Novel experimental technique to confirm fish hosts for "large river mussels".

by

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Abstract

Unionid mussels are declining in North America. The larval stage (glochidium) represents a life-history bottleneck because glochidia are parasitic and require an appropriate fish host. However, appropriate fish host species for many mussel species are still unknown, making it difficult to implement effective conservation measures. Large-river migratory fish are suspected to be important hosts for mussels, but are difficult to work with and few have been positively confirmed as hosts in laboratory trials. I applied standard fisheries techniques to successfully capture, haul, and hold two problematic, large-river fish species; *Alosa chrysochloris* (skipjack herring) and *Alosa alabamae* (Alabama shad). I describe a new technique (gill excision) which facilitated the use of problematic fish for host assays and allowed me to provide the first laboratory conformation of fish host identity for *Reginaia ebenus* and *Elliptio crassidens*, as well as confirming that glochidia can successfully metamorphose from dead fish gills.

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Introduction:

Freshwater Unionid mussels (mussels) have a complex reproductive strategy in which parasitic glochidia require a vertebrate host (usually fish) to metamorphose into a juvenile mussel. Because of this host requirement, the larval stage represents a bottleneck that limits many mussel populations (Strayer 2008). The regional distribution of mussel species is linked to the distribution of their host fish (Watters 1992, Schwalb et al 2011, Schwalb et al 2013, Haag and Warren 1998). Within a region, extinction and recolonization rates of local mussel populations are strongly impacted by larval life-history traits (Vaughn 2012). Within a site, coexistence or competitive exclusion of mussel species with shared host taxa may be driven by differing efficiencies in host infection and relative abundance of shared host taxa (Rashleigh and DeAngelis 2007). Host specificity can affect how a mussel population responds to a decrease in abundance, or complete loss, of specific fish-host species. Some mussel species are generalists, able to utilize a wide range of fish taxa as hosts, whereas others are specialists, able to utilize only a small number of closely related fish taxa. Therefore after the loss of a specific host species, generalist mussels can continue to utilize remaining fishes as hosts, whereas a specialist that loses its host species will not be able to reproduce, persisting until the population succumbs to old age (Douda et al. 2012).

A major contributing factor in the decline of freshwater unionids is the construction of dams and other impoundments on natural river systems (Vaughn and Taylor 1999; Watters 1996). Impounded reaches in a river system can lead to lower fish species richness when compared to free flowing sections (Santucci et al 2005) due to disruption of migratory patterns.

It has long been recognized that dams block migration of anadromous fish (Coker 1930), and populations of many migratory fish are not nearly as abundant and as widespread as they once were, particularly in heavily-dammed river systems (Ely et al 2008). This may not create a bottleneck for generalist mussels that can continue to utilize the remaining fish species. However, specialist mussels lose the ability to recruit if their required host is eliminated from the local fish community. Many mussel species that are suspected to be specialists on large-river migratory host fish have suffered declines above dams and some have even been extirpated (Kelner and Sietman 2000, Haag and Warren 2010, Hinck et al 2012).

Reginaia ebenus (ebony shell) is a large-river mussel typically found at depths between 4 to 7 meters in sandy gravel substrate and areas of swift current protected from excessive silt deposition (Williams et al 2008). It is a short-term brooder (tachytictic), typically gravid between April and September (Surber 1912; Howard 1914a; Coker et al. 1921), and suspected to use *Alosa chrysochloris* (skipjack herring) as its host fish based on observations of naturally infected fish (Watters 1994; Surber 1913; Howard 1914b). This host relationship, however, has never been confirmed in a controlled laboratory setting.

Skipjack herring is a euryhaline species typically found in deep, clear waters with swift current (Boschung et al. 2004). It is native to the Gulf of Mexico and associated river drainages, and migrates upriver in spring in order to spawn between March and April (Etnier and Starnes 1993). This species has historically been found in the Mississippi River drainage as far north as Minnesota. However, dam construction in the Mississippi River has severely impeded its migration routes, reducing populations in the upper Mississippi River (Kelner and Seitman 2000; Neebling and Quist 2008). Skipjack herring are now considered critically imperiled in Wisconsin and of special concern in Minnesota, where they were considered extirpated for many

years (Natureserve 2013). In Alabama, skipjack herring have been collected in nearly every river system and are considered secure (Mettee et. al. 1996, Natureserve 2013)

The distribution of ebony shell mirrors that of skipjack herring. Previously abundant in the upper Mississippi River system, populations that are now cut off from their presumed migratory host species are in danger of extirpation (Kelner & Sietman 2000; Coker 1930; Natureserve 2013). This pattern is not limited to the northern states. In the Mobile River basin of Alabama, the historic distribution of ebony shell included the main stem of the Coosa River up to Three Island Shoals. Today, however, ebony shell can no longer be found in the Coosa River above Jordan Dam (Gangloff 2003); presumably due to the inability of skipjack herring to migrate upstream past hydroelectric dams. However, ebony shell is still common in the Coastal plain reaches of large Mobile Basin Rivers (downstream of hydroelectric dams) where it is typically the most common mussel species (Lydeard et al 1999, Williams et al. 2008) and is considered stable in Alabama (Williams et al. 1993).

Although ebony shell is assumed to be a specialist, and declines of the species are generally assumed to be associated with the interference of skipjack herring migratory patterns (Payne and Miller 2000), the relationship between ebony shell and skipjack herring has never been confirmed with laboratory trials. Nor has any other fish host for ebony shell been confirmed with laboratory trials (Howard 1914c). Skipjack herrings are very difficult to transport and hold in captivity, and, to our knowledge, have never been successfully utilized in laboratory host trials for any mussel species. For example, in a previous study looking at potential host fish for *Elliptio crassidens,* Hauswald (1997) was unable to keep skipjack herrings alive in transit, much less utilize this species in laboratory trials. In this study, I hypothesize that ebony shell is a specialist and utilizes skipjack herring as its primary host.

My objectives were as follows:

1) Evaluate the efficacy of standard fisheries techniques to capture and haul skipjack herring from river to laboratory with minimal mortality.

2) Evaluate techniques to infect and hold skipjack herring during host trials.

3) Evaluate the host specificity of ebony shell by testing the suitability of 14 fishes, across 7 families, as hosts.

Methods:

Collection and holding of study animals

Collection of an array of potential host fishes and gravid unionids presented logistical challenges related to time of availability, and acclimation needs. Stream fishes were most easily collected in summer and early fall to avoid high water events associated with spring and late fall. Skipjack herring were most easily collected in early spring, when they concentrated below dams during upstream spawning migrations. Ebony shell do not become gravid until late spring/early summer and could only be collected during low river stage. Short term brooders such as ebony shell typically abort their broods when disturbed or handled. Thus the full complement of fish species to be tested had to be acclimated to laboratory conditions and ready to use as soon as ebony shell were collected. This entailed a lengthy period of collection and holding in the lab for most species.

Specimens of 14 fishes of 7 families were collected for use in laboratory host trials. pretty shiners (*Lythurus bellus*), fathead minnows (*Pimephales promelas*), blackspotted topminnows (*Fundulus olivaceous*), orangefin shiners (*Notropis ammophilus*), largescale stonerollers (*Campostoma oligolepis*), striped shiners (*Luxilus chrysocephalus*), and darters (*Etheostoma* sp. and *Percina* sp.) were collected via seining and backpack shocking from

Hillabee and Choctafaula creeks which are tributaries of the Tallapoosa river. Ebony shell is not known to be present in these streams (Williams et al 2008, Gangloff 2003). All fish were collected in fall, 2010, returned to the lab in aerated coolers and held in a recirculating aquaria system with dechlorinated tap water at approximately 18 °C for ≥ 7 months.

Bluegill (*Lepomis macrochirus*), and largemouth bass (*Micropterus salmoides*) were collected via electroshocking from a water supply reservoir at the E. W. Shell Fisheries Research Center (Auburn University) that has few to no mussels present (personal observation). Channel catfish (*Ictalurus punctatus*, "Marion strain") were obtained from a separate experiment on the North Auburn Fisheries Station. Bass, bluegill, and catfish were held in insulated rectangular fiberglass tanks (Length 59" x Width 21.5" x Height 14") with a baffle dividing the length in order to create circular water flow. The tanks were filled with dechlorinated tap water and maintained at approximately 21°C. Fish were fed frozen brine shrimp, bloodworms, flake food, commercial fish pellets and fathead minnows as appropriate. Water quality (Ammonia, Nitrite, and Nitrate) was measured at least once per week using Tetra® EasyStrips. These fish were held for ≥ 7 months prior to being used in experiments.

The large river fish species tested in this study; freshwater drum (*Applodinotus grunniens*), gizzard shad (*Dorosoma cepedianum*), and skipjack herring (*Alosa chrysochloris*), are seldom utilized in host studies due to challenges associated with collecting and holding them in captivity. In this study, all three large-river species were held in large, outdoor, fiberglass tanks prior to host trials. Two of the tanks were rectangular (3.5m L x 1.8m W x 0.9m H,) with a volume of 5,700 liters). The rectangular tanks were equipped with a central, longitudinal baffle and airlifts were used to generate a circular water flow (Fig. 1a, b). The third tank was circular (diameter = 3 meters, depth = 1.2 meters) with a volume of 8,500 liters (Fig. 2). Internal bio

filters were installed in both tanks to maintain water quality. The circular tank was part of a larger recirculating system that had a total volume of 23,000 liters. Water was pumped into the circular tank from an external biofilter with a volume of 2,300 liters and provided circular flow around the central baffle. The rectangular tanks were located under an awning whereas the circular tank was located a short distance away in direct sun.

Freshwater drum were collected via hook and line from Lake Guntersville in Guntersville, AL during December of 2010 and hauled back to the lab in a 3,600 liter rectangular fish hauler filled with water from the catch site. Upon return to the South Auburn Fisheries Research Station they were held in one of the 5,700 liter rectangular tanks at a stocking density of 2.64 fish/m^3. The tank was filled with dechlorinated tap water and water quality was monitored weekly for ammonia, nitrite, and nitrate using Tetra® EasyStrips. Drum were held in these tanks for ≥ 6 months prior to infection and maintained on a diet of *Corbicula* and crayfish.

Gizzard shad were collected via electroshocking from the Chattahoochee River below Eagle Phenix Dam in Columbus, GA. Gizzard shad (5) were returned to the lab on March 15 2011 in a 3,600 liter rectangular fish hauler filled with river water at the collection site and salted with Kent Marine Sure-Haul and sodium chloride according to product directions. Gizzard shad were subsequently held in the round tank salted to 3-5 ‰ with Morton Pool Salt for ~12 weeks prior to infection. They were not directly fed during this time although fathead minnows were added to the tank as food for co-occurring skipjack herring (see below), and they may have preyed on minnows during this time. Also, detritus from occasional algal blooms had settled on the bottom and served as a food source for gizzard shad.

To my knowledge, skipjack herring have never been successfully utilized in previous host trials due to the inherent difficulty of handling and holding this species. Thus, methods for

this species will be described in more detail compared to the other two large river fish species utilized in this study. Skipjack herring were collected from the Chattahoochee River just below Eagle Phenix Dam in Columbus GA and identified according to Boschung et al 2004). In our initial collecting and hauling attempt we coordinated with the Georgia Department of Natural Resources to obtain fish that were collected as part of an annual fish survey. During this initial collection, I noted that electroshocking was not very effective for skipjack herring capture as the fish were nearly always deep and immediately sank rather than floating to the surface. After several hours of shocking I had only collected 6 fish. I also noted that the 3,600 liter rectangular hauling tank was not suitable for skipjack herring. Upon arrival at the lab (~ 1 hr. hauling time), two of the six skipjack herring were dead, and the surviving fish had lesions near their mouths and along the sides of their bodies, presumably from beating themselves against the tank corners and walls.

For subsequent attempts (March 24th and 29th), I therefore switched to collecting skipjack herring with hook and line, using small white jigs. To reduce physical trauma to the fish during transport caused by swimming into corners and sloshing of water, I switched to using a 1900 liter, round, fish hauler loaned from the Warm Springs Fish Hatchery (Fig. 3). This tank was filled with water close to the top and sealed with a water-tight lid. This kept sloshing to a minimum which has been shown to be important in reducing stress in hauled fish (Winkler 1987). Skipjack herring were reeled to the boat as quickly as possible and immediately placed in a 570 liter oval stock tank with no corners or protrusions. The stock tank was situated on the deck of the boat and was filled with water from the collection site. Within ~30 minutes of capture, skipjack herring were transferred to the round hauling tank filled with river water at the boat ramp. In order to reduce the amount of osmoregulatory stress experienced by the fish

captured for this trial I salted the water using Kent sure-Haul, a commercial product containing a mixture of hauling salts, combined with sodium chloride (Morton Pool salt) to a final salinity of ~5‰. The use of salts in hauling water has been shown to reduce the amount of osmoregulatory stress experienced by hauled fish as well as to help them recover more quickly (Murphy and Willis 1996; Collins and Hulsey 1963; Mazik et al. 1991; Swanson et al. 1996; Wydoski and Wedemeyer 1976, Weirich and Tomasso 1991). Oxygen was gently bubbled into the hauling tank during collection and subsequent hauling. The time of travel from leaving the boat ramp to arriving at the station was approximately 1 hour. Once the skipjack herring arrived at the South Auburn Fisheries Station they were distributed among the large circular tank and the two rectangular tanks. Netting material was placed over both rectangular tanks to keep fish from jumping. The circular tank had a cover made from off-white shade cloth (SYNTHESIS COMMERCIAL 95™) to prevent fish from jumping and to shade the tank and prevent it from overheating. All tanks were filled with dechlorinated tap water and sodium chloride was added to maintain 3-5 ‰ salinity (Morton Pool salt) in order to lessen the stress from transferring fish from hauling tank to holding tank water. Water quality was monitored biweekly in these systems and partial water changes were implemented whenever ammonia rose above 0.5 mg/L or nitrites rose above 1mg/L. Skipjack herring were fed fathead minnows biweekly.

Gravid ebony shell were collected from the Alabama River in Wilcox County on June 15 and 24 of 2011. Mussels were collected using a hookah breathing apparatus (Keene engineering) and tactile searching of the substrate at approximately 1.5 to 2.5 meters deep. Mussels were checked for gravidity on the bank of the river by gently opening the shell with reverse pliers and looking for swollen creamy white or pink to red gills (Williams et al 2008), and gravid individuals were transported to the laboratory (approximately 2.5 hours travel time) in a cooler

with a small amount of ice. On June 6th, 18 of 31 collected mussels were transported wrapped in moist paper towels, while the remaining 13 mussels were transported in individual gallon Ziploc® bags of river water. Upon arrival at the laboratory, the bags were immediately submerged in a recirculating aquarium system maintained at 18°C and an airstone was placed into each individual bag. Mussels wrapped in paper towels were kept moist and cool. All mussels were held overnight prior to use in host assays. Mussels collected on June 23 were all transported in Ziploc bags in coolers with a small amount of ice, and held overnight in the same manner as for the previous collection.

Host Trials

Approximately one week before trials, fish were transferred to their respective trial tanks to acclimate. Trial tanks were filled with dechlorinated tap water and salinity was gradually decreased to < 0.5 ‰ during the acclimation period. Trials for all fish species other than freshwater drum, gizzard shad, and skipjack herring were conducted in Aquatic Habitat (AHAB) research tanks of two sizes (1.5 L, and 3 L) according to individual fish size. AHAB tanks were held within two, independent, recirculating systems holding 36 tanks each. Within each recirculating system, water flowed out of each tank through a 105µm filter cup to retain sloughed glochidia and juveniles (Fig. 4), and into a sump/biofilter. From there water was subjected to coarse mechanical filtration and ultraviolet light sterilization. After sterilization, water passed through a secondary 100µm filter to catch any stray glochidia or juveniles that had bypassed the previous filters. Water then flowed into a manifold system that distributed water into each tank to start the cycle over again. Water temperature was maintained at 18-21° C by means of an in-line chiller. Water quality was monitored from a sample collected from the sumps weekly using

Tetra® EasyStrips for ammonia, nitrites, and nitrates with water changes being performed as needed.

Freshwater drum trials were conducted in a cone tank system (Fig. 5) that consisted of 4 cone tanks that were 55 liters in volume with all four outflows going into a 100 liter sump/biofilter. The gizzard shad trials were conducted in a similar system consisting of 4 cone tanks that were 150 liters in volume and emptied into a common 200 liter sump. Water was pumped from the common sumps through a manifold back into the cone tanks. The inflow line for each tank was angled against the side of the cone tank to create a circular flow. The cone tanks were fitted in a double stand pipe configuration which allowed the water exiting the cone tanks to come from the bottom. This water then flowed out of each individual cone tank though a drain pipe and into a 100µm nylon filter sock attached to the outflow of each drain pipe to catch any sloughed glochidia or juveniles. Cone-tanks were held at ambient room temperature (19-21^{o} C), and water quality was monitored in each cone tank system bi-weekly using Tetra® EasyStrips for ammonia, nitrites, and nitrates.

Prior to using the cone tank systems for the gizzard shad and drum we tested their suitability for skipjack herring. All skipjack herring held individually in 55 or 150 liter cone tanks died within 24 hrs. I therefore decided to hold infected skipjack herring in the previously described rectangular outdoor tanks. Partial water changes with dechlorinated tap water were used to reduce salinity in these tanks during the acclimation period. The water temperature range for the skipjack herring tanks during the trial was 24-26°C and was monitored throughout the trial using a HOBO™ Pendant temperature data logger. Water quality was monitored in each tank weekly for ammonia, nitrites, and nitrates using Tetra® EasyStrips, and water exchanges were performed as needed.

Although all of the mussels returned to the lab had swollen gills, indicating fertilization, the stage of embryonic development was unknown. Therefore, on the day following collection, I extracted a small amount of fluid from a gill of each mussel using an 18 gauge needle and examined the contents under 10X magnification. Embryonic stage was placed into one of four categories: 1) fertilized eggs (observable cell division, but not developed into glochidia), 2) early stage glochidia (embryos shaped like glochidia but not fully formed), 3) free glochidia (fully formed, not bound in membranes), or 4) bound glochidia (fully formed, enclosed in a membrane). Only broods dominated by free glochidia, were utilized for host trials. Glochidia for host trials were collected by flushing excised gills into a beaker, using a syringe with a 20 gauge needle and artificial freshwater (AFW). Glochidia viability was evaluated by means of a salt test (Fritts et al. 2014). Glochidia in the beaker were diluted to 2000 ml with AFW. The solution was mixed with a plunger to suspend glochidia, while avoiding the creation of a vortex, and 100 µL subsamples were withdrawn and placed on a counting tray. Subsamples were examined under a dissecting microscope at 10X and the number of open and closed glochidia was counted for each subsample. A few drops of 35‰ artificial saltwater (Instant Ocean® Sea Salt) were added to each subsample, and the number of open and closed glochidia in each subsample was recorded for a second time. Glochidial viability was recorded as the proportion of glochidia snapping shut after addition of salt water:

Proportion of viable glochidia) = [(number open initially) – (number open after NaCl)] ÷ (total number of glochidia).

The total number of viable glochidia in a given brood or batch (multiple broods combined) was calculated as:

$$Vn = G/s * V * P$$

Where Vn = total number of viable glochidia in a brood or batch, G = number of glochidia (open plus closed) in each subsample, s = combined volume of subsamples (ml), V = sample volume (ml) and P = proportion of viable glochidia.

Skipjack herring were infected on June 16, 2011 and all other fish were infected on June 24, 2011. All fish species were inoculated by immersion for 15 minutes in an aerated glochidial bath of dechlorinated tap water containing 2,000 glochidia / L. Skipjack herring were infected in a bath made using combined broods of 4 mussels, while all other fishes were infected using baths made from the combined broods of 2 mussels. Multiple fish were held within a single glochidial bath, and any given glochidial bath was only used once. Bath volume varied according to fish size and sensitivity to handling. Fishes to be tested in AHAB tanks were inoculated in 2 L beakers or 19 L buckets. Drum and gizzard shad were inoculated in large, 140 L coolers with the lids closed during inoculation. Skipjack herring were inoculated in large, round tanks covered with shade cloth material and containing 570 L of water from the tanks in which they were housed. After the standard 15 minute inoculation period, fish were returned to the same host trial tanks to which they had been acclimated (see previous section).

To determine whether glochidia were successfully encapsulated on host fish, gills of all fish species except for skipjack herring were analyzed for presence or absence of encapsulated glochidia 4-6 days post infection (Zale and Neves 1982). One fish from each of the other species was anesthetized using a bath made with AQUI-S 10 at the recommended rates specified by the manufacturer (5-10ml/100L). Both sets of gills were then, inspected under a dissecting microscope at 10-15X by gently prying back the operculum. Individuals of a given species were inspected sequentially until one fish was observed with encapsulated glochidia. Once a fish had been inspected it was placed into a bath of fresh water and allowed to recuperate from the

anesthesia and handling event before being placed back into their respective tanks. Because I saw no evidence of encapsulated glochidia on any fish of any species other than Skipjack herring (Table 2), I did not monitor for sloughed glochidia or juvenile production after Day 6.

Because of their sensitivity to confinement and handling, skipjack herring were housed in large communal tanks and were not examined for glochidial encapsulation within the first week of infection. To check for juvenile production, I siphoned a 3.2 m^2 portion of the bottom of each communal tank on day 10 post infection. Siphoned water was passed through a 100µm filter sock to collect sloughed glochidia and/or juveniles and filtrate preserved in 75% ethanol. Subsamples were examined under a dissecting microscope equipped with cross-polarized lighting (Johnson 1995). Even with the use of cross-polarized lighting, it quickly became apparent that detection of juvenile mussels would be very difficult due to the sediment, detritus, and fish waste accumulating on the tank bottoms.

Therefore, on day 11 a single skipjack herring (TL 37.5cm and WW 305.6g) was removed from one of the tanks to inspect the gills for encapsulated glochidia and determine if subsequent siphoning was warranted. The fish was anesthetized in a 140 L cooler dosed with AQUI-S 10 (5-10ml/100L) to minimize stress during handling. Gills were inspected in the same fashion as for the other species and the fish was placed in an aerated water bath free of AQUI-S 10 to recuperate from the anesthesia. However, the fish never recovered and died within 5 minutes of being placed in the recuperation bath. Because I had observed encapsulated glochidia, I immediately excised its gill arches and preserved half of them in 70% ethyl alcohol for documentation. The remaining gill arches were placed in a 1.5 L AHAB tank within the same system used for the non-large river fish species, with a 105 µm filter cup situated under the outflow of the tank. The filter cup was examined every 1-2 days for sloughed glochidia and/or

juveniles for 8 days post excision. Juveniles were differentiated from sloughed glochidia by valve movement and the presence and movement of a foot. The remainder of the skipjack herring from both tanks were inspected for encapsulated glochidia at 19 days post infection.

Because I had observed encapsulated glochidia on Day 11, I siphoned a separate 3.2 m^2 portion of the tanks on Day 12 post infection and then siphoned the entire tank bottoms on Day 19. The Day 12 sample contained a substantial amount of detritus, and was preserved in ethanol for later analysis. The Day 19 sample was relatively clean since the entire tank bottom had already been siphoned once, and I examined this sample immediately for active juveniles rather than preserving it.

Results:

The combination of hook and line sampling, use of a circular hauling tank, use of hauling salts, and provision of bubbled oxygen, proved very effective for the successful capture and transport of skipjack herring. Using these techniques, survival from capture to arrival at the lab ranged from 96% (23/24 fish) to 86% (25/29 fish) (Table 1). Using the initial technique of electroshocking and hauling in a rectangular hauler, I captured only 6 skipjack herring with 4 surviving the transport to the lab. It was noted that the fish hauled in the round hauling tank upon arrival at the lab looked better and had fewer lesions on their bodies than fish hauled in the rectangular tank.

Water quality monitoring showed that our large, outdoor recirculating system had high nitrite levels (>10ppm) for roughly 13 days after fish were introduced into this system. Through the use of frequent water changes using dechlorinated tap water (approximately 10% of total system volume 2-3X per day) throughout this initial period nitrite levels were brought down to around ~3ppm and continued to decrease towards zero as the biofilter became established. To

minimize stress and effects of high nitrites I maintained a salinity level of around 4-5‰ using sodium chloride (Boyd 1990)

Feeding activity of the skipjack herring varied between the two different tank types used. In the rectangular tanks located under the awning actively feeding skipjack herring were seldom witnessed, while in the large round tank located in the direct sun, skipjack herring were frequently observed swimming up and grabbing a minnow before returning to the depths of the tank while a portion of the cover was lifted during feeding events.

Results from host trials

Five of the mussels collected on June 16th and held in bags of creek water released a portion of their gill contents while being held overnight. Gill extractions the following day on all mussels revealed 12 with fertilized eggs that had not yet developed into glochidia, 8 with early stage glochidia, and 11 mussels with free, mature glochidia. Broods were randomly assigned to one of three batches consisting of 4, 4, and 3 combined broods respectively. The first batch exhibited a salt test viability of 85% whereas the subsequent batches exhibited declining viabilities 52% and 41%. Only the first batch was used to infect skipjack herring. Because the skipjack herring required a large volume glochidial bath, there were not enough glochidia left over to infect additional fish species.

Ten of the 20 mussels collected on June 23rd released at least some of their gill contents while being held in bags overnight. Three mussels broods were dominated by fertilized eggs, 6 broods were dominated by glochidia in membranes, 2 broods were dominated by free, mature glochidia, and the remaining broods were released as conglutinates. The two broods dominated by free, mature glochidia were combined to create glochidial baths to infect all fish species other than skipjack herring. Glochidial viability of baths made from these two broods was 67%.

Gill inspections (Table 2) performed at 4-6 days post infection on all species other than skipjack herring revealed no individuals with encapsulated glochidia. In contrast, the gill inspection performed on the single skipjack herring on Day 11 post infection revealed numerous encapsulated glochidia (Fig. 5). The excised gills from this fish produced 1,160 active juveniles over the course of 8 days (Fig. 6). Gills of the remaining 7 skipjack herring in the communal holding tank that were examined on Day 19 revealed no encapsulated glochidia and thus were never excised and held for juvenile production (Table 2).

Siphoning of the tanks prior to Day 11 revealed no sloughed glochidia or juveniles, but the large amount of detritus on the tank bottoms prevented an accurate analysis of the samples. Samples collected via siphoning after Day 11 were cleaner but still difficult to analyze. However I did observe one juvenile with its foot extended from samples taken on day 19 post infection indicating that juveniles were successfully produced by live fish in the communal tanks.

Discussion:

As far as I am aware (Science Citation Index; keywords: *Fusconaia ebena*, *Reginaia ebenus*, host study, life history, skipjack herring, *Alosa chrysochloris*), this study represents the first successful collection, holding, and use of skipjack herring for a mussel host study. Previous researchers either didn't try to use skipjack herring due to difficulty in holding this species (Surber 1913) or were unsuccessful in their attempts. For example, although not explicitly described in Hauswald (1997), the author hauled skipjack herring in rectangular coolers filled with river water (no hauling salts) with aeration. All skipjack herring died before reaching the lab (Hauswald personal communication).

It is very important to minimize stress during the process of capturing, hauling, and holding of fish for research. Stress takes many different forms and can have many different

effects. Some fish tend to swim into tank corners and cause physical harm to themselves as they beat against tank walls. Stress associated with temperature shock can suppress the immune system (Noga et al 2000). Stress associated with handling or capture, can cause adrenaline to be released into the bloodstream, ultimately leading to loss of ions in the blood and problems with maintaining osmoregulatory homeostasis. (Ports et al. 2006). Stress can also increase oxygen consumption rates and deplete energy reserves (Barton and Schreck 1987; Harmon 2009). Scope for Activity (SFA) is the energetic difference between standard and active metabolism (Fry 1971; Fry 1947) and represents the energy available for growth, reproduction, swimming, and dealing with stress. Stress depletes the SFA, leading to a negative feedback between stress and energetic health (Barton and Iwama 1991).

The high survival of skipjack herring through standard hauling and holding procedures demonstrated that they are not as sensitive to handling as commonly assumed. In this study, we collected fish by hook and line rather than using standard electroshocking techniques because it was a much more efficient way to collect large numbers of skipjack herring (see Methods). I was concerned that handling associated with landing of fish would be more stressful than electroshocking. However, all fish collected in this manner survived collection, holding in the boat, and transport to the on-shore hauling tank. Subsequent high survivorship showed that skipjack herring can be successfully collected and held for host assays as long as the following standard fisheries techniques are carefully utilized and followed: 1) use of round hauling tanks, 2) use of professional hauling salts, 3) use of bubbled oxygen during hauling, and 4) use of large tanks with circular flow for holding.

One thing I overlooked in this study was the amount of time necessary to establish sufficient bio-filtration in our large outdoor recirculating system. I didn't set up our biofilter far

enough ahead of time, due mainly to freezing temperatures prior to collection of skipjack herring in March. I also salted our large outdoor recirculating system down to match the hauling tank waters' salinity and this caused what little biofilter I had established to crash before it eventually acclimatized to higher salinities. This led to harmful spikes in ammonia and nitrites. In subsequent trials I established the bio-filtration well ahead of time by seeding it with an ammonia solution and adding salt well ahead of the fish collection date so that a salt-tolerant biofilter was already well established by the time I added fish to the system.

Although I was able to successfully hold skipjack herring in large communal tanks, the tanks were not optimal for host studies for several reasons. Air blowers or large pumps are required to maintain constant water flow, generating high utility bills. The tanks themselves are expensive and require a large amount of space, limiting the number available for host studies. Feeding and holding a large number of fish within a tank generates a lot of waste, which makes siphoning juveniles from the bottoms of the tanks problematic. Also, the housing of multiple infected fish in a communal tank prevents the collection of transformation data from individual fish.

Gill excision represents a valuable, new technique to confirm problematic, large-river fishes as hosts for mussels. I was able to produce 1,160 active ebony shell juveniles from half of the excised gills of a skipjack herring, demonstrating that it is a viable technique. The main benefits of this technique are that it allows problematic large river fishes to be successfully utilized in host trials using a minimum number of large tanks, it allows for the collection of transformation data from individual fish, and it allows for the collection of data from fish dying during traditional host-trials that would otherwise simply be discarded. Future studies demonstrating the success of this technique across a range of fish taxa, and comparing "live gill"

to excised gill production are warranted. Also, research is needed to determine the length of time glochidia need to develop on gills before they are competent to metamorphose from excised gills.

This study represents the first laboratory determination of a host species for ebony shell. It appears to be a specialist on skipjack herring based on the evidence from this and previous studies. Surber (1913) attempted artificial infections of ebony shell with a range of fishes obtained in the vicinity of Fairport Iowa (species list not provided in paper), but with no success. He did not attempt artificial infections with skipjack herring likely due to difficulty of handling. However he was able to obtain specimens of skipjack herring that were naturally infected with glochidia that he identified as ebony shell based on morphology. Howard (1914c) nominally identified the glochidia of ebony shell encapsulated on wild-caught largemouth bass (*Micropterus salmoides*), black crappie (*Pomoxis nigromaculatus*), and white crappie (*Pomoxis annularis*). However, subsequent attempts to confirm these hosts through laboratory methods ultimately failed. Similar to Howard (1914c), I did not observe successful transformation of ebony shell on largemouth bass. These results illustrate the danger in identifying hosts based solely on observations of wild-caught animals. Glochidia may be misidentified based on morphological characteristics only, and observations of encapsulation do not necessarily indicate successful metamorphosis.

Specialization of ebony shell on a large-river migratory fish species may explain the recent decline and current distribution patterns of this species. In Alabama ebony shell have been extirpated in the Coosa River upstream of Jordan Dam likely due to the inability of skipjack herring to migrate upstream past the dams (Gangloff 2003). The inability of skipjack herring to migrate upstream past dams (Coker 1930) could also explain the existence of relic populations

such as those observed upstream of Lock and Dam 19 on the upper Mississippi River (Kelner and Seitman 2000). Maintenance and restoration of ebony shell populations will require restoration of longitudinal connectivity within river systems currently fragmented by dams. Although some small dams may be removed completely, other methods to increase fish passage may be more practical in many situations. For example Ely et al. (2012) showed that "attraction flow" created by pumps in lock chambers facilitated the upstream migration of Alabama shad (*Alosa alabamae*) through lock and dam systems in the Apalachicola River. This, and similar strategies, may allow for passage of migratory fish species through some existing dams without requiring dam removal or installation of expensive fish passage structures.

Ch. 2) Use of gill excision to identify hosts for *Elliptio crassidens*: metamorphosis success and time of excision.

Introduction:

Dams have been implicated in the decline of many freshwater mussel species native to North America, particularly those which rely on migratory fish species as hosts for their parasitic larvae (Watters 1996; Vaughn and Taylor 1999). Dams facilitate mussel declines by 1) interfering with dispersal potential of mussel larvae when host fish are present but constrained in their historic migration patterns (Watters 1996), and 2) by reducing or eliminating recruitment altogether when preferred host fish are prevented from migrating past dependent mussel beds (Douda et al. 2012; Hinck et al. 2012; Larinier 2000). In some cases loss of fish hosts results in the creation of relic populations: populations of large, old individuals with little to no recruitment that persist until they die out of old age.

Elliptio crassidens (elephantear) (Lamarck, 1819) is a freshwater mussel species found in creeks and rivers with a moderate current (Heard 1979). In larger impounded rivers it is typically found in the tail waters of dams as well as stable areas composed of firm sand and mud where there is little silt deposition (Williams et al. 2008). It is a short-term brooder (tachytictic), reported to be gravid from April to June in the Mississippi River (Coker et al., 1921) and from April through August in the Apalachicola Basin (Brimbox and Williams, 2000; O'Brien et al., 2003). Similar to *Reginaia ebenus* (ebony shell; Chapter 1) recruitment of elephantear populations is thought to be dependent on availability of large-river, migratory fish hosts. *Alosa chrysochloris* (skipjack herring) is a suspected host based on historic observations of natural infection (Howard 1914a). It is typically anadromous but can complete its life cycle in fresh water if blocked from the sea (Etnier and Starnes 1993), spawning from early March through late

April on sand and gravel bars within large rivers. *Alosa alabamae* (Alabama shad), a congener of skipjack herring, has also been suggested as a host species (Hauswald 1997). Alabama shad is an anadromous fish species that migrates upriver from late February through April to spawn in habitats with coarse sand /gravel substrates and moderate current.

Historically, elephantear was widely distributed in North America, reaching from Pennsylvania west to Wisconsin and from Quebec Canada all the way south to the panhandle of Florida (Parmalee and Bogan 1998). In the early 1900's it was so abundant in certain reaches of the Cumberland River that it was considered a nuisance species due to its shell being unsuitable for use in the button industry. Culled shells from the button industry were so abundant, they were considered for use as flux in metal foundries along the river (Wilson and Clark 1914). Since that time, elephantear has declined in the northern portion of its range (Natureserve 2014).

The northern decline of elephantear is thought to be due in large part to the inability of its host fish to migrate northward through numerous dams and impoundments. In Minnesota, skipjack herring populations declined with the initiation of dam construction along the Mississippi River in 1913. The species was considered extirpated from Minnesota after 1928, but has been collected in low numbers since 1986 (www.dnr.mn.us; Bell Museum of Natural History, fish collection database). Skipjack herring is currently endangered in Wisconsin (www.dnr.wi.gov) and considered "unrankable" in Missouri (http://mdc4.mdc.mo.gov/applications/mofwis/Mofwis_Summary.aspx?id=0100086) due to a lack of information and/or substantially conflicting information about status or trends. Alabama shad were previously numerous enough to support commercial fisheries as far north as Iowa, but are now considered globally imperiled (Natureserve, 2014). Alabama shad are currently depleted in most of the Mississippi River basin (Lee et al. 1980, Page and Burr 1991) and have been extirpated from

Iowa (Harlan and Speaker 1969). Missouri still hosts some of the last spawning populations in the Mississippi River (Pflieger 1997) but the species is not abundant and considered to be imperiled in the state (Missouri Natural Heritage Program). Similar to its suspected hosts, elephantear is now endangered in Minnesota, Missouri, and Wisconsin, and is threatened in Illinois (Cummings 2011, USFWS 2006). In Alabama, elephantear and skipjack herring are still abundant in the Tennessee River and parts of some other drainages (Williams et al. 2008; Natureserve 2014), but Alabama shad are rare (Mirarchi et al 2004).

Although circumstantial evidence suggests that the decline of northern elephantear populations is due to dams and impediment of suspected host species migration, elephantear has never been successfully produced in host trials from any fish species (e.g. Hauswald). This is likely due to elephantear being a host specialist: they do not transform on easily cultured fish species and their suspected hosts have never been successfully utilized in host trials due to difficulties in handling and holding. In the previous chapter, I demonstrated that skipjack herring could be successfully collected and held if established fish hauling protocols were scrupulously utilized and followed. I also demonstrated the utility of a promising new technique – gill excision – for conducting host trials with problematic fish species.

The success of this technique raised further questions regarding its viability for other fish and mussel species, as well as the optimal time during which gills should be excised in order to ensure transformation of encapsulated glochidia. There is presumably a "window of opportunity" in which the glochidia are competent enough to metamorphose but have not all dropped off of the gill tissue. Prior to this time, glochidia may not be sufficiently developed to metamorphose from dead gills. In this chapter I describe my attempts to refine the gill excision

technique using elephantear and two of its suspected host fish species: skipjack herring and Alabama shad.

Objectives were as follows:

1) Determine if the standard fisheries techniques successfully utilized for hauling, holding and infecting skipjack herring will also work for Alabama shad.
2) Determine whether skipjack herring and Alabama shad are viable hosts for elephantear.
3) Determine the period of time within which gills can be excised from a host and produce juvenile mussels.
4) Determine the length of time glochidia will metamorphose from gills after excision.

Methods:

Collection and holding of study animals

We utilized the same large, outdoor holding tanks described in chapter one for this study. In order to establish a functioning biofilter that was already adjusted to enhanced salinity levels prior to stocking fish in the tanks, we filled all tanks with dechlorinated tap water 3 weeks prior to the first fish collection and brought them up to a salinity of 3-5 ppt with Morton Pool salt. Commercially available ammonia (RED MAX brand) was added daily to each tank to maintain a concentration of 3 ppm until a nitrite pulse was observed, indicating the establishment of a functioning biofilter microbial community. Ammonia additions were then halted.

Three species of fish representing 2 families (one suspected host family and one suspected non-host family) were collected for use in this host trial. Bluegill (*Lepomis macrochirus*) were collected at the Auburn University South Auburn Fisheries Research Station (SAFRS) via trapping in a reservoir that did not contain mussels. Bluegill were held in a fiberglass tank (Length 1.5m x Width .55m x Height .35m) with a volume of 290 liters and a 52

liter biofilter at ambient room temperature (~20°C) for approximately 1 month prior to infection. Bluegill were fed frozen bloodworms and flake food three times per week and water quality was checked weekly with aquarium water test strips. Water exchanges were performed if nitrates exceeded 40 mg/L.

Alabama shad were collected below the Jim Woodruff Lock and Dam (JWLD) during their annual spring spawning run on March 22, 2012 via electrofishing by the Georgia Department of Natural Resources. The JWLD impounds the confluence of the Chattahoochee, and Flint Rivers. Fish were transported to SAFRS in a 1900 liter, round, fish hauler (Warm Springs Fish Hatchery, Warm Springs, GA) filled with water from the collection site that was treated with Kent Sure-haul and Morton Pool salt to reach a final salinity of ~5‰. Oxygen was gently bubbled into the tank through an ultra-fine pore ceramic plate air diffuser that sat nearly flush with the bottom of the tank to minimize contact with the fish being hauled. Before shad were placed into the round fish hauler, they were weighed and measured by the Georgia DNR as part of a separate study. Water quality parameters (salinity, dissolved oxygen, and temperature) in the hauling water were measured just prior to, during, and just after transport to SAFRS. Length of the trip was 4 hours and 40 minutes. Upon arrival (3:00pm CST) at SAFRS the number of live and dead shad was recorded, and live shad placed into a large 8500 liter round (3m diameter, 1.2m height) fiberglass holding tank incorporated within a larger recirculating system (total volume of ~23000 liters). The holding tank was equipped with a central biofilter constructed from two 120 liter trashcans filled with BIO-FILL™ (Aquatic eco-systems) connected at the bottoms and weighed down with cinder blocks. The internal biofilter had a 2" PVC pipe fitted inside the two trashcans to serve as an airlift inside to provide water flow across the bio filter media. The internal biofilter was utilized for backup bio filtration/aeration in the

case of pump failure for the main system. The internal bio filter also acted as a central baffle that caused the inflow from the larger recirculating system to create a circular current within the holding tank. The holding tank was covered with off-white shade cloth (SYNTHESIS COMMERCIAL 95™) stretched across a PVC frame to prevent fish from jumping out and to keep the system relatively cool in full sun. All of the other tanks within the larger recirculating system were also covered with corrugated plastic roof panels to keep the temperature down and reduce algal blooms. The bio filtration for the overall recirculating system was provided by two external sumps that were filled with BIO-FILL™ (Aquatic eco-systems) bio media and had a volume of 2,300 liters combined (.65m wide x .47m deep x 3.65m long each). Water quality was assessed weekly and partial water changes were implemented whenever nitrates rose above 40mg/L. Alabama shad were fed a biweekly ration of mosquito fish (*Gambusia affinis*) collected from an earthen pond at the SAFRS.

Skipjack herring were collected in late March, 2012 during the annual spring spawning run in the tail waters of the Eagle Phenix Dam in Columbus Georgia. Forty four skipjack herring were captured by hook and line using light colored, plastic grubs and jig heads on March 20. Skipjack herring were quickly reeled in and immediately placed in an oval (~570 liter) stock tank filled with water from the catch site. To avoid overcrowding in the stock tank, skipjack herring were taken back to the boat ramp and placed in a round hauler in batches of 10 or less. The round fish hauler (~1,900 liters) was filled with water from the catch site and salted down with Kent Sure-Haul and Morton pool salt (uniodized) to a salinity of approximately 5‰. Oxygen was bubbled into the hauler using an ultra-fine ceramic plate air diffuser that was flush with the bottom of the tank to reduce contact and injury of the fish being transported. Once all 44 fish had been transported to the hauler they were driven back to the SAFRS (~1 hr. transport time).

Upon arrival at the SAFRS, survival and mortality was recorded and live fish were split between two, outdoor, rectangular tanks (3.5m L x 1.8m W x 0.9m H) situated under an awning. Tanks had a volume of 5,700 liters each and were covered with 1" mesh fish netting to prevent skipjack herring from jumping from the tanks. Both tanks had been drained and thoroughly cleaned of all sediment using a wet-dry vacuum prior to filling. Each tank was fitted with an internal central longitudinal baffle made from lengths of 10" diameter PVC pipes and corrugated plastic roof panels. Airlifts made from lengths of 4" diameter PVC pipe fitted with a 90° fitting at the top horizontal to the surface of the tank produced a circular current around the central baffle. A 920 liter, external bio filter was connected to each rectangular tank via a 1.25" diameter PVC airlift with a distribution pipe leading down into the bottom of the bio filter which caused the water to pass upward through the bio media. The water then flowed back into the main rectangular tank though a 2" diameter PVC overflow. The bio reactors were filled with KALDNES® filter media, and larger bio barrels to provide plentiful surface area for the nitrifying bacteria. A large airstone was affixed to a 2" diameter PVC standpipe running up the center of these bio reactors creating an upwelling current which agitated and aerated the bio media. Water quality parameters were measured weekly and partial water changes (approximately 30-50%) were implemented whenever nitrates rose above 40mg/L. Skipjack herring were fed bi-weekly ration of mosquito fish collected from an earthen pond at the SAFRS.

Elephantear were collected on April 3rd of 2012 from Chewacla creek in Macon County by tactile searching and brought to the bank. Mussels were gently pried open with reverse pliers and identified as gravid if the outer gills appeared creamy white and slightly inflated compared to non-marsupial gills (Britton and Fuller, 1980; Williams et al., 2008). Nine gravid mussels were identified and placed into individual 1 gallon Ziploc® bags filled with creek water

(temperature 21.1°C pH: 6.8). Bags were held in a cooler and transported (~20 minutes) back to the SAFRS. Upon arrival at the laboratory the bags were placed into a recirculating aquarium system maintained at 18°C and an airstone was placed into each bag to provide aeration. Three of the mussels began releasing glochidia within several hours of arriving at the lab and were utilized for host trials that same day. The remaining mussels were held (in bags) overnight in the system. Only two still retained glochidia the following day and were suitable for use in host trials.

Host trials

One week prior to initiation of the host trials, fish were transferred among tanks such that rectangular tank #1 held eight skipjack herring and eight Alabama shad while rectangular tank #2 held seven skipjack herring and seven Alabama shad. These fish were used in the host trials. The remaining fish were transferred to the large circular tank. To avoid negative effects of salinity on glochidia, the salinity of the two rectangular tanks was gradually lowered via water changes over a one week period until salinities were approximately <1 ppt.

To initiate host trials, glochidia were extracted from gravid mussels by puncturing the marsupia with a 22 gauge needle and then flushing out the glochidia (Dodd et al. 2005) into separate beakers brought up to a final volume of 2,000 mL with AFW. Glochidial viability of each brood was evaluated by means of a salt test (Fritts et al. 2014). Each 2,000 mL batch of glochidia was mixed using a plunger type stirrer to keep the glochidia uniformly suspended in the solution while avoiding a vortex. Ten 200 µL subsamples were withdrawn and placed into a 10-well glass plate. Each subsample was examined under a dissecting microscope, and the number of open and closed glochidia recorded. Several drops of 35‰ synthetic salt water were then added to each subsample and the number of open and closed glochidia was quantified again.

The proportion of viable glochidia in each brood was calculated as: [(number open initially) – (number open after NaCl)] ÷ (total number of glochidia)

The total number of viable glochidia in each brood was calculated as:

$$Vn = [(G/S) * V] * P$$

Where Vn = total number of viable glochidia in a brood, G = number of glochidia (open plus closed) in all subsamples combined, S= combined volume of all subsamples (ml), V = total volume of sample (ml), and P = proportion of viable glochidia.

Infection

Fish were infected on two consecutive days. On April 3, 2012 a 560 L (280 L holding tank water plus 280 L dechlorinated tap water) glochidial bath was made to a concentration of 4,000 viable glochidia per liter using the combined glochidia from 3 elephantear broods. Seven skipjack herring, and seven Alabama shad were held simultaneously in the bath for 15 minutes. The bath was covered with 1" fish netting and a layer of shade cloth to minimize disturbance of the fish. Two large air stones provided aeration and helped keep glochidia in suspension. Following the 15 minute holding period, fish were transferred back to the original holding tank and the glochidial bath discarded

On April 4, 2012 a second glochidial bath was made as previously described, and eight skipjack herring, eight Alabama shad, and five bluegill infected simultaneously. Following the 15 minute holding period, skipjack herring and shad were transferred back to their original holding tank. Bluegill were transferred to individual 1.5 L AHAB tanks in a recirculating system. AHAB tanks were filled with dechlorinated tap water and maintained at 18-21°C. Water flowing out of each tank passed through 105 µm filter cups to catch any sloughed glochidia or juvenile mussels, then drained into a common biofilter. From the biofilter, water

was pumped through a coarse mechanical filter fitted with an ultraviolet (UV) sterilization, through a chiller, through a fine (100 µm) filter, and then through a manifold system for redistribution through the AHAB tanks.

To determine the window of opportunity within which glochidia could successfully metamorphose from excised gill tissues of skipjack herring and Alabama shad, we excised the gills of one infected fish/species/tank every 2-3 days from day 5 through day 36 or until we ran out of live fish, whichever came first. To excise gills we sacrificed the fish via quick severing of the spinal cord with scissors. The first two gill arches on each side of the fish were then removed with a pair of scissors and forceps and placed immediately into a 1.5 L AHAB tank that was labeled according to fish species and date of excision.

Gills were not excised from bluegill. Filter cups for all bluegill in the AHAB system were checked for sloughed glochidia and juvenile mussels 2 days post infection and every day thereafter up until day 16. On day 16 all bluegill were removed from their tanks and their gills inspected. Encapsulated glochidia were not observed on any bluegill at this time (see results), but they were returned to their tanks and filter cups were checked again on day 23 as a precaution.

Filter cups associated with the AHAB tanks containing excised gills were checked 1 days post excision and every other day thereafter for sloughed glochidia and juvenile mussels. All filter cups were monitored until no glochidia or juveniles were found during 5 sequential inspections. Filter cups were analyzed by first rinsing the contents of the filter cups into a counting tray and inspecting the counting tray under a dissecting microscope (10X magnification). Juveniles were identified as such 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). Dead glochidia or juveniles were recorded as empty shells.

To determine whether juveniles metamorphosed off of live fish in the holding tanks prior to gill excision, we siphoned a 1 m^2 area of the bottom of tank # 2 twenty five days after infection. Siphoned water was first filtered through a 100 µm filter bag to retain glochidia and juveniles (130-150 µm length x 141-160 µm height). Material collected on the 100 µm filter was then passed through a 200 µm filter screen to remove large particles. The filtrate was then brought to a volume of 125 mL and mixed with a plunger. Juveniles and glochidia were enumerated in 15 consecutive 5 ml subsamples, using a dissecting microscope equipped with cross-polarized lighting to make the shells stand out from sediment and debris. Juvenile density of the holding tank (#/m^2) was calculated by the following formula:

$$D = J * (V/v) / SA$$

Where D = juvenile density; J = total # juveniles counted; V = total volume of sample; v = volume of subsamples; and SA = surface area of holding tank sampled.

Results:

Hauling success

Use of a round, circular hauler and hauling salts proved successful for Alabama shad as well as for skipjack herring. Survival from capture to arrival at the SAFRS was 96% (29/30 fish) for Alabama shad after a 5 hour haul and 97% (43/44 fish) after a 1 hour haul for skipjack herring (Table 3).

Glochidial viability of gravid elephantear

The salt test assay indicated glochidial viabilities of 91, 96, and 96% for the three mussel broods used on April 3rd, and 94, and 97% for the two mussel broods used on April 4th. Brood size ranged from 386,000 to 1,506,000 viable glochidia per mussel (Table 4).

Transformation Success

Bluegill produced an average of 83.8 ± 49.2 sloughed glochidia per fish, but no viable juveniles. Over 92% of all glochidia had sloughed off of the bluegill by the second day post infection (Table 5). On day 16, direct observation revealed no encapsulated glochidia within the gill filaments of any bluegill, nor had any sloughed glochidia or juveniles accumulated in filter cups during the subsequent week (June 23 observation; Table 5).

Gill arches of Alabama shad excised on days 5, 6, 7 and 8 after infection produced no juveniles. Gills excised on days 10, 13, and 14 produced 2, 4, and 9 active juveniles respectively. Gills excised on days 17, 20, and 23 produced a total of 326, 328, and 293 active juveniles respectively. Gills excised on day 28 produced only 5 empty shells. Gills excised on days 30 and 36 did not produce any live, dead, or questionable juveniles (Fig. 1). Similarly, transformation efficiency of excised gills (active juveniles / total gill production after excision) was zero for gills excised prior to day 10, less than 1% from gills excised on days 10-14, 50-60% for gills excised on days 17-23, and NA for gills excised on or after day 28 (Fig. 1).

Empty shells (dead) were typically produced by gills over a period of 2-3 weeks after excision, but the majority of active juvenile production occurred within the first 10 days of excision, with peak juvenile production observed within the first 4 days of excision (Fig. 2). Relatively large numbers of "questionable" juveniles were produced by gills excised on days 13

and 14, while juveniles produced by gills excised on days 17, 20, and 23 were nearly all active (Fig. 2).

Gill arches of skipjack herring that were excised on days 5 - 13 produced no active juveniles. Gills excised on day 16 produced a total of 85 active juveniles with a transformation efficiency of 28% (Fig. 3). Questionable juvenile production dominated the first 3 days after excision. Active juvenile production occurred over a ten day period with peak production occurring on days 6-8 post excision. Empty shell production was observed for 19 days post excision (Fig. 3). Due to mortality of skipjack herring during the holding period prior to the period of observed juvenile production, no infected skipjack herring remained in the holding tanks after day 16.

Siphoning the bottom of holding tank # 2 on day 25 following infection yielded an estimated juvenile density of 187 / m^2, or 1,134 juveniles per the entire 6.3 m^2 of tank bottom. Because the tank sides sloped at the bottom and juveniles likely crawled partly up the sides of the tank, this should be viewed as a conservative estimate of juvenile production. Regardless of the total number of juveniles produced, observation of active juveniles on the holding tank bottom demonstrates that juveniles were produced by live fish, most likely by both species. Because all deaths of infected fish occurred prior to the period of active juvenile production, and dead fish were quickly removed and discarded, juveniles were most likely produced from live fish rather than from the gills of fish that died in the tanks.

Discussion:

After capture by electrofishing, Alabama shad were weighed in a plastic battery box before being transferred into the round hauler. Despite this initial rough handling, the survivorship of the Alabama shad was high. This showed that, similar to skipjack herring,

Alabama shad are less delicate than commonly assumed. Alabama shad can successfully be captured, hauled, and held for use in host fish assays as long as the following standard fisheries techniques are followed: 1) use of round hauling tanks, 2) use of professional hauling salts, 3) use of bubbled oxygen during hauling, and 4) use of large tanks with circular flow for holding.

The collection of gravid females from our local population of elephantear coincided with the reported periods of gravidity in the Mississippi river between April and June (Coker et al. 1921) as well as in the Apalachicola river basin between April and August (Brim Box and Williams 2000; O'Brien et al., 2003). Although some of the mussels held in Ziploc bags released glochidia into their bags within several hours of collection, it did not seem to affect the quality of their glochidia as viability of all broods was > 90%.

To our knowledge this is the first laboratory determination of host fish identity for elephantear. Skipjack herring have long been suspected as a host (Howard 1914a, Fuller 1974), but never confirmed due to the inherent difficulty in working with this fish species. In a previous study attempting to identify fish hosts for elephantear, 7 different families (*Catostomidae, Centrarchidae, Clupeidae, Cyprinidae, Ictaluridae, Percidae,* and *Sciaenidae*) were tested but none produced juveniles (Hauswald 1997). Similar to that study, we observed no production of elephantear from bluegill. The *Clupeidae* species tested by Hauswald (1997) was *Dorosoma cepedianum*, whereas we tested skipjack herring and Alabama shad. Our results combined with that of Hauswald (1997) suggests that elephantear is a host specialist on large river migratory fish of *Clupeidae*, particularly those in *Alosa*.

Determination of migratory *Alosa* as hosts for elephantear has important implications for conservation. While elephantear currently has lowest concern status on the IUCN red list (Natureserve 2014) and is considered stable across its entire range (Williams et al. 1993),

northern populations in Wisconsin and Minnesota have virtually disappeared (Natureserve 2014; Hauswald 1997; Mathiak 1979; Sietman 2003). Additionally, many extant populations in the southern part of its range appear to be relic populations with little to no recruitment (Miller and Payne 2000, Gangloff 2003). Persistence of old individuals coupled with a lack of recruitment can be explained by blockage of upstream migration of large-river migratory fish hosts by dams (Kelner and Sietman 2000; Watters 1996; Fuller 1974). My study suggests that installation of fish passage mechanisms for skipjack herring and Alabama shad have the potential to restore elephantear populations upstream of dams.

The gill excision technique described in this study is a valuable tool for conducting host assays with problematic fish species that require large tanks for holding. While large tanks can be successfully siphoned for juveniles if kept very clean, there are disadvantages to this method. Most importantly, large tanks take up a lot of space and many research facilities are space-limited. This requires that either multiple individuals of the same species be held in the same tank, or, as was the case in this study, multiple species may be held in the same tank. Siphoning of tank bottoms precludes the collection of transformation data from individual fish. Gill excision allows for collection of data from individual fish even if those fish were housed together in multi-species tanks.

A critical aspect of the gill excision technique, however, is that it is only successful within a limited time period. This window of opportunity starts when encapsulated glochidia have developed for a sufficient amount of time that they can metamorphose from excised gills, and ends when they have all metamorphosed from the gills of live, infected fish. In this study, the window of opportunity for gill excision lasted for nearly two weeks (days 10 – 25 post infection) for Alabama shad, with greatest transformation success occurring from gills excised

on days 17-23, from which 50-60% of encapsulated glochidia successfully metamorphosed into active juveniles. Due to mortality of skipjack herring in our trial we were unable to determine the full window of opportunity but did show that the optimal period of excision began at 16 days post infection.

Surprisingly, gills did not rapidly decompose to the point where all encapsulated glochidia metamorphosed or sloughed off within a few days. Although the majority of active juveniles were produced within the first four days, successful metamorphosis occurred for as long as 1-2 weeks after excision and dead shells sloughed off of gills for 3 weeks or more, post excision. We hypothesized that encapsulated glochidia were somehow slowing the decomposition rate of gill tissue, giving them time to complete metamorphosis. This hypothesis is tested in Chapter 3.

Ch. 3) Effect of glochidial infection on decomposition rate of excised fish gills

Introduction:

Parasites can modify their environment (host) in various ways in order to increase the likelihood of their survival and reproduction. Some parasites are able to modify the behavior of their hosts in ways that make them more susceptible to predation by their final host (Combes 2001; Moore 2002; Lafferty 1999). Other parasites are able to modify their environment down to the cellular level in order to make their ultimate survival and reproduction more likely. For example, certain intracellular parasites such as *Cryptosporidium parvum, Leishmania spp., Trypanosoma cruzi, Thelleria spp., Toxoplasma gondii,* and *Plasmodium spp.* are able to inhibit apoptosis of cells in which they inhabit (Carmen et al. 2007).

During experiments described in the previous two chapters, an interesting observation was made regarding the gill tissue that had been excised and held within the AHAB tanks. Gill tissue seemed to degrade very slowly in the AHAB system and was still recognizable as gill arches with filaments for > 7days after excision. This raised several questions concerning potential effects of encapsulated glochidia on parasitized gill tissue. Perhaps the glochidia were somehow altering their environment in a way that prevented or slowed down the gill tissue breakdown which would allow them to persist in the tissue until metamorphosis. This would be advantageous to glochidia whose host died before the glochidia were able to metamorphose from live gills.

In this chapter I tested whether or not encapsulated glochidia slow the decomposition rate of the excised gill tissue. I hypothesized that gill tissue infected with glochidia would decompose more slowly than uninfected gills.

Methods:

Collection and holding of study animals.

I utilized *Lampsilis straminea* (southern fatmucket) and one of their hosts, *Micropterus salmoides* (largemouth bass), in this study because both species are abundant, easily collected, and easily held in a laboratory environment. Largemouth bass were collected from a water supply reservoir on the North Auburn Fisheries Research Station (NAFRS) that contains few to no Unionids. This increased the chance that our wild-caught host fish were naïve and had not acquired immunity to mussel glochidia (Rogers and Dimock 2003). Small fish (approx. 5-6") were used to facilitate capture, hauling and holding. Fish were captured by use of a large seine net and transported back to the South Auburn Fisheries Research Station (SAFRS) in large 140 L coolers.

Bass were held individually in 3 L tanks within a recirculating AHAB tank system for approximately 1 month prior to infection. Water flowing out of each AHAB tank passed through a filter cup (105µm mesh) to retain any sloughed glochidia or juveniles. Water then drained into a common sump containing bio barrels that served as a biofilter. From there, water was pumped through a coarse mechanical filter that also contains a UV sterilization bulb and then through a fine mechanical filter (100µm) to catch any stray glochidia or juveniles that had bypassed the initial filter cup. Filtered water was then distributed to AHAB tanks via a manifold.

Pond temperature during the time of fish collection was 11ºC. Water in the AHAB system was originally brought down to this temperature to avoid temperature shock to the fish, and subsequently raised 1 degree per day until the system reached 21ºC and then held constant for the remainder of the trial. Bass were fed mosquito fish (*Gambusia* sp.) on a bi-weekly basis.

Water quality was monitored on a weekly basis using aquarium water test strips and water exchanges were performed when nitrate levels exceeded 40 mg/L.

Gravid *Lampsilis straminea* were collected in April, 2013 from Line Creek (Union Springs, Bullock County, Alabama) by tactile searching of the substrate. Mussels were embedded in a sandy silt substrate approximately 1-2 meters deep in a fairly swift current. Southern fatmuckets are sexually dimorphic, allowing for easy identification of females. Mussels were identified as gravid by the presence of swollen marsupial gills, creamy white to tan in color (Williams et al. 2008). Three gravid mussels were collected and placed in 1 quart Ziploc bags filled with water from the collection site. The bags were held in a cooler and transported (~1 hour) back to the Auburn University South Auburn Fisheries Research Station (SAFRS). Upon arrival the bags were placed in a recirculating aquarium system maintained at 18ºC with an airstone placed into each bag to provide aeration and water circulation. These mussels were held overnight and used the following day for the trial.

Host trials

Glochidia were extracted from the three gravid mussels using a 22 gauge needle attached to a syringe filled with Artificial Fresh Water (AFW). Water was injected into marsupia to flush glochidia into individual 1,000 mL beakers (one beaker per mussel) and the resultant glochidial solutions were diluted up to 1,000 mL.

Glochidial viability of each brood was determined via a standard salt test (Fritts et al. 2014). Each 1,000 mL beaker was mixed using a plunger style stir rod to prevent a vortex and to ensure even distribution of glochidia throughout the beaker. Ten 200 µL subsamples were withdrawn from a given beaker and placed into a 10-well glass plate. The number of open and closed glochidia were counted and recorded in each subsample using a dissecting microscope.

Several drops of 35‰ synthetic salt water were then added to each subsample and the number of open and closed glochidia was counted again. The proportion of viable glochidia in each brood was calculated as: [(number open initially) – (number open after NaCl)] ÷ (total number of glochidia).

The total number of viable glochidia in each brood was calculated as:

$Vn = [(G/S) *V]* P$

Where Vn = total number of viable glochidia in a brood, G = number of glochidia (open plus closed) in all subsamples combined, S= combined volume of all subsamples (ml), V = total number of mL in solution, and P = proportion of viable glochidia.

Infection

All three beakers containing glochidia were combined to make a master stock solution. One liter of the master stock solution was diluted to 19 L to create a glochidial bath at a concentration of 4,000 glochidia/L. Six largemouth bass were placed in this stock solution for 15 minutes before being transferred back to their respective AHAB tanks. Another group of 6 bass was placed in a bucket containing 19 L of AFW with no glochidia in order to expose them to similar handling stress while remaining free of glochidia infection. After 15 minutes, uninfected largemouth bass were also returned to their respective tanks and all tanks labeled as to whether they contained infected or uninfected fish. Fish were then held in AHAB tanks for 7 days to allow sufficient time for encapsulation, but not enough time for metamorphosis. Previous assays showed *L. straminea* glochidia metamorphosed from live largemouth bass 14-16 days after infection at ~21 C (M. Hart, unpublished data).

Gill excision

At day 8 post infection, all fish were removed from their AHAB tanks and quickly euthanized by severing of the spinal cord with surgical scissors. Once fish were dead the three outermost gill arches on each side of each fish were excised using forceps and cut in half to produce 12 "gill units" (Figure 1). The gill units were then returned to their respective AHAB tanks and fish were discarded. Three gill units were taken out of each AHAB tank on days; 0, 3, 6, and 12 days post excision and preserved in a 10% neutral buffered formalin solution (NBF). To quantify decomposition, each preserved gill unit was examined under 40X magnification, and the number of identifiable lamellae on three individual gill filaments was recorded. (Figure 1). The results of this analysis were analyzed using a Two-way ANOVA (Minitab 16).

Results:

Glochidial viability of L. straminea

Glochidial viabilities of the three broods used to make up the master stock solution were 94, 88, and 67%. Brood sizes ranged from 32500, to 111500 viable glochidia per gravid mussel (Table 6).

Juvenile production

No juveniles were produced from infected gills excised on day 8 and held in the AHAB chambers for up to twelve additional days.

Gill decomposition

Gill units from all time treatments appeared to the naked eye to be in good condition (identifiable gill filaments). However upon examination with a microscope (40X) it became apparent that the tissue had decayed substantially. After day 6 post-excision, identifiable lamellae were no longer present (Figure 2).

Results of gill unit lamellae counts

There was no significant difference in lamellae counts between infected and non-infected gills through time (Fig. 3; $p = 0.472$)

Discussion:

My hypothesis that encapsulated glochidia slowed the decomposition rate of gill filaments was rejected. Within 6 days gill lamellae had decomposed to the point where they could no longer be identified, regardless of treatment. Gill units at every time treatment however still appeared to be in "good shape" with the naked eye and also appeared to be decomposing slowly as observed in Chapters 1 and 2.

The slow breakdown of gill tissue observed in chapters I and II may have been due to constant flow of clean water over the gills. During the gill decomposition experiment, one of the manifold valves became clogged and the associated AHAB tank lost flow for ~ 3 days. The gill tissue in this tank quickly became covered in bacteria and fungi, and degraded to the point where it became unidentifiable as gill arches with filaments.

This chapter and the chapters preceding it have shown that glochidia can successfully metamorphose off of dead gill tissue from their host fish in the lab. In nature, metamorphosis of glochidia from an infected fish that died is possible, but would likely require specific conditions. First, the infected fish would have to die at a time that coincides with the window of opportunity as described in chapter 2. The timing and length of this window likely varies with species and temperature, but I show that it can last for a week or more. Second, based on the anecdotal observation of this chapter, the fish may have to die and be retained in a riffle or other location that provides a constant flow. Additionally, the fish may have to die with its gills flared so as to expose them to water flow. A congruence of all of these circumstances may be unlikely, but I

have occasionally observed recently dead fish, with gills flared, in flowing sections of local streams. It is unknown whether these conditions occur often enough to provide substantial recruitment from dead gills in natural systems. However, as discussed in previous chapters, even if not likely to occur in nature, the ability of glochidia to metamorphose from dead gills allows for a valuable research tool in mussel host assays. Fish that happen to die in routine host trials may still be able to provide valuable data.

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Table 1. Skipjack herring survival from collection site to lab.

Collection Date	# Collected	# Surviving transport to lab
3/18/2011	6	4
3/24/2011	24	23
3/29/2011	29	25

Table 2. Results of gill inspections for encapsulated glochidia of *Reginaia ebenus* on all fish taxa tested. Y represents successful encapsulation, N represents no encapsulation.

Fishes

	# Inspected	Encapsulation (Y/N)	Day after infection
Cyprinidae			
Lythurus bellus	6	N	4-6
Notropis ammophilus	2	N	4-6
Campostoma oligolepis	3	N	4-6
Luxilus chrysocephalus	3	N	4-6
Pimephales promelas	6	N	4-6
Fundulidae			4-6
Fundulus olivaceous	6	N	4-6
Percidae			4-6
Etheostoma sp. and Percina sp.	6	N	4-6
Centrarchidae			4-6
Lepomis macrochirus	6	N	4-6
Micropterus salmoides	6	N	4-6
Ictaluridae			4-6
Ictalurus punctatus	6	N	4-6
Sciaenidae			4-6
Applodinotus grunniens	4	N	4-6
Clupeidae			4-6
Dorosoma cepedianum	4	N	4-6
Alosa chrysochloris	1	Y	11
Alosa chrysochloris	7	N	19

Table 3. Alabama shad and skipjack herring survival from collection site to lab.

Fish species	Collection Date	# Collected	# Surviving transport to lab
Alosa alabamae	3/22/2012	30	29
Alosa chrysochloris	3/20/2012	44	43

Table 4: Mussel length, percent viable glochidia, and viable brood size of mussels utilized for glochidial baths on two dates.

Date used	Mussel length (mm)	% viable glochidia	Brood size (# viable glochidia)
4/3/2012	105	90.8	548,000
4/3/2012	107	96.0	1,506,000
4/3/2012	100	96.0	386,000
4/4/2012	106.3	94.2	1,108,000
4/4/2012	104.4	97.1	948,000

Table 5: Number of sloughed glochidia from Bluegill infected with elephantear. No juveniles were observed.

	Days post infection							
Bluegill ID	2	6	8	10	12	14	16	23
1	78	9	0	0	0	0	0	0
2	146	0	0	1	0	0	0	0
3	4	15	2	0	1	0	0	0
4	51	0	0	0	0	0	0	0
5	108	2	1	0	1	0	0	0

Table 6: Percent viable glochidia, and viable brood size of mussels utilized for glochidial bath.

Date used	% viable glochidia	Brood size (# viable glochidia)
4-15-2013	88.1	111,500
4-15-2013	93.5	87,000
4-15-2013	67.0	32,500

Figure 1. a) 5700 liter rectangular fiberglass tanks used to hold large river fish. b) Baffle, and airlifts used to generate circular water flow. Arrows show direction of flow.

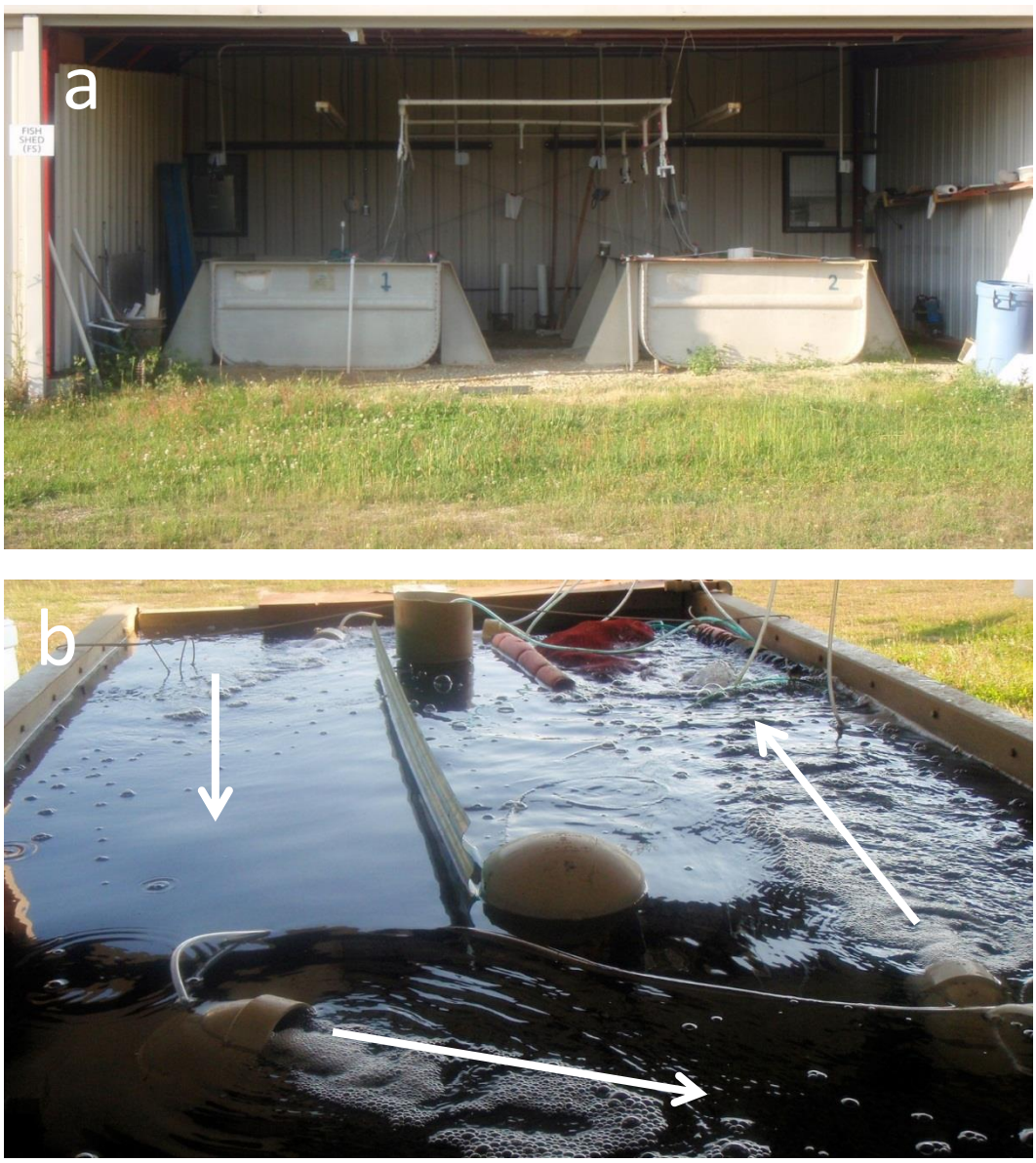

Figure 2. 8500 liter round fiberglass tank and cover used to hold large river fish.

Figure 3. 1900 liter round fish hauler with oxygen tanks used to haul fish from collection site to lab.

Figure 4. Picture and schematic diagram showing collection of sloughed glochidia and juveniles from AHAB tanks. Arrows show direction of water flow.

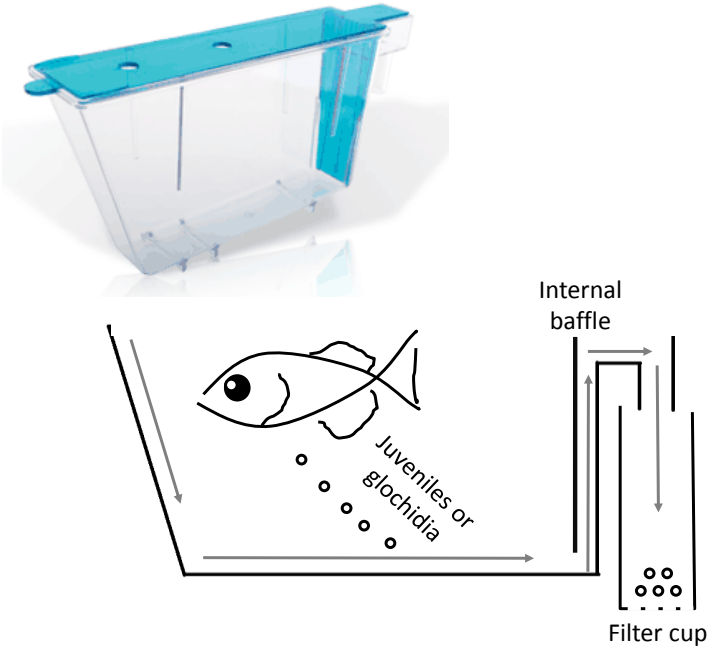

Figure 5. Schematic diagram showing collection of sloughed glochidia and juveniles from cone tanks. Arrows show direction of water flow.

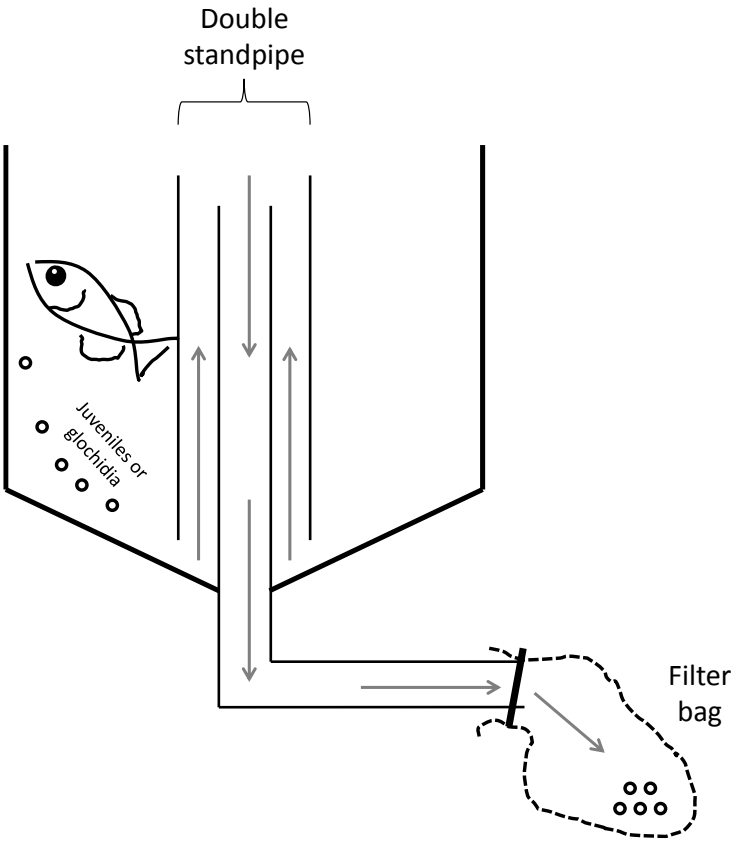

Figure 6. Photo of infected skipjack herring gills under cross polarized lighting. Light colored "discs" are encapsulated glochidia.

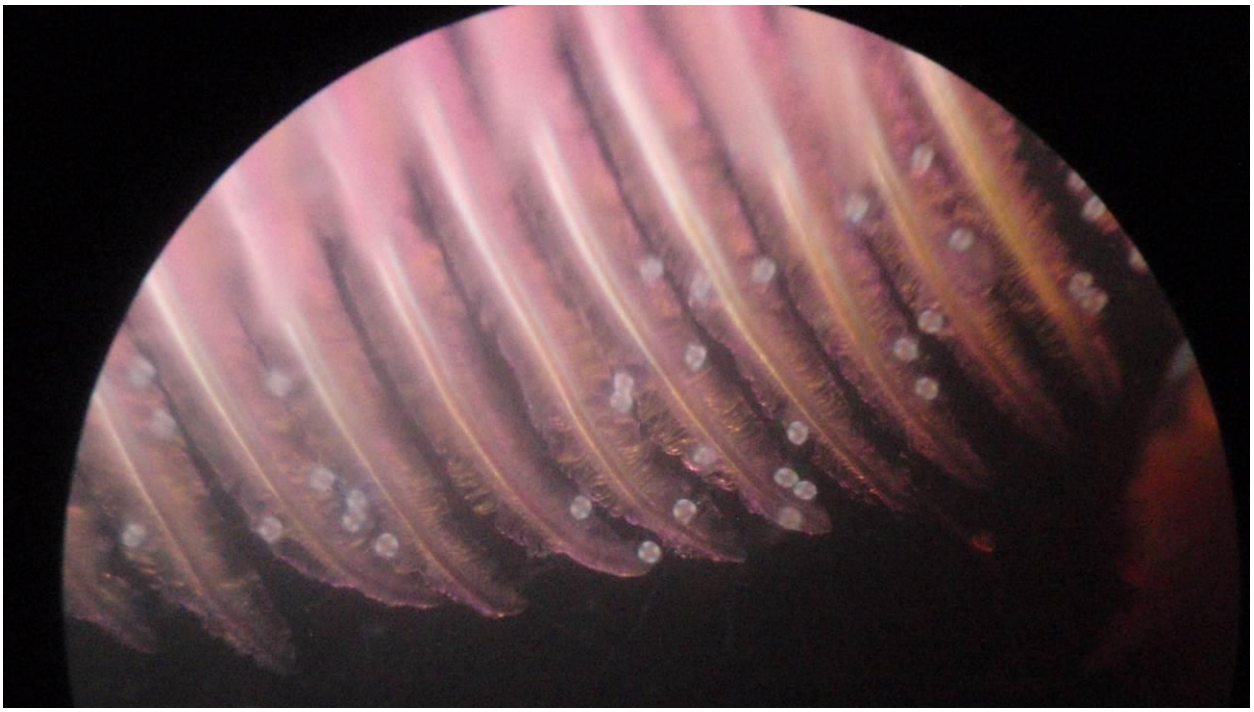

Fig. 7. Number of juveniles produced from skipjack herring gills on each day following excision.

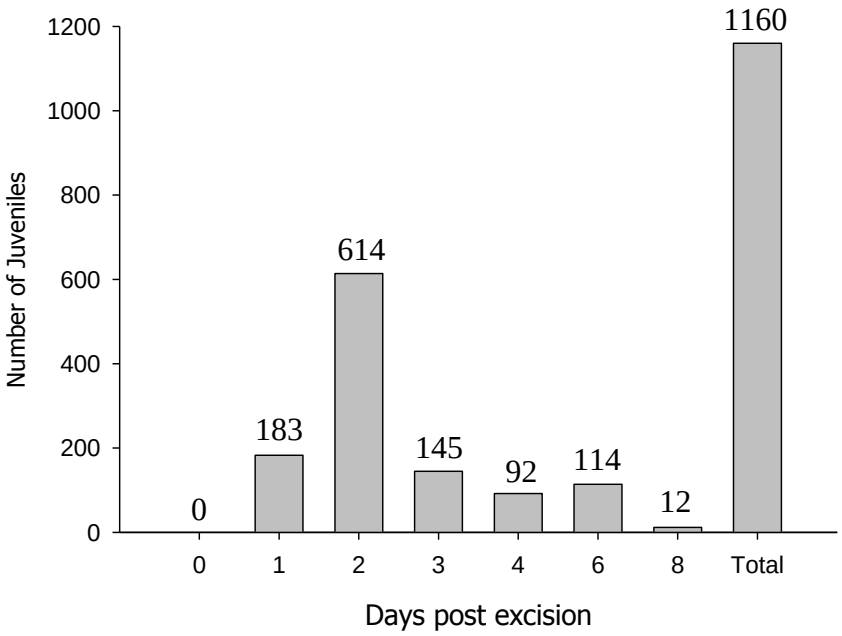

Figure 8: Total juvenile production (bars) and transformation efficiency (circles) from Alabama shad gills excised on different days. "X" symbols denote gills from which no juveniles were produced. Numbers above bars denote total # juveniles produced from each gill set.

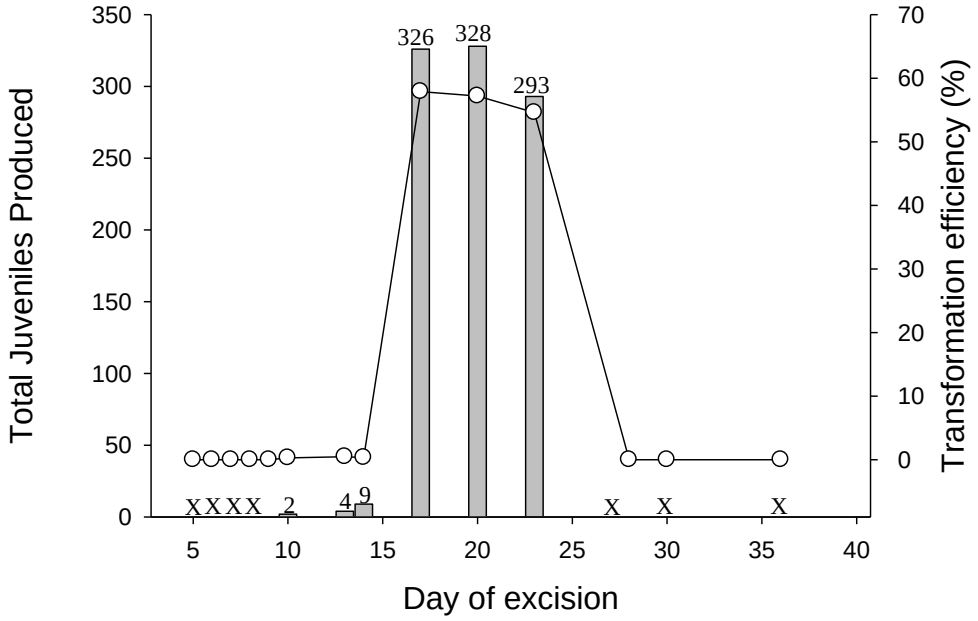

Figure 9: Daily juvenile production from Alabama shad gill arches excised on: a) Day 10, b) Day 13, c) Day 14, d) Day 17, e) Day 20, and f) Day 23 after infection.

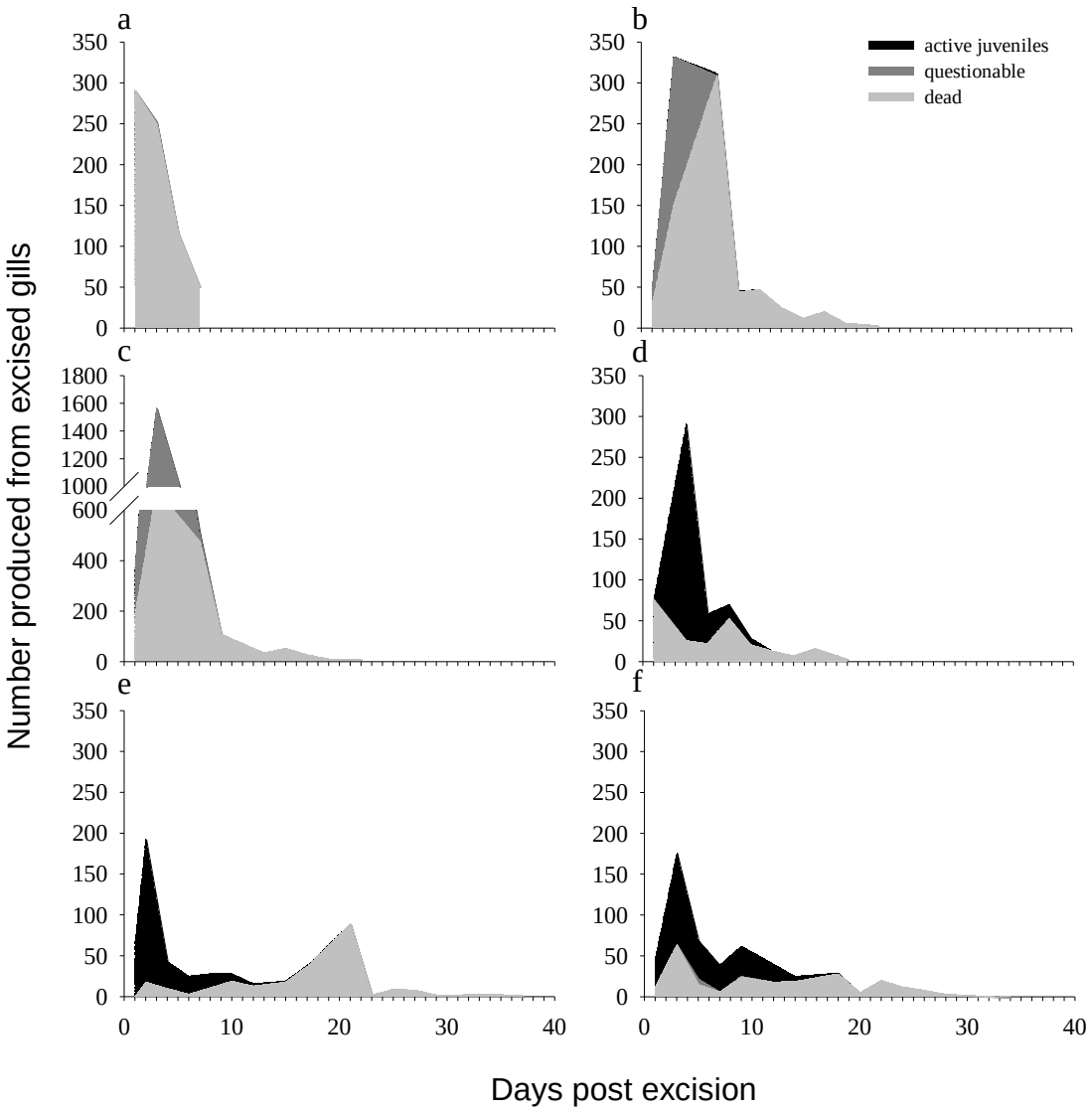

Figure 10: Total juvenile production (bars) and transformation efficiency (circles) from skipjack herring gills excised on different days. "X" symbols denote gills from which no juveniles were produced. Number above bar denotes quantity of juveniles produced from that gill set.

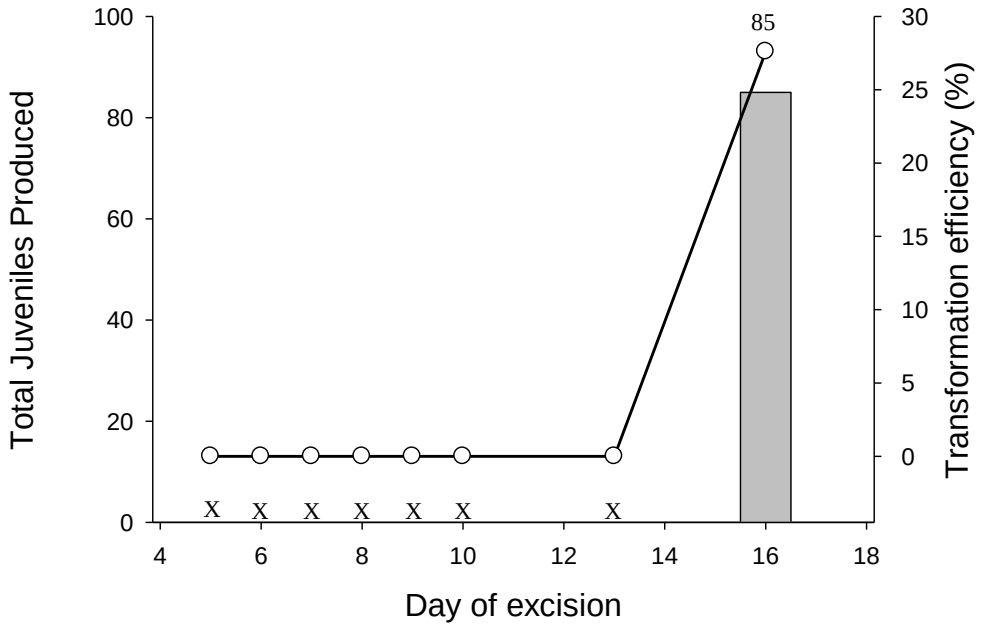

Figure 11: Daily juvenile production from skipjack herring gill arches excised on Day 16 after infection.

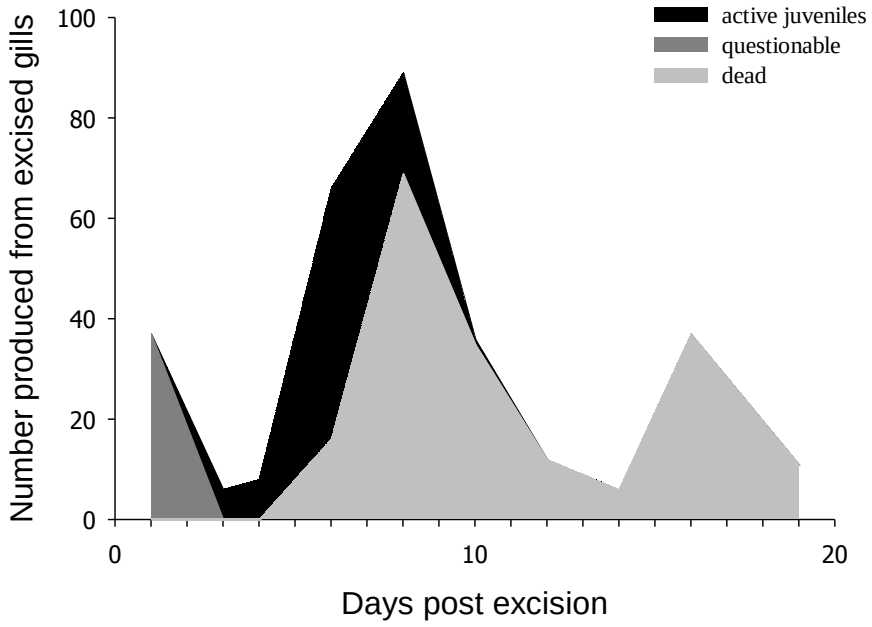

Figure 12: a) Image showing representation of a "gill unit" analyzed in this study. Red ovals demonstrate general areas where individual filaments were selected for lamellae counts. b) Close up image of filament on a gill unit. Yellow oval shows individual lamellae that were counted.

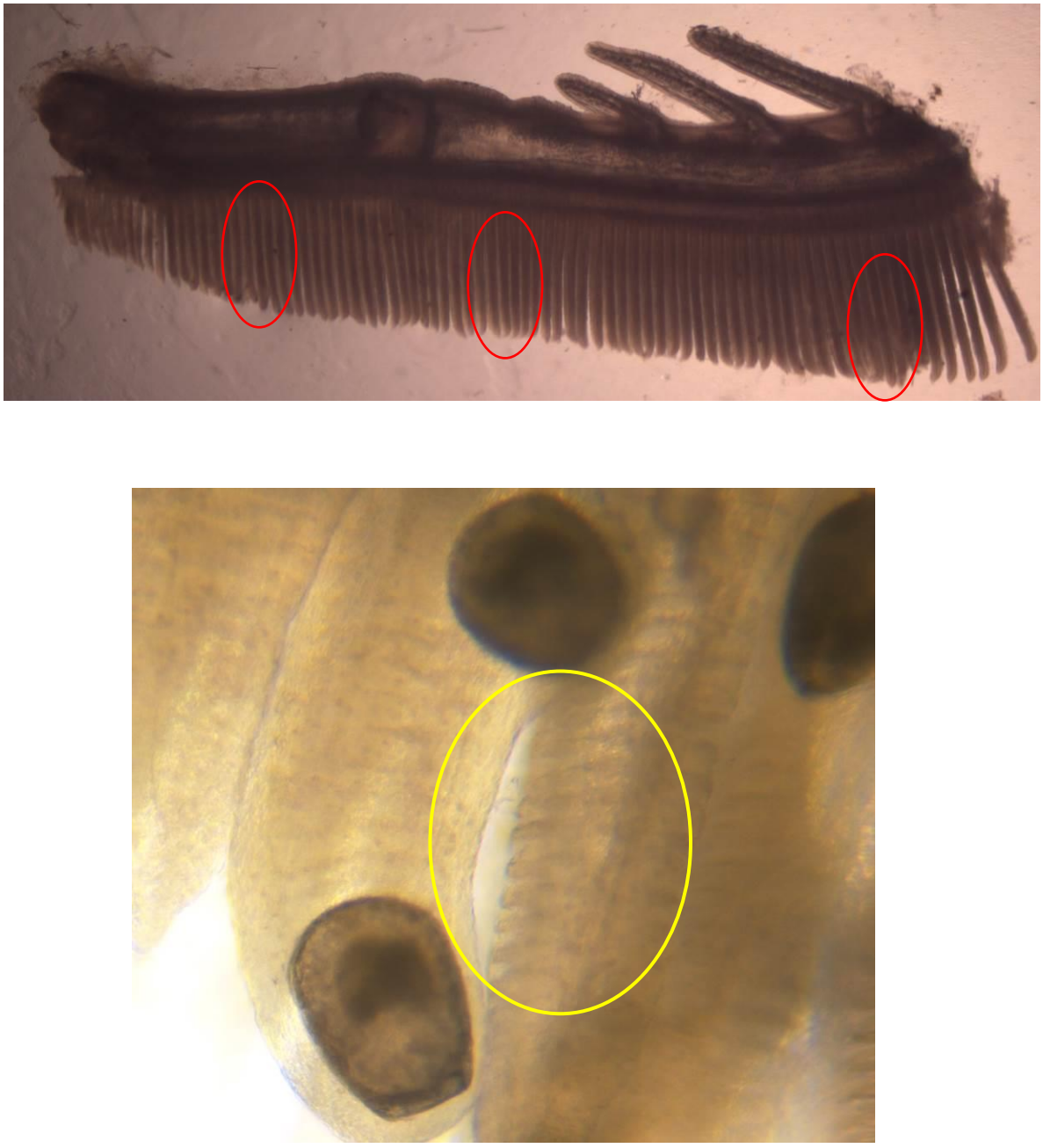

Figure 13. Images showing decomposition of gill filaments on gill units across time treatments.

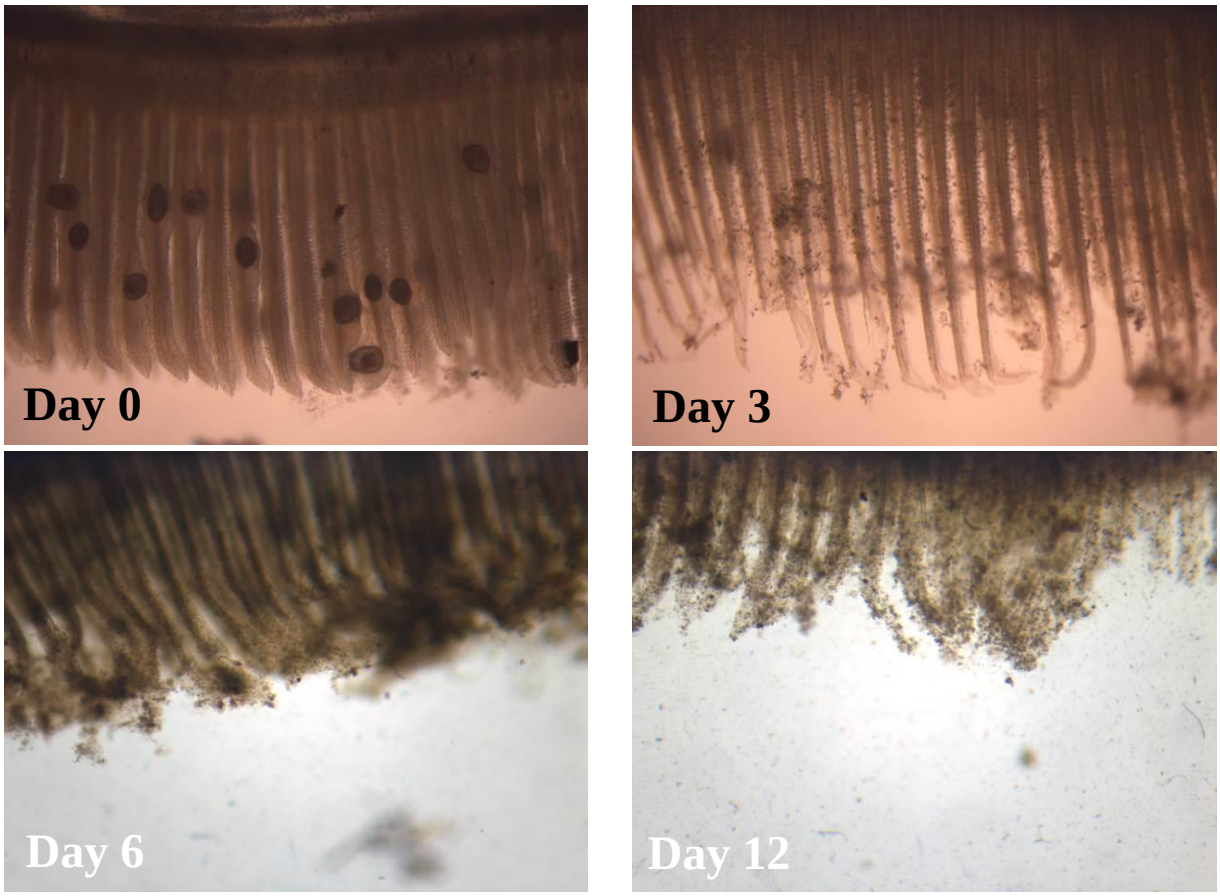

Figure 14. Average number of lamellae per gill unit in infected (white) and uninfected (black) units for 12 days following excision. Red x's denote days in which lamellae were unidentifiable.