in both trials, and it is not unusual to see 90% mortality over the first month of culture; this is witnessed even with common and relatively easily cultured mussel species. We remain optimistic that both species can be grown out in the right culture conditions. The potential exists for tens of thousands of juveniles to be produced using the in vitro method.

Beaker culture system development

Newly metamorphosed freshwater mussels are very small and delicate, so that captive culture presents challenges for handling, for maintenance of suitable microhabitat, water quality, and food, and for the avoidance of competitors and predators in culture. Two commonly used approaches to laboratory culture are sediment-free recirculating systems (mucket buckets; Barnhart 2006) and the static sediment boxes described above. Both of these approaches can yield positive results with some species, though there are drawbacks to consider. A third approach was pioneered this year at USGS Columbia Environmental Research Center in Columbia, Missouri, and at MSU. Flow-through diluter systems used for toxicology experiments (similar to Mount and Brungs 1967) were adapted to deliver water and food to culture mussels. These diluter systems periodically refresh the solutions in a series of screened beakers (0.25-1 liter) containing juvenile mussels and a 1-3 mm layer of sand substrate. The sand is sieved to smaller particle size than the juveniles being cultured. Flow of water is provided in gentle pulses of 2-3 minutes duration, at 1-2 hour intervals, with each pulse of flow replacing the volume of the beaker by overflow through a screened opening. At other times, the water in the beakers is static. Substrate conditions are maintained by sieving the juveniles from the sand at 1-2 week intervals and transferring them to clean beakers and fresh sand. Food is cultured marine microalgae, kept ice-cold until just before mixing with culture water and delivery to the beakers. The combination of periodic renewal of water, fresh food, mainly static conditions, and the presence of clean substrate for burrowing, appears to give improved growth and survival with a variety of taxa. Another major advantage is the separation of groups into independent replicate beakers, reducing the likelihood of disease or blooms of competitors or predators affecting the entire batch of mussels being cultured. The small beakers are also easily removed for observation or for subsampling juvenile mussels. We are presently using this approach to culture *A. confragosus* juveniles.

PREPARED BY: Chris Barnhart
Missouri State University

DATE: October 21, 2015

APPROVED BY: _____
Andrea K. Crews, Federal Aid Coordinator
Oklahoma Department of Wildlife Conservation

\ _____
Wildlife Division Administration
Oklahoma Department of Wildlife Conservation

Literature Cited

Barnhart, M. C. 2003. Culture and restoration of special concern mussel species. Final Report to Missouri Department of Conservation & U.S. Fish and Wildlife Service, Endangered Species Grant E-1-35, Year 3 of 3. 55 pages.

Barnhart, M.C. 2006. A compact system for rearing juvenile freshwater bivalves. Aquaculture 254:227-233. doi:10.1016/j.aquaculture.2005.08.028.

Barnhart, M.C. 2010. Reproduction and fish hosts of the Ouachita Rock Pocketbook, *Arkansia wheeleri*. Final Report for Arkansas Game and Fish Commission. 19 p.

Barnhart, M. C., W. R. Haag & W. N. Roston. 2008. Adaptations to host infection and larval parasitism in the Unionoida. Journal of the North American Benthological Society 27(2): 370-394.

Dodd, B.J., M.C. Barnhart, C.L. Rogers-Lowery, T.B. Fobian, and R.V. Dimock Jr. 2005. Cross-resistance of largemouth bass to glochidia of unionid mussels. Journal of Parasitology 91(5):1064-1073.

Douda, K., J. Sell, L. Kubíková-Peláková, P. Horký, A. Kaczmarczyk, and M. Mioduchowska. 2014. Host compatibility as a critical factor in management unit recognition: Population-level differences in mussel-fish relationships. Journal of Applied Ecology 04/2014; 51(4). DOI:10.1111/1365-2664.12264

Graf, D.L. and Cummings, K.S. 2007. Review of the systematics and global diversity of freshwater mussel species (Bivalvia: Unionoida). Journal of Molluscan Studies 73: 291-314.

Harris J.L., W. R. Posey II, C. Davidson, J. Farris, S. Oetker, J. Stoeckel, B. Crump, M. Barnett , H. Martin, M. Matthews, J. Seagraves, N. Wentz, R. Winterringer, C. Osborne, and A. Christian. 2010. Unionoida (Mollusca: Margaritiferidae, Unionidae) in Arkansas, third status review. Journal of the Arkansas Academy of Science 63: 50–86

Inoue, K., A. L. McQueen, J. L. Harris and D. J. Berg. 2014. Molecular phylogenetics and morphological variation reveal recent speciation in freshwater mussels of the genera *Arcidens* and *Arkansia* (Bivalvia: Unionidae). Biological Journal of the Linnean Society, 2014.

Mount, D. I., & Brungs, W. A. 1967. A simplified dosing apparatus for fish toxicology studies. Water Research 1:21-22.

Ortmann, A. E. and B. Walker. 1912. A new North American naiad. Nautilus 25:97-100.

Posey, W.R. II. 1997. Location, species composition, and community estimates for mussel beds in the St. Francis and Ouachita rivers in Arkansas. M.S. thesis, Dept. of Biological Sciences, Arkansas State University, State University, AR. 178pp.

Roberts, A.D. & M.C. Barnhart. 1999. Effects of temperature, pH, and CO_2 on transformation of glochidia of the flat floater mussel, Anodonta suborbiculata. Journal of the North American

Benthological Society 18(4): 477-487.

Seagraves, J. 2006. The reproductive biology and habitat characterization of the endangered Ouachita rock pocketbook, Arkansia wheeleri, (Ortmann and Walker, 1912). Masters Thesis, Arkansas State University, Jonesboro, 64 pp.

U.S. Fish and Wildlife Service. 2004. Ouachita Rock Pocketbook (*Arkansia wheeleri* Ortmann and Walker, 1912) Recovery Plan. Albuquerque, New Mexico.

Vaughn, C.C. and M. Pyron, 1995. Population ecology of the endangered Ouachita rock-pocketbook mussel, *Arkansia wheeleri* (Bivalvia, Unionidae), in the Kiamichi River, Oklahoma. Am. Malac. Bull 11(2):145-151.

Table 1. Metamorphosis of *A. wheeleri* and *A confragosus* on individual golden shiner at 14ºC and 22ºC. Fish were monitored individually in AHAB system. Data are mean per fish (minimum-maximum), n=3 fish in each treatment group. The time course of drop-off from these fish is shown in Figures 2 and 3.

Species and Temperature	Attached glochidia per fish	Sloughed glochidia per fish	Juveniles recovered per fish	Metamorphosis success of attached glochidia (%)
A. confragosus 14ºC	207.0 (172-246)	197.7 (171-220)	9.3 (1-26)	2.9% (0-10.6%)
A. confragosus 22ºC	326.0 (198-522)	290.3 (108-515)	35.7 (7-90)	12.7% (0-45.5%)
A. wheeleri 14ºC	158.7 (133-210)	156.3 (126-210)	2.3 (0-7)	1.3% (0-5.3%)
A. wheeleri 22ºC	102.3 (33-187)	93.3 (33-185)	9.0 (0-25)	7.5% (0-29.7%)

Table 2. Metamorphosis of *A. wheeleri* and *A confragosus* on groups of 100 golden shiner at 14C. Each cohort is from a separate group of fish.

Species and Cohort	Total attached glochidia per 100 fish	Total sloughed glochidia recovered	Total juveniles recovered	Metamorphosis success of attached glochidia (%)
A. confragosus (MSU Cohort #151)	4529	4300	229	5.1%
A. wheeleri (MSU Cohort #152)	5378	4818	560	10.4%

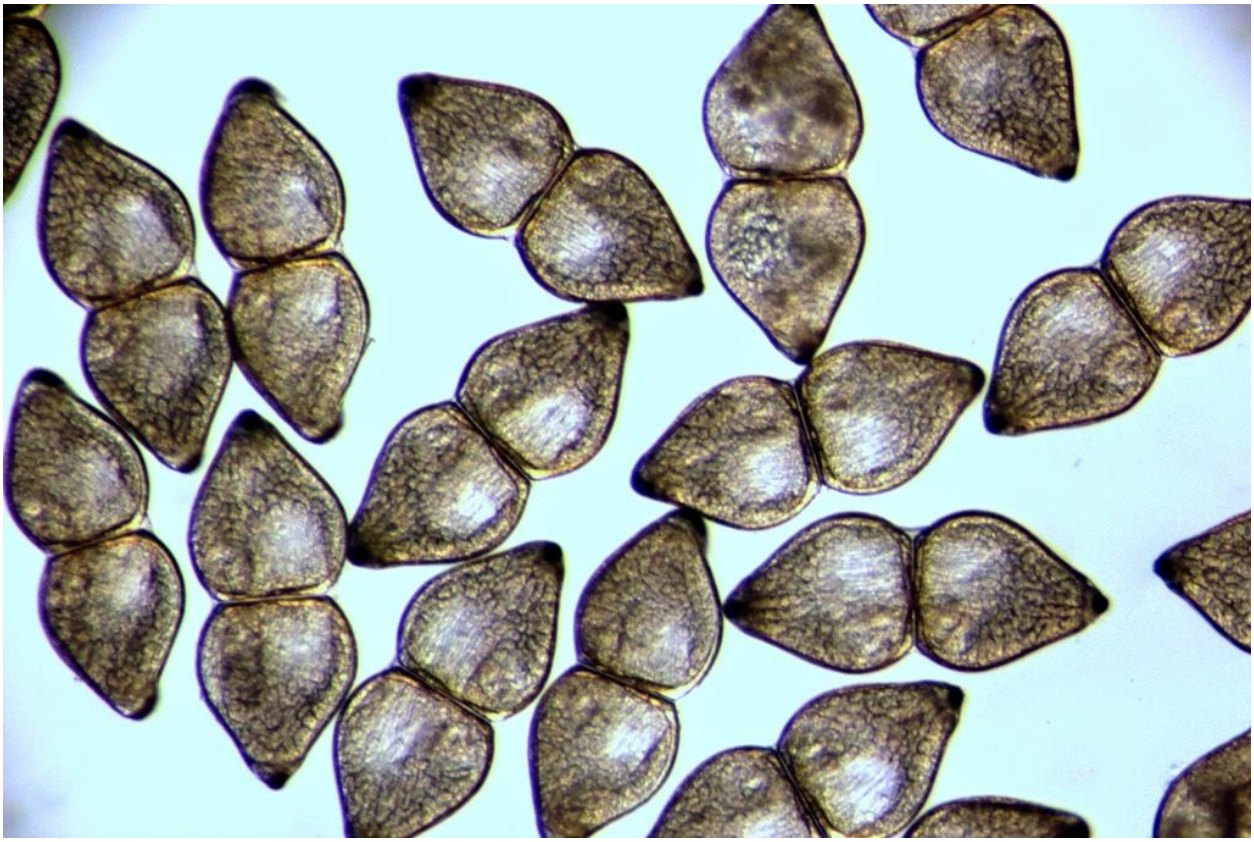

Figure 1. Glochidia from the Little River *A. wheeleri* collected 12-10-14. Fertilization and viability were nearly 100%. Photos of the female mussel, the glochidia and culture operations are available at this URL: https://goo.gl/photos/k8UZAdApvxCNhUQG6

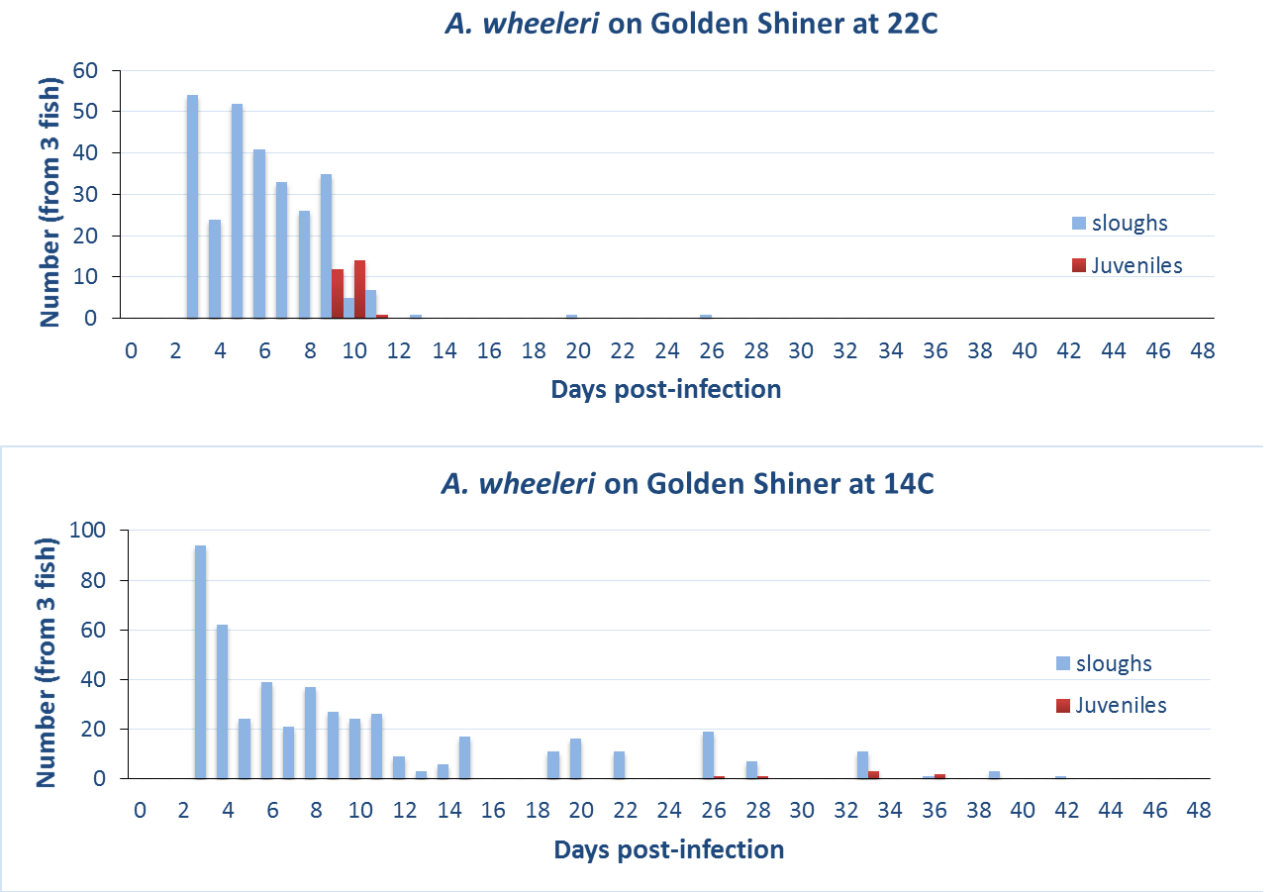

Figure 2. Time course of drop-off of sloughs (unmetamorphosed or dead larvae) and metamorphosed juveniles of *Arkansia wheeleri*. Each graph represents the average of catch from 3 fish.

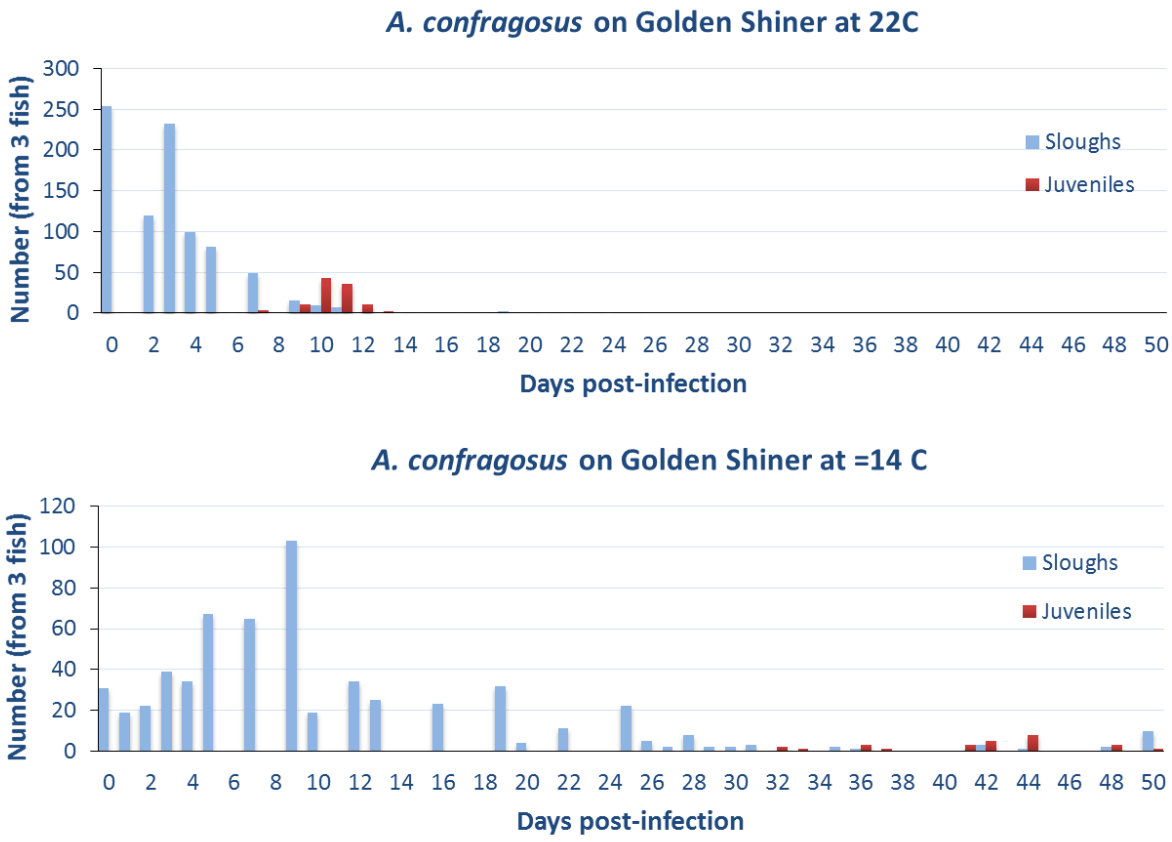

Figure 3. Time course of drop-off of un-metamorphosed larvae (sloughs) and metamorphosed juveniles of *Arcidens confragosus*. Each graph represents the average of catch from 3 fish.

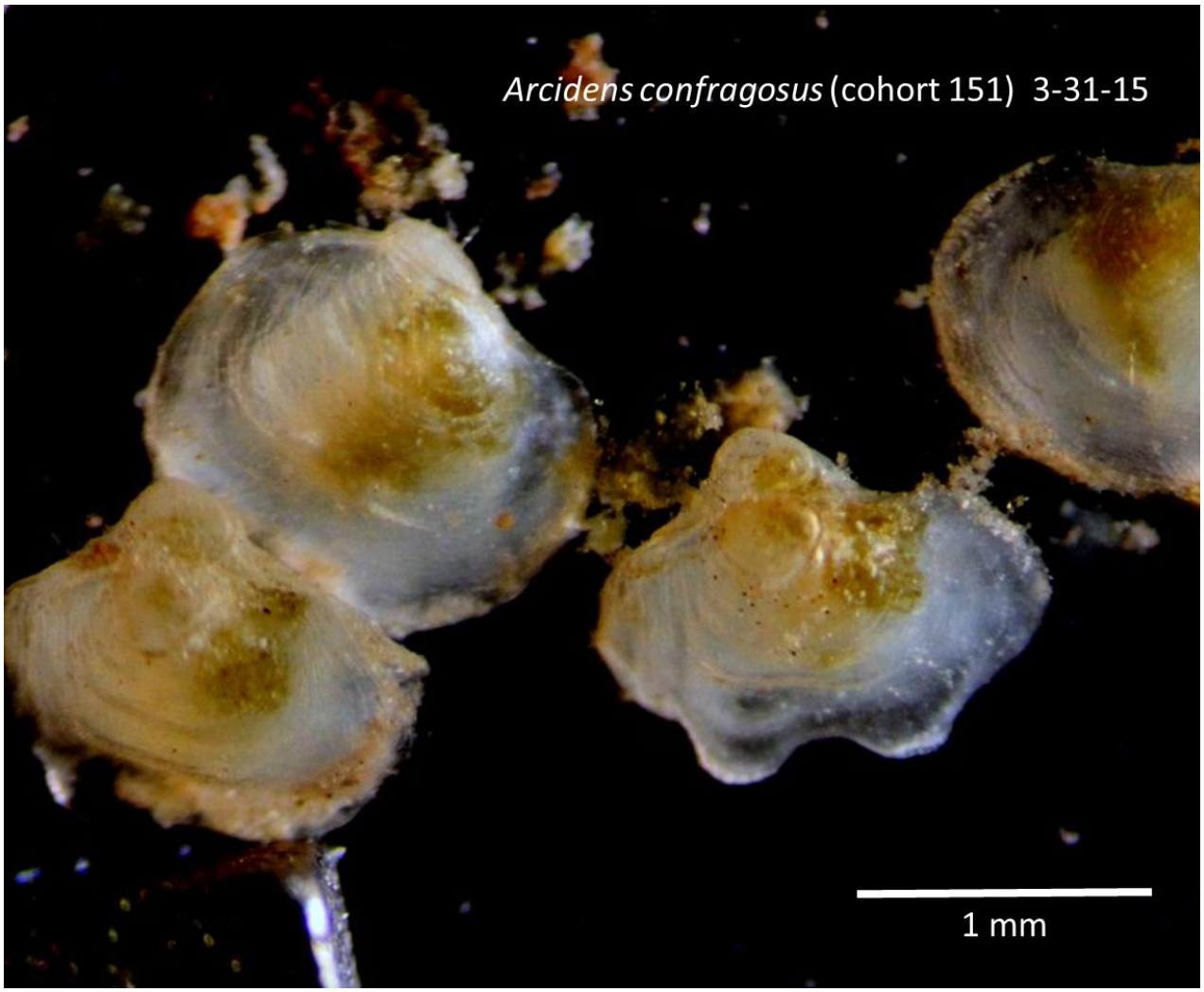

Figure 3. *Arcidens confragosus* 2-mo juveniles after culture in static sediment boxes and flow trays. Golden color is the digestive gland. The developing 'beak sculpture' is evident as a scalloped shell margin. More photos and video can be viewed at https://goo.gl/photos/k8UZAdApvxCNhUQG6