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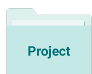

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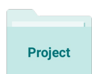

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Novel technique to identify large river host fish for freshwater mussel propagation and conservation

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ABSTRACT

Skipjack Herring (*Alosa chrysochloris*) has long been proposed as the sole host for *Reginaia ebenus* (Ebonyshell) and *Elliptio crassidens* (Elephantear), but these relationships were unconfirmed because of difficulties with maintaining this fish species in captivity. We confirmed the suitability of Skipjack Herring as host for both mussel species, and we also showed that Alabama Shad (*Alosa alabamae*) is an additional suitable host for *E. crassidens*; both fish species produced large numbers of juvenile mussels. No other fish species tested (n = 12) were suitable hosts for either mussel species. Our results, combined with results from other studies, suggest these mussel species are specialists on genus *Alosa.* Traditional methods for host identification were problematic for herrings because of their sensitivity to handling and the large volumes of water required to maintain them in captivity. In addition to traditional methods, we confirmed the suitability of these fishes as hosts using a novel technique in which fish gills infected with glochidia were excised from sacrificed fishes and held in recirculating holding tanks with flow until metamorphosis was complete. Completion of metamorphosis on excised gills required glochidia spend at least 11–17 d encapsulated on live fishes before gill excision. This technique may be useful for other large or sensitive fishes that do not lend themselves well to traditional methods for host identification. Confirmation of *Alosa* spp. as primary hosts for *R. ebenus* and *E. crassidens* supports the idea that dams and other river modifications that disrupt migrations of these fishes are key factors in the range restrictions of these mussel species.

1. Introduction

Development of the larvae (glochidia) of most freshwater mussels requires a brief period during which they are parasites on fishes. Host specificity ranges from generalists which develop on a taxonomically wide array of fish species, to specialists which successfully parasitize only one or a few closely related fishes. Knowledge of host use is essential for understanding mussel ecology and for designing effective conservation strategies.

The standard method for determining mussel host use involves laboratory trials in which a wide array of potential host fish species are inoculated with glochidia from gravid female mussels (*e.g.*, Zale and Neves, 1982; Haag and Warren, 1997; Fritts et al., 2012). Suitable host fish species are considered those that facilitate glochidial development resulting in production of metamorphosed juvenile mussels. This method has the advantages of controlling infestation densities, directly evaluating immunological compatibility of mussel-fish pairings, and allowing for replication and repeatability. In contrast, examination of naturally occurring glochidial infestations on wild fishes cannot evaluate whether an observed infestation ultimately will result in glochidial metamorphosis, or conversely, whether a lack of an observed infestation indicates an unsuitable host or simply a lack of contact between fish and glochidia. Furthermore, positive identification of encapsulated glochidia on wild fish can be difficult. A disadvantage of laboratory host trials is that fishes must be kept alive in captivity for weeks to months. This is particularly problematic for large, migratory fish species and those that are sensitive to handling. Consequently, such species are rarely included in host trials and their relative importance as mussel hosts is poorly understood.

Skipjack Herring (*Alosa chrysochloris*) are purported as a primary host for at least two large river mussel species, *Elliptio crassidens* and *Reginaia ebenus*, but these relationships are based only on observations of naturally occurring glochidial infestations or incomplete inoculation trials (Surber, 1913; Howard, 1914, 1917). Both of these mussel species

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Table 1
Results of host trials for *Reginaia ebenus*. Y represents successful encapsulation, N represents no encapsulation observed. Day after inoculation is the time period within which gills were inspected for encapsulation. Production of juvenile *R. ebenus* on *Alosa chrysochloris* is depicted in Fig. 2.

Taxa	Common Name	# Inspected	Glochidia present? (Y/N)	Day after inoculation
Cyprinidae				
Lythrurus bellus	Pretty Shiner	6	N	4–6
Notropis ammophilus	Orangefin Shiner	2	N	4–6
Campostoma oligolepis	Largescale Stoneroller	3	N	4–6
Luxilus chrysocephalus	Striped Shiner	3	N	4–6
Pimephales promelas	Fathead minnow	6	N	4–6
Fundulidae				
Fundulus olivaceus	Blackspotted Topminnow	6	N	4–6
Percidae				
Percina nigrofasciata	Blackbanded Darter	6	N	4–6
Centrarchidae				
Lepomis macrochirus	Bluegill	6	N	4–6
Micropterus salmoides	Largemouth Bass	6	N	4–6
Ictaluridae				
Ictalurus punctatus	Channel Catfish	6	N	4–6
Sciaenidae				
Aplodinotus grunniens	Freshwater Drum	4	N	4–6
Clupeidae				
Dorosoma cepedianum	Gizzard Shad	2	Y[1]	5
		1	N	7
		1	N	19[2]
Alosa chrysochloris	Skipjack Herring	1	Y	11
Alosa chrysochloris		7	N	19

[1] Low number of glochidia relative to *A. chrysochloris* and glochidia were poorly encapsulated.
[2] Fish not inspected prior to day 19.

have declined in rivers in which Skipjack Herring spawning migrations have been blocked or restricted by dams, but they remain common in rivers that continue to support large Skipjack Herring populations (Kelner and Sietman, 2000; Gangloff, 2003; Haag, 2012). This pattern supports the proposed role of Skipjack Herring as an important host for these mussel species, but these relationships have not been confirmed by laboratory studies because of the difficulty of maintaining *Alosa* spp. in captivity. Furthermore, the breadth of host use by these two mussel species and the extent of their dependence on Skipjack Herring is unknown. More comprehensive knowledge of host use for these species is necessary to understand the reasons for their recent decline in some streams and to inform management strategies.

Observation of juvenile production is essential for confirmation of mussel hosts and requires holding fish in captivity for weeks to months. Development of techniques to evaluate host suitability in the absence of a live host would provide much needed options for assessment of host species. Previous observations suggest that juvenile mussels may be obtained from excised gills of fish that died during the host trial, if the gills are maintained in favorable conditions (R. Bringolf, unpublished). Currently little is known about the factors that influence metamorphosis success after a host has died.

We conducted laboratory host trials for *E. crassidens* and *R. ebenus* with emphasis on Skipjack Herring and a related species, Alabama Shad (*Alosa alabamae*). We report on our use of standard fish hauling/holding techniques that resulted in high survival of *Alosa* spp. and other sensitive fishes during capture, transport, and maintenance in captivity. We examined the breadth of host use across 14 fish species from 7 families for *R. ebenus*, and across 3 species from 2 families for *E. crassidens*. We also report a novel technique, gill excision, which allows glochidial metamorphosis to continue after death of the host.

2. Materials and methods

2.1. Mussel collection and glochidial extraction

We collected 32 gravid female *R. ebenus* from the Alabama River, Wilcox Co., Alabama, on 15 June 2011 and 20 gravid females on 23 June 2011. Mussels were transported in coolers to the South Auburn Fisheries Research Station (SAFRS) and held in a recirculating aquarium system with dechlorinated tap water at 18 °C. One day after collection, we assessed the glochidial development stage of gravid females by extracting a small subsample of the gill contents with an 18 gauge needle and syringe and examining the material under 10× magnification. Following examination, we flushed the gills of all 11 females with mature glochidia (fully formed and not enclosed by membranes) and randomly assigned each brood to one of three composite batches consisting of four, four, and three combined broods, respectively. Viability of each composite batch was then determined by exposing subsamples to a saturated solution of NaCl. The traditional method scores glochidia as nonviable if they are either closed before exposure to, or fail to close after exposure to, the NaCl solution (Fritts et al., 2014). However, mature glochidia of *R. ebenus* often exhibited a conspicuous snapping behavior in which the shell valves periodically opened and closed in the absence of NaCl. Unlike the traditional NaCl test, glochidia that were initially closed could not be automatically scored as nonviable because they might open spontaneously at a later time. Thus, we ignored glochidia that were closed initially and calculated viability as the percentage of open glochidia that closed their shell valves in response to salt exposure. We subsequently collected 20 gravid female *R. ebenus* from the same site on 23 June 2011. Only two of these females had mature glochidia, which were combined into a composite batch and examined for viability. The remaining 18 broods were discarded.

We collected ten gravid *E. crassidens* from Chewacla Creek, Macon Co., Alabama, on 26 March 2011 and 3 April 2012 (five individuals on each date), transported them in coolers to SAFRS, and placed them in a recirculating aquarium system at 18 °C. Glochidia were flushed from all five females per date within 24 h of arriving at the lab. Viability was estimated using the same methodology as for *R. ebenus*, except we estimated viability of individual broods rather than for combined broods. Also, because glochidia of *E. crassidens* exhibited little to no snapping behavior in the absence of NaCl solution, we used the traditional method for scoring viability. After calculating viability, we combined broods of *E. crassidens* into two composite batches containing the broods of three and two females, respectively.

2.2. Fish collection and holding

Small-bodied fishes for host trials (Table 1), were collected by seining or backpack electrofishing from small to medium streams of the Mobile Basin in eastern Alabama. Bluegill (*Lepomis macrochirus*), Largemouth Bass (*Micropterus salmoides*), and Channel Catfish (*Ictalurus punctatus*) were collected from ponds or commercial hatcheries. Fishes were transported in coolers to SAFRS. Small fishes were held in a recirculating aquaria system, and Bluegill, Largemouth Bass, and Channel Catfish were held in rectangular fiberglass or plastic tanks; all fishes were held at 18–21 °C and fed three times/week.

Large river fishes for host trials were collected by electrofishing or hook and line and subsequently handled using standard fish hauling/holding techniques that incorporated rounded tanks, aeration, and salting (e.g. Wright and Kraft, 2012; C. Eschevarria, USFWS Warm Springs National Fish Hatchery, pers. comm.). We collected *Alosa* during their spawning runs in March, 2011 and 2012. We first attempted to capture Skipjack Herring and Gizzard Shad by electrofishing in the Chattahoochee River below Eagle Phenix Dam, Columbus, Georgia. Gizzard Shad (*Dorosoma cepedianum*) were readily collected in this manner, but Skipjack Herring were deep and sank quickly after being shocked. Consequently, we captured Skipjack Herring at this location in 2011 and 2012 by hook and line using small white jigs. Alabama Shad were collected in 2012 by electrofishing from the Apalachicola River below Jim Woodruff Lock and Dam, Gadsden County, Florida, by the Georgia Department of Natural Resources. We collected Freshwater Drum (*Aplodinotus grunniens*) in December, 2010 from the Tennessee River (Lake Guntersville, AL) by hook and line.

Immediately after capture, Skipjack Herring, Alabama Shad, Freshwater Drum, and Gizzard Shad were placed in a 570 L oval stock tank filled with aerated river water, and they were transferred to a 1900 L round fish hauling tank within 30 min. The hauling tank was filled with river water aerated with compressed pure oxygen and supplemented with Kent Sure-Haul (Kent Marine, Franklin, Wisconsin; 0.21 L Sure-Haul granules/1000 L water) and non-iodized NaCl (Morton pool salt) resulting in a salinity of ∼5‰. Kent Sure-Haul is a commercial formulation of salts and defoaming agents that reduces fish stress and water foaming during hauling and crowded conditions. The hauling tank was filled nearly to the top and sealed with a lid to minimize sloshing during transport (Winkler, 1987). Transport time from collection sites to SAFRS ranged from ∼1–3 h.

After arrival at SAFRS, *Alosa* spp., Freshwater Drum, and Gizzard Shad were transferred to one of two, 5700 L rectangular tanks or an 8500 L circular tank. All tanks were outdoors and were equipped with a central baffle with circular flow established with pumps or airlifts. Each tank was covered with coarse netting or shade cloth to prevent fish escape and to reduce water temperature. Water temperature never exceeded 26°C in any tank, and salinity was maintained at 3–5 to reduce stress after hauling. We monitored water quality weekly with Tetra EasyStrips (United Pet Group, Blacksburg, VA) and made partial water changes when ammonia or nitrites exceeded 0.5 mg/L or 1.0 mg/L, respectively. We fed Skipjack Herring and Alabama Shad with Fathead Minnows (*Pimephales promelas*) or Mosquitofish (*Gambusia* spp.) twice weekly. Freshwater Drum were fed Asian Clams (*Corbicula fluminea*) and Redswamp Crayfish (*Procambarus clarkii*) approximately weekly. Gizzard Shad foraged on detritus that accumulated on the bottoms of the large tanks from occasional algal blooms. Salinity in the large tanks was gradually reduced to < 0.5 over a 1–2 week acclimation period before initiation of host trials to avoid potential negative effects of salinity on glochidia.

2.3. Host trials

Host trials for small-bodied fish species were conducted in 1.5 or 3.0 L tanks within a recirculating AHAB system (Pentair Aquatic Habitats, Apopka, Florida). Water from the tanks flowed first through a removable 105 μm screen to retain sloughed glochidia and juvenile mussels, then into a sump/biofilter followed by ultraviolet sterilization, a secondary 100 μm filter, and finally into a manifold that returned water to the tanks. Fishes were placed in tanks (one individual/tank) filled with dechlorinated tap water and allowed to acclimate for about one week before conducting host trials. We monitored water quality weekly and made water changes as described previously.

Host trials for Freshwater Drum and Gizzard Shad were conducted in an array of four cone-bottom tanks each of which were 55 L (for drum) or 150 L (for shad), and each held a single fish. Cone-bottom tanks were fitted with a double stand pipe that allowed collection of sloughed glochidia and juveniles from the bottom of the tank, which were retained in a 100 μm nylon filter bag. Filtered water then drained into a common 100–200 L sump/biofilter and was pumped back to the tank via a manifold and an inflow line that was angled against the side of each tank to create circular flow. Tanks were maintained at room temperature (19–21 °C) during the trials. Acclimation, twice weekly water quality monitoring, and water changes were conducted as described previously.

We attempted to use the cone-bottom tanks for Skipjack Herring, but all individuals held in either tank size died within 24 h. Consequently, we conducted host trials for this species and Alabama Shad in the two 5700 L rectangular holding tanks. Fish were distributed equally among both tanks and acclimated for one week as described previously, but the number of fish varied among trials according to availability. Water temperature was 24–26 °C during the trials and water quality was monitored and adjusted at least weekly as described previously.

2.4. Reginaia ebenus trials

Host suitability for *R. ebenus* was evaluated on 14 fish species from seven families, including Skipjack Herring (Table 1). All species were inoculated in a suspension of approximately 2000 viable glochidia/L. We inoculated Skipjack Herring in a 570 L inoculation suspension on 17 June 2011, with the combined broods of four females. The remaining fish species were inoculated using the combined broods of two females collected on 23 June 2011. Freshwater Drum and Gizzard Shad were inoculated in 140 L coolers. All other fish species were inoculated in 19-L buckets or 2-L beakers, depending on fish size.

All fishes were placed in the inoculation suspension for 15 min, and the solution was aerated vigorously to keep glochidia suspended in the water column. Multiple individuals and species of smaller fishes were inoculated simultaneously in common chambers, but the inoculation suspension for each chamber was used only once to avoid exposing subsequent fishes to lower glochidial concentrations. After inoculation, fishes were returned to holding tanks for the remainder of the experiment. Water temperature of the indoor tanks (AHAB and cone tanks) was maintained at 18–21 °C using a combination of air conditioning and in-line chillers. Water temperature of the large, outdoor holding tanks was maintained at 24–26 °C as described previously.

Four to six days after inoculation, we examined the gills of all fishes in the AHAB and cone tanks for encapsulated glochidia. At that time, we saw no glochidia on any individual of any species, except for Gizzard Shad, and these trials were terminated. We examined two Gizzard Shad on day 5, both of which had a small number of poorly encapsulated glochidia but died apparently due to handling stress. Subsequently, one of the remaining Gizzard Shad died on day 7. The other Gizzard Shad survived until day 19.

Because of their sensitivity to disturbance and handling, we did not examine Skipjack Herring gills, nor did we siphon the large outdoor tanks for sloughed glochidia within the first 9 d after inoculation. On day 10, we siphoned half of the bottom of each holding tank (3.2 m^2) through a 100-μm filter bag, and examined nine, 6 mL subsamples of the filtrate under a dissecting microscope with cross-polarized lighting. Even with cross-polarized light, the large amount of detritus from the

tank bottom made it difficult to detect sloughed glochidia or juveniles. Consequently, on day 11 we removed and examined a single Skipjack Herring from one of the tanks to check for encapsulated glochidia. This fish had encapsulated glochidia on its gills but it died soon after examination. We immediately excised its gill arches and placed half of the gills in a 1.5-L AHAB tank; the remaining gills were preserved in 70% ethanol. The filter screen of the AHAB tank containing the gills was then examined daily for sloughed glochidia and juveniles over a one week period. Juveniles were differentiated from sloughed glochidia by valve movement and/or the presence of a foot. A second dead Skipjack Herring was found on day 12 but its gills were partially decomposed and no encapsulated glochidia were observed. Two additional dead Skipjack Herring were found on day 19 but gills were not examined due to decomposition. The remaining seven, live Skipjack Herring had no encapsulated glochidia on their gills on day 19, but one individual had numerous, small lesions on the gill lamellae.

Because encapsulated glochidia were observed on day 11, we siphoned the other bottom half (3.2 m^2) of each tank on day 12 and the entire bottoms (6.4 m^2) on day 19. The day 12 samples contained substantial amounts of detritus and were examined as described previously for day 10. The day 19 samples were relatively clean due to previous siphoning. Day 19 samples were diluted to 800 and 500 mL for Tanks 1 and 2, respectively and ten, 6 mL subsamples from each dilution were examined under a microscope equipped with cross polarized light.

2.5. *Elliptio crassidens* trials

Host suitability for *E. crassidens* from Chewacla Creek was evaluated on Skipjack Herring, Alabama Shad, and Bluegill. All species were inoculated in a suspension of approximately 4000 viable glochidia/L. We inoculated seven Skipjack Herring and seven Alabama Shad in a 564 L inoculation suspension using the combined broods of three females. Fishes were placed in the suspension for 15 min while the suspension was aerated vigorously to keep glochidia in suspension. All 14 fish were then placed together in a 5700 L holding tank. The following day, we inoculated an additional eight Skipjack Herring, eight Alabama Shad, and five Bluegill in a 473-L inoculation suspension using the combined broods of two female *E. crassidens*. Fishes were placed in the inoculation suspension for 15 min as described previously. After inoculation, Skipjack Herring and Alabama Shad were placed together in the second 5700 L holding tank, and Bluegill were placed individually in 1.5-L AHAB tanks.

We examined filter cups from Bluegill AHAB tanks 2 days after inoculation and every day thereafter until day 16. On day 16, we inspected the gills of all Bluegill. No glochidia were found on any Bluegill, but we returned all individuals to their tanks until day 23 and examined filter cups again on this date as a precaution.

We monitored glochidial inoculations on Skipjack Herring and Alabama Shad primarily by gill excision because of concerns about the efficacy of detecting juveniles or glochidia in material siphoned from the bottom of the large holding tanks (see *R. ebenus*). We excised the gills of one infected Skipjack Herring and one Alabama Shad from alternate holding tanks every 2–3 days from day 5 until day 36 or until we ran out of live fish. After severing the fish's spinal cord, we removed the first two gill arches on each side of the fish and placed the gills immediately in a single 1.5-L AHAB tank; gills from each fish were placed in separate AHAB tanks. We examined filter cups from these AHAB tanks 1 d after excision and every 2 d thereafter. We ceased inspection when no glochidia or juveniles were found for 5 consecutive examinations. Juveniles were identified by the presence of a foot and were recorded as active juveniles (locomotion observed) or questionable juveniles (closed and with tissue, but no observed movement or activity). Empty shells were recorded as "dead" because we could not distinguish sloughed glochidia from dead juveniles. Metamorphosis success for each individual fish was calculated as the total number of active juveniles/the total number of individuals recovered (active juveniles + questionable juveniles + empty shells).

We determined if juvenile metamorphosis occurred on live fish before gill excision by siphoning a 1 m^2 area of the bottom of one of the holding tanks 25 d after inoculation. For *E. crassidens*, we addressed in the following ways the problems with excess detritus accumulation encountered in the *F. ebenus* trials. First, before the host trials, we scrubbed the tank bottoms and removed detritus that had accumulated between experiments. Second, during trials, siphoned water from the tank bottom was passed through a 200 μm filter screen to remove larger particles (glochidia and juveniles were 130–150 μm length × 141–160 μm height) before sample collection on a 100 μm filter sock. The filtrate was brought to a volume of 125 mL, and juveniles and glochidia were counted in 15, 5-ml subsamples under a dissecting microscope with cross-polarized light. Juvenile density in the sample (number/m^2) was calculated as $J \cdot (V/v)$, in which, J = total number of juveniles in all subsamples, V = total volume of sample, and v = combined volume of subsamples.

3. Results

3.1. Mussel brood characteristics

Of the 32 gravid *R. ebenus* collected on June 15, 11 broods were dominated by mature glochidia, 8 by early stage glochidia, and the remaining 12 by cleaved embryos that had not yet developed into glochidia. Of the three composite batches of broods, batch 1 (4 broods combined) exhibited 86% viability and was used to inoculate Skipjack Herring. The remaining two composite batches had glochidial viabilities of 53% and 41%, respectively, and were discarded. The glochidial batch of 2 combined broods from the June 23 collection had 67% viability and was used to inoculate all other fish species. Note that because of the snapping behavior of *R. ebenus* (see Methods), viability was likely to have been underestimated.

All five *E. crassidens* females collected on March 26 were brooding early stage embryos and were therefore not examined for viability or used for experiments. Of the five females collected on April 3, one contained early stage embryos and mature glochidia, and the remaining four contained mature glochidia only. Viability of all *E. crassidens* broods was > 90%. Both composite batches of E. crassidens broods were used to inoculate fish.

3.2. Transport and holding of *Alosa* spp.

We captured a total of 127 *Alosa* spp. (97 Skipjack Herring, 30 Alabama Shad). Survival from capture to arrival at SAFRS averaged 94%, and ranged from 86 to 98%. Survival was comparable between Skipjack Herring captured by hook and line (mean survival = 93%) and Alabama Shad captured by electrofishing (97%). Survival during host trials appeared higher for Alabama Shad than Skipjack Herring. In the *R. ebenus* trial, 3 of 11 Skipjack Herring died of unknown causes and a fourth died after handling. In the *E. crassidens* trials, 7 of 15 Skipjack Herring died, but only 1 of 15 Alabama Shad died of unknown causes. The remaining fish were sacrificed for gill excision.

3.3. Host use of *R. ebenus*

No individuals of any fish species had encapsulated glochidia on the gills 4–6 d after inoculation except for Gizzard Shad and Skipjack Herring. The two Gizzard Shad examined on day 5 had few encapsulated glochidia on the gills and died after handling. An additional Gizzard Shad that died on day 7 had no encapsulated glochidia. The single Gizzard Shad that survived until day 19 had no encapsulated glochidia, and no glochidia or juveniles had accumulated in its associated filter bag by day 19. The single Skipjack Herring examined on day 11 had numerous encapsulated glochidia (Fig. 1). The excised gills

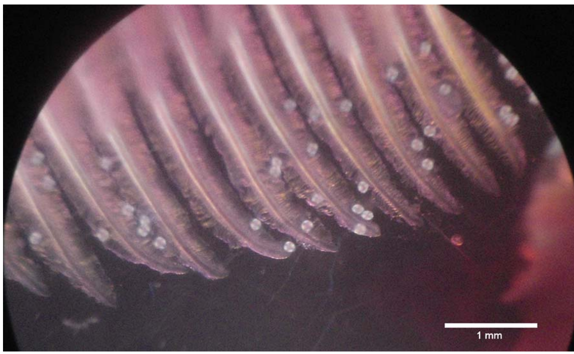

Fig. 1. Glochidia of *Reginaia ebenus* encapsulated on the gills of Skipjack Herring (photographed under cross polarized lighting, 25×). Light colored objects on gill filaments are encapsulated glochidia measuring ~125 μm diameter.

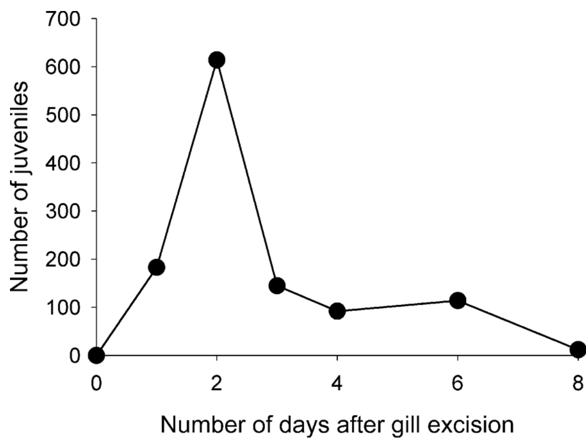

Fig. 2. Production of live juvenile *Reginaia ebenus* from a single set of excised Skipjack Herring gills excised 11 d after inoculation.

from this fish produced a total of 1160 juvenile mussels over 8 d, with the highest number of juveniles observed 2 d after excision (Fig. 2). The 7 remaining Skipjack Herring examined on day 19 had no encapsulated glochidia.

We found a single dead glochidium and no juvenile mussels in material siphoned from the bottom of the tanks on day 10, and we found no glochidia or juveniles on day 12. Large amounts of detritus made detection and counting of juveniles difficult, and thus these results may represent the minimum number of juveniles metamorphosing. On day 19, we found two recently dead juveniles (with tissue in the shell) and 7 empty shells in one tank, and a single live juvenile in the other tank.

3.4. Host use of *E. crassidens*

Fecundity of female *E. crassidens* averaged 819,000 (± 199,339 se; N = 5 females) glochidia/female. No juvenile mussels were produced on Bluegill. An average of 84 (± 22 se) sloughed glochidia were recovered from each Bluegill, but 92% of sloughed glochidia were recovered by 2 d after inoculation. Single glochidia were recovered as late as day 12, and no fishes had encapsulated glochidia on day 16.

Production of live juvenile mussels from excised gills of Alabama Shad and Skipjack Herring was dependent on when gills were excised (Fig. 3). Gills of Alabama Shad excised 5–8 d after inoculation produced no live juvenile mussels even though encapsulated glochidia were observed. Gills excised on days 10–14 also contained encapsulated glochidia but produced very few live juveniles and metamorphosis success was < 1%. Peak juvenile production occurred on gills excised on days 17–23; total juvenile production from these individuals ranged from 293 to 328/fish and metamorphosis success ranged from 50 to 60%.

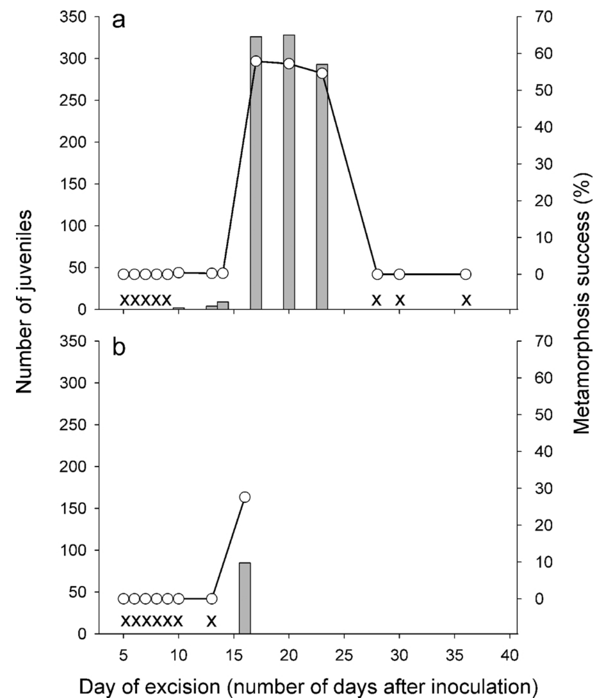

Fig. 3. Production of live juvenile *Elliptio crassidens* (bars) and metamorphosis success (circles) from (a) Alabama Shad and (b) Skipjack Herring gills excised on different days following inoculation. Observations for each day of excision represent gills from different individual fishes, and represent the sum of all juveniles produced over multiple days following that excision; X denotes gills from which no juveniles were produced.

Gills excised on day 28 had no observed encapsulation and produced only five empty shells. Gills excised on days 30 and 36 had no observed encapsulation and produced no juveniles or shells. Gills of Skipjack Herring excised 5–13 days after inoculation produced no juvenile mussels even though they contained encapsulated glochidia. Gills excised on day 16 produced 85 live juveniles with metamorphosis success of 28%. No live Skipjack Herring remained after day 16. For both host species, production of live juveniles occurred up to about 10 d after gill excision but peaked about 2–4 d after excision for Alabama Shad (Fig. 4), and about 6–8 d for Skipjack Herring (Fig. 5). It is possible that metamorphosis success for both species is overestimated to some extent because our estimates do not include glochidia that may have been sloughed in holding tanks before excision.

Examination of material siphoned from the bottom of one of the holding tanks on day 25 yielded an estimate of 187 live juveniles/m^2 and a total of 1134 juveniles in the entire tank (6.3 m^2 total area). Because Alabama Shad and Skipjack Herring were held together in this tank, it was not possible to determine the number of juveniles produced by each species.

4. Discussion

Host trials with large river fishes are difficult. Fishes, particularly *Alosa* spp., were sensitive to repeated handling and examination required by standard host suitability test methods, and the large volume of water necessary to hold these fishes made it difficult to examine tank bottoms for juvenile mussels. Gill excision represents an important methodological advance that helps to overcome challenges associated with conducting host trials with large or sensitive fishes because it does not require that fish remain alive during the entire encapsulation/metamorphosis period and allows for production and harvest of juveniles using small-volume (e.g. 1.5 L) tanks.

For *E. crassidens*, we harvested large numbers of metamorphosed juvenile mussels from excised gills and from inspection of material on the bottom of the holding tank. This shows that gill excision can

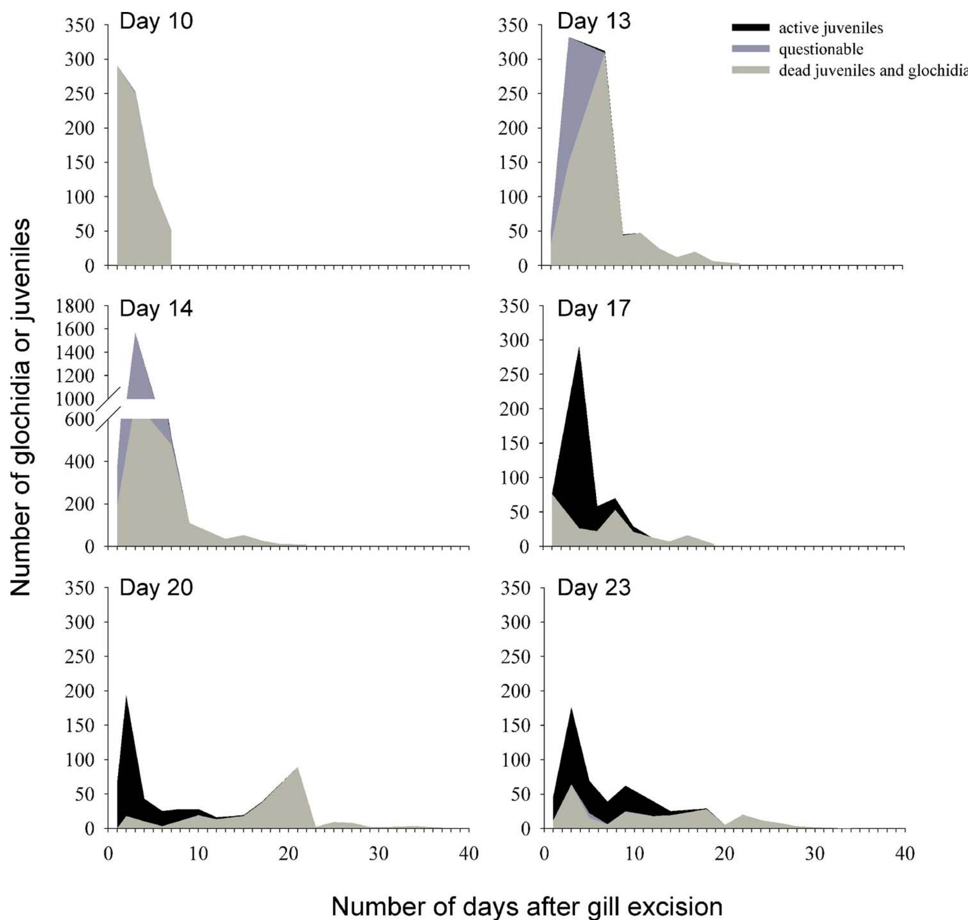

Fig. 4. Daily production of *E. crassidens* juveniles and glochidia from excised gills of Alabama Shad. Day at top of each panel indicates the day gills were excised (number of days after inoculation) and the x-axis indicates the number of days after excision.

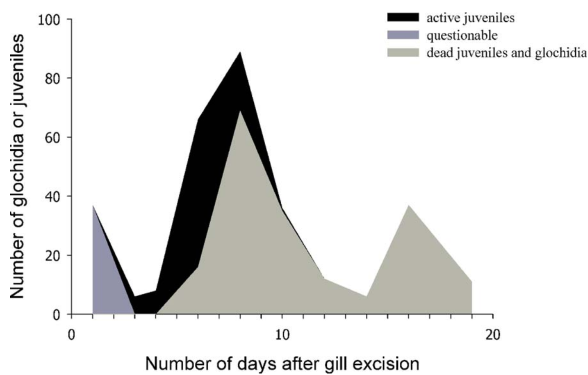

Fig. 5. Daily production of *E. crassidens* juveniles and glochidia from Skipjack Herring gills excised 16 d after inoculation.

provide results comparable to traditional approaches for host identification, in which juveniles excyst normally on live fishes after the end of the parasitic period. Our results from gill excision involving *R. ebenus* were not corroborated by traditional methods; we harvested large numbers of juveniles from excised gills but virtually none from the tank bottom. Our failure to find juveniles on the tank bottom on days 10 and 12 could be because of the large amount of detritus in the samples or because this represented the onset of juvenile excystment (as identified by excised gills) and few juveniles were present at that time. The rarity of juveniles on day 19 is more puzzling. Samples collected on this day had much less detritus and were easier to examine. Assuming juvenile production from the two fish remaining in tank 1 and five fish remaining in tank 2 was comparable to production from the single individual from which we excised gills (only half of the gills were examined), we would have expected about 5500 and 11,500 juveniles to be present in each tank, respectively, numbers that should have yielded higher detection in our samples despite the presence of detritus. We cannot explain this discrepancy, and further corroboration of our results for *R. ebenus* is desirable.

Several other aspects of gill excision need to be evaluated regarding the extent to which this technique accurately represents natural host relationships. First, it is possible that gill excision weakens the host immune response to glochidial infection and thus may produce false positives regarding host suitability. We did not excise gills of any non-suitable host species to directly test this possibility, but our results from *Alosa* show that such a scenario is unlikely. Glochidia did not metamorphose on gills that were excised too early, indicating that blood flow or other factors present in live fish are necessary for juvenile development sufficient to allow completion of metamorphosis on excised gills (e.g., see Fritts et al., 2013). This minimum period of encapsulation on live fishes varied between species for unknown reasons but ranged from 11 to 17 d. Glochidia were rejected from all non-suitable host species in less than 4–6 d. Therefore, if gill excision dampens the immune response, avoidance of rejection would require gill excision soon after inoculation, and it is unlikely that such a short period of encapsulation on live fish would be sufficient to allow later metamorphosis from excised gills. Second, it is unknown whether gill excision alters the timing of juvenile release. It is possible that gill excision hastens or slows release, but we were unable to precisely determine the normal timing of release from live fishes because of the difficulty of inspecting tank bottoms for juveniles. Finally, longer-term survival and viability of juveniles produced on excised gills should be evaluated if this technique is used to produce juveniles for restoration of wild populations.

Despite questions remaining about the technique, gill excision offers important advantages over traditional methods for conducting host

trials with large or sensitive fishes. First, gill excision allows for greater and more controlled replication. Inoculated fishes initially can be held communally in one or a few large tanks, but gills from individual fishes then can be placed in small AHAB tanks to assess variability in metamorphosis success among species and individuals. In contrast, traditional methods would require a large number of holding tanks, which are expensive and require large amounts of space. Second, quantification of juvenile production is easily accomplished with excised gills held in AHAB units, but this is extremely difficult in large tanks. Third, gill excision can shorten the amount of time fishes must be kept alive in captivity. Even using appropriate holding techniques, maintaining fishes throughout the natural period of glochidial metamorphosis can be challenging regardless of the fish species involved.

A critical aspect of the gill excision technique is that it is successful only if excision occurs within a specific time period during which glochidia have developed sufficiently such that metamorphosis and release can be completed on excised gills. For *E. crassidens* on Alabama Shad, this window of opportunity lasted about two weeks, from 10 to 25 d after inoculation, but the period of highest metamorphosis success was even shorter, lasting from day 17–23. The window of opportunity for *E. crassidens* on Skipjack Herring and for *R. ebenus* appears to be similar, but we were unable to determine the precise time period due to fish mortality and other factors. Our observations also suggest that water flow is likely necessary for gill excision to be successful. In a subsequent experiment, flow through some AHAB tanks was inadvertently shut off and excised gills quickly became coated in bacteria/fungus (M. Hart et al., unpublished data). Similar observations have been made in other studies (R. Bringolf, unpublished data). Flow was maintained throughout our study, and gills showed little evidence of bacterial or fungal growth for > 1 week after excision.

Our results provide the first direct confirmation of Skipjack Herring as host for glochidia of *R. ebenus* and *E. crassidens*, and we provide the first report of the suitability of Alabama Shad as host for *E. crassidens*. Previous conclusions about the suitability of Skipjack Herring for both species were based on observations of natural infestations or incomplete artificial inoculations. As far as we can ascertain, the proposal of Skipjack Herring as host for *E. crassidens* is based entirely on the observation of a single Skipjack Herring that carried a heavy natural infestation of encapsulated glochidia identified as *E. crassidens* (Howard, 1914); little research was conducted with *E. crassidens* because it had no value in the pearl button industry of the early 1900s. In contrast, *R. ebenus* was one of the most valuable species for buttons, and identification of its host received much attention (Surber, 1913; Howard, 1917). These studies provided support for the suitability of Skipjack Herring as a host for Ebonyshell but the relationship was not confirmed because of the difficulties of working with this fish.

In addition to confirming host suitability of *Alosa* spp. for *R. ebenus* and *E. crassidens*, our observations, along with results of other studies, support the idea that these two mussel species are strict specialists on *Alosa* spp. In our study, all glochidia of *R. ebenus* were rejected quickly by 11 other fish species in six families. Similarly, Howard (1917) reported rejection of all *R. ebenus* glochidia within three days on the following species [it is unclear whether these species were tested by Howard or represent fishes tested previously by Surber]: Mooneye (*Hiodon tergisus*); Shovelnose Sturgeon (*Scaphirhynchus platorhynchus*); Bowfin (*Amia calva*); Channel Catfish (*Ictalurus punctatus*); Brown and Black Bullhead (*Ameiurus nebulosus* and *A. melas*); Flathead Catfish (*Pylodictis olivaris*); White and Black Crappie (*Pomoxis annularis* and *P. nigromaculatus*); Green Sunfish (*Lepomis cyanellus*) and Bluegill (*L. macrochirus*); Largemouth Bass (*Micropterus salmoides*); Yellow Perch (*Perca flavescens*); and Freshwater Drum (*Aplodinotus grunniens*). Together, these represent 21 species in nine families that are unsuitable hosts. Previously, Howard (1914) reported limited natural infestations of *R. ebenus* (1–4 glochidia/fish) on a single Largemouth Bass, two individuals of White Crappie, and a single Black Crappie among thousands of fishes examined. He attributed these infections either to incidental attachments on non-suitable species or misidentification of glochidia (see previous), and subsequent work failed to confirm these fishes as hosts.

Except for Skipjack Herring, the only other fish species that was not clearly an unsuitable host for *R. ebenus* was Gizzard Shad (*Dorosoma cepedianum*), which, like *Alosa* spp., is a member of the family Clupeidae. In our study, Gizzard Shad retained glochidia slightly longer than other species, but even these fishes appeared to have rejected most or all glochidia by day 7. Howard (1917) also reported that Gizzard Shad "gave some indications of being favorable [hosts]", but these indications were not specified, and the results were considered inconclusive. Howard (1917) further suggested that the very fine gill rakers of this species (an adaptation for filter feeding) might prohibit natural infestation of the species by mussel glochidia. Many mussel species that specialize on a single species or genus also show marginal use of other species within the same family. For example, glochidia of *Hamiota altilis* and *Villosa vibex* metamorphosed robustly and consistently on black basses (*Micropterus* spp) but showed low and inconsistent metamorphosis on Green Sunfish (*Lepomis cyanellus*), all of which are in the family Centrarchidae (Haag et al., 1999). We suspect that the equivocal results for Gizzard Shad are a manifestation of the same phenomenon, and at best, this species is a marginally suitable host for *R. ebenus*.

The conclusion of strict specialization on *Alosa* spp. is also supported for *E. crassidens*. In our study, Bluegill clearly was an unsuitable host, but we were unable to test other species. However, Hauswald (1997) infected 20 fish species in seven families (Catostomidae, Centrarchidae, Clupeidae, Cyprinidae, Ictaluridae, Percidae, and Sciaenidae) with glochidia of *E. crassidens* and found no evidence of host suitability on any species, including Gizzard Shad.

Regardless of the degree of specialization, the robust production of juveniles of *R. ebenus* and *E. crassidens* by *Alosa* spp. shows that these fishes are important for maintenance of populations of both mussel species. Surber (1913) examined natural glochidial infestations on dozens of fish species (for many mussel species), but the highest percentage of infected fishes and among the highest intensity of infestations he observed were of putative *R. ebenus* on Skipjack Herring (5 out of 5 fishes infected during the apparent peak in glochidial release in August; 1895–3740 glochidia/fish). The single observation of *E. crassidens* on Skipjack Herring also was reported to be a heavy infestation (Howard, 1914). These apparently characteristic, heavy infestations demonstrate the great juvenile production potential of *Alosa* for these mussel species, and the absence of heavy infestations on other fishes suggests that these mussel species have highly efficient glochidial transmission strategies that target *Alosa*. However, transmission strategies for these species are unknown.

Our confirmation of *Alosa* spp. as hosts for *R. ebenus* and *E. crassidens* supports the idea that dams and other river modifications that interrupt herring migrations are a key factor in the decline of these mussel species (Kelner and Sietman, 2000; Gangloff, 2003; Haag, 2012). Many streams continue to harbor large populations of both species that consist mainly of individuals of advanced age with little or no evidence of recruitment in the last 50 years (e.g., Hauswald 1997). These remnant populations can be expected to disappear unless herring migrations can be restored. Where dam removal is impractical, changes in dam operation or infrastructure may facilitate increased fish passage and restoration of herring migrations. For example, "attraction flows" created by pumps in lock chambers were successful in allowing upstream migration of Alabama Shad on the Apalachicola River (Ely et al., 2008). In addition to Skipjack Herring and Alabama Shad, interruption of Alewife (*Alosa pseudoharengus*) migrations by dams appears responsible for decline of the Alewife Floater (*Anodonta implicata*) and perhaps other mussel species of the Atlantic Coast (Smith 1985). Other Atlantic Coast species of *Alosa* (*A. aestivalis*, *A. mediocris*, and *A. sapidissima*) should be considered as potential hosts for mussel species that have declined in regulated rivers.

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