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The Role of Freshwater Drum as a Host of Freshwater Mussels, Unionidae

Michael S. Martin
Missouri State University, Martin340@live.missouristate.edu

THE ROLE OF FRESHWATER DRUM AS A HOST OF FRESHWATER

MUSSELS, UNIONIDAE

A Masters Thesis

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By

Michael Stephan Martin

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ABSTRACT

The Freshwater Drum, *Aplodinotus grunniens*, serves as fish host to multiple mussel species. Some mussel species are "drum specialists" in the sense that Freshwater Drum is apparently the only host that they utilize. Freshwater Drum diet consists partly of mussels, which it crushes with specially adapted pharyngeal teeth. The Freshwater Drum's habit of feeding on mussels should favor adaptations to use the species as a host. Mussels that use Freshwater Drum as host could adapt to attract the fish. I predicted that many other species will have some ability to use Freshwater Drum as host because of its habit of seeking bivalve prey. I also predicted that species that use Freshwater Drum as their sole host should 1) have high metamorphosis success (%M) and 2) exhibit adaptations to avoid predation while attracting a predaceous host. I investigated the host potential between nine species of freshwater mussel and Freshwater Drum with laboratory host tests. These tests quantified the %M of two mussel species (*T. truncata* and *L. fragilis*) that are known to use Freshwater Drum as a host and quantified the %M of two new mussel-host relationships (*O. reflexa* and *L. abrupta*). The results show that Freshwater Drum exhibit a very high %M when hosting "drum specialist" mussels with little variability between individual fish; however, when Freshwater Drum host mussels that are not "drum specialists" the %M is much lower and more variable between individual fish.

KEYWORDS: drum specialist, host, adaptations, metamorphosis percent, variability

This abstract is approved as to form and content

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M. Christopher Barnhart
Chairperson, Advisory Committee
Missouri State University

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Approved:

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M. Christopher Barnhart, PhD

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Alicia Mathis, PhD

_____
Debra Finn, PhD

_____
Julie Masterson, PhD: Dean, Graduate College

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Life-Cycle of Unionoidea

Freshwater mussels in the family Unionidae have a complex mode of reproduction that is unique among bivalves (Howard and Anson 1922). Female mussels brood eggs in hollow gill chambers called marsupia (singular *marsupium*). Males release spermatozeugmata (packets of sperm) into the water column (Bauer 1987). Water containing spermatozeugmata fertilizes the eggs in the mussel's dorsal passages. After fertilization, eggs develop into a microscopic larval stage known as the glochidium (plural *glochidia*). Glochidia are obligate parasites on a vertebrate host, usually a fish (Kat 1984). Glochidia develop in the marsupium and are held there for weeks to months before being released by the female mussel.

Mussels use a variety of methods for releasing the glochidia from the marsupium. Some species expel conglutinates (packets of eggs containing glochidia) into the water, while others inflate extensions of their mantle flaps surrounding the gills, producing a "lure" which imitates the intended host fishes' natural prey (Kraemer 1970; Barnhart et al. 2008). The intended host fish identifies the conglutinate or mantle flap as a possible item of prey and the fish attempts to ingest the imitation. When the fish strikes at the conglutinate or mantle flap, the gill and the eggs containing the larval mussels break open and the fish is infected with glochidia (Watters 1999). Attachment occurs as a glochidium closes its valves on fish tissue, usually gills. If a glochidium attaches to the correct species, it becomes encapsulated by the fish's epithelial cells for a period of days to weeks. While encapsulated the glochidium metamorphoses into a juvenile mussel. When

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the metamorphosis is complete, the capsule breaks down and the metamorphosed juvenile is released from the fish and drops to the sediment as a free-living juvenile mussel.

Host Specificity

Mussel hosts encompass a taxonomically and ecologically wide range of fishes from drift-feeding minnows to large top predators. One mussel species is a specialist on the mudpuppy, an aquatic salamander (Howard 1915). Host use varies widely among mussel species from specialists that use only one fish species as host, to generalists that are able to parasitize nearly any fish species (Haag 2012). Generalists typically have only a small fraction of the larvae metamorphose, whereas, specialists that use only one host fish or a few close relatives usually have high success on those hosts (Watters 1996; Crownhart 2009). The attachment of glochidia to a potential host is indiscriminate, as glochidia will attach to any fish that are encountered; however, when glochidia attach to a non-compatible host, the larvae either fail to be encapsulated, or the capsule is shed (sloughed) from the gills within 2-7 days (Kat 1984). In some cases the shed larvae are alive, and in others they are killed within the capsule before being shed. This host specificity is the result of innate immunity of fishes to certain parasites (Reuling 1919; Dodd et al. 2005, 2006; Bowers 2006).

The standard measure of the compatibility between mussel and fish species is a quantification of the metamorphosis success (%M) of the glochidia that attach to the fish. %M is calculated as the total number of recovered juveniles divided by the sum of total recovered juveniles and sloughed glochidia. A high %M indicates a greater level of mussel-host compatibility, whereas a low %M indicates a low level of mussel-host

compatibility (Crownhart 2009; Douda et al. 2016). Mussels coevolve with their fish hosts, gaining adaptations that allow bypass of the fish's innate immune system. Logically, adaptations to particular fish species can only evolve if the glochidia of the mussel commonly encounter that host. Freshwater Drum are a predatory fish species that are known to eat bivalves. Some mussels are known as "drum specialists" because Freshwater Drum is the only species that the mussel uses as a host. Drum specialists have a high %M that may be a result of the close interaction of the two in the mussel/predator relationship.

Thesis Objectives

Life history studies have been identified as a critical mussel conservation need (Neves 1993; Haag 2012). One of the key aspects of mussel life history is the identification of the mussel species' vertebrate host. Currently, suitable hosts for approximately half of North American mussel species remain unknown, and many of the reported mussel-host relationships require further investigation (Williams et al. 2008). Identification of fish hosts is the highest priority listed under the basic biology research goals of the National Strategy of Freshwater Mussel Conservation (NNMCC 1998; FMCS 2016). Research in this area of life history is needed for the conservation and management of mussels, as well as for the management of the host fishes. Information concerning mussel-host compatibility is also essential in the expanding field of mussel propagation. Artificial propagation of mussels on host fish is becoming one of the primary tools used in efforts to restock rare mussels to their native ranges, from which many have been extirpated. Of the approximately 297 mussel species living in North

America, 213 are listed as either endangered, threatened, or a species of conservation concern (Hove et al. 1998). The results of this project could expand the known spectrum of mussel-host relationships, as well as providing information that may influence the management of Freshwater Drum and their role as a host for mussels.

The first component of this study was host testing to determine compatibility between Freshwater Drum and nine species of Unionid mussels. Additionally, a feeding experiment was conducted on Freshwater Drum of different sizes to determine if there is a correlation between Freshwater Drum size and the size of mussels they are capable of consuming (Chapter 1). The second chapter of this study investigated the fish hosts of a particular mussel, the Threehorn Wartyback (*Obliquaria reflexa*). I tested compatibility between nine potential fish species and Threehorn Wartyback, whose primary fish host is currently unknown (Chapter 2).

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CHAPTER 1: HOST TESTS WITH FRESHWATER DRUM

Introduction

Freshwater Drum (*Aplodinotus grunniens*) is the only known fish host for several North American mussel genera, including *Potamilus, Leptodea, Truncilla,* and *Ellipsaria* (Barnhart et al. 2008). Freshwater Drum belongs to the fish family Sciaenidae, which includes: Croakers, Sea Trout, Corbina, Red Drum and Black Drum. It is the only member of Sciaenidae to inhabit freshwater for the entirety of its life. This is a taxonomically unique species, being the only member of its genus. Like many fishes, drum can also be known by multiple common names such as: Sheepshead, Shepherd's Pie, Gray Bass, Grunter, Bubbler, Croaker, and Wuss Fish. Freshwater Drum is one of the most widespread native fish species in North America. The species native range spans from the western border of the Appalachian Mountains to the eastern edge of the Rocky Mountains and from Hudson Bay to the Rio Usumacinta Basin of Guatemala (Trautman 1981; Ross 2001). This species typically lives in medium to large rivers and impoundments. Adults can be found in the larger pools of streams and in depths exceeding ten meters in reservoirs. It is a benthic species that populates a variety of habitats. Large numbers of Freshwater Drum can be found in backwaters and areas of slack current with silty to rocky substrate (Barney 1926; Pfleiger 1975).

Freshwater Drum breed seasonally in open water. Spawning occurs from late May to July and is triggered by an increase in water temperature to approximately 20° C (Swedberg and Walburg 1970). Females produce 40,000 to 60,000 eggs that are released into the water column, where they are fertilized by the males. There is no parental involvement after spawning (Etnier and Starnes 1993). The pelagic eggs float to the

surface and are carried by the current until they hatch 48 to 96 hours later (Daiber 1953).

Larvae are about 3mm at hatching and adhere to surface film for approximately 3 days

until they are capable of swimming freely. Surviving larvae are considered juveniles at

15mm. Freshwater Drum can reach a total length of 85mm in their first year and up to

150mm by the end of their second year. On average, males reach sexual maturity at age

5, whereas, females reach sexual maturity at age 6 (Butler and Smith 1950; Priegel 1969).

The largest Freshwater Drum ever recorded was caught in 1972 at Nickajack Lake,

Tennessee, USA and weighed 24.9KG (IGFA 1985).

The diet of the Freshwater Drum is generally benthic and becomes more diverse

as the fish grows into sexual maturity. Freshwater Drum in Harlan County Reservoir,

Nebraska, USA were reported to be strict zooplanktivores during the larval (<19 mm in

length) stage (Sullivan et al. 2012). This study corroborates the findings of Swedberg and

Walburg (1970) at Lewis and Clark Lake, South Dakota, USA. After the fish reach

approximately 12mm, zooplankton constitutes the majority of the diet (Sullivan et al.

2012). Larger juveniles feed largely on the larval stages of aquatic insects (Daiber 1952).

The diet of adults can be very diverse, encompassing four main categories: fish, insects,

crayfish, and mollusks (Shields and Beckman 2015). Adults are equipped with heavy

pharyngeal teeth that aid in the consumption of mollusks such as snails and freshwater

mussels. The proportion of these food categories varies with seasonal changes and food

availability (Griswold and Tubb 1977; Schael et al. 1991).

The introduction of Zebra mussel (*Dreissena polymorpha*) in North America has

impacted the diet of Freshwater Drum, with dreissenids forming as much as 60% of their

diet in some situations (Watzin et al. 2008). A 2007 sampling study in Lake Winnebago,

Wisconsin, USA revealed that most of the larger Freshwater Drum sampled were found to have Zebra mussels in their gut content, with larger Freshwater Drum often having their digestive tract completely engorged with crushed Zebra mussel shells (Davis-Foust 2012). The arrival of dreissenids in Lake Winnebago resulted in a major dietary shift for the Freshwater Drum population from fish and insects to mollusks, given that the native mussel population of the lake was historically low. Similar dietary shifts in Freshwater Drum were previously reported upon the invasion of Asiatic clams (*Corbicula fluminea*) into impoundments, where the diet of larger fish had shifted from predominantly dipteran larvae and copepods to being almost exclusively composed of Asiatic clams (Wrenn and Shoals 1968). Food resource utilization in fish tends to relate to body size and fish near the transitional stage from small to large, or at about 9-12 inches; often display the greatest diet diversity (Werner and Gilliam 1984).

Freshwater Drum serve as exclusive host to mussel species in several genera in the family Unionidae. This relationship can only be possible as the result of many generations of close interaction and coevolution between Freshwater Drum and the mussel species that they host. Malacologists have established that Freshwater Drum potentially host at least 15 freshwater mussel species in North America (Sietman et al. 2018). These mussel-host relationships have been established by both observing natural infections and through laboratory infections of freshwater mussels on fish. Known mussel species that use Freshwater Drum as a host include: Pink Papershell (*Potamilus ohiensis*), Giant Floater (*Pyganodon grandis*), Fawnsfoot (*Truncilla donaciformis*), Threeridge (*Amblema plicata*), Butterfly (*Ellipsaria lineolata*), Fragile Papershell (*Leptodea fragilis*), Scaleshell (*Leptodea leptodon*), Washboard (*Megalonaias nervosa*), Pink

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Heelsplitter (*Potamilus alatus*), Bleufer (*Potamilus purpuratus*), Rock Pocketbook (*Arcidens confragosus*) , Deertoe (*Truncilla truncata*), Fat Pocketbook (*Potamilus capax*), Inflated Heelsplitter (*Potamilus inflatus*), and Higgins Eye (*Lampsilis higginsii).* Many of these mussels are considered to be "drum specialists" because Freshwater Drum is the only known fish species that the mussel uses as host. Drum specialists typically have unusually small glochidia and high fecundity (Barnhart et al. 2008; Sietman et al. 2018).

I hypothesize that if Freshwater Drum frequently attempt to prey on many species of freshwater mussels, it is possible that the glochidia of many species regularly encounter drum and may have adapted to use it as a host. I tested this hypothesis with laboratory host tests. Seven freshwater mussel species not previously tested with drum, as well as two species considered to be drum specialists, were tested in host trials on Freshwater Drum. The mussel species I tested were: Fatmucket (*Lampsilis siliquoidea*), Pink Mucket (*Lampsilis abrupta*), Black Sandshell (*Ligumia recta*), Neosho Mucket (*Lampsilis rafinesqueana*), Round Pigtoe (*Pleurobema sintoxia*), Threehorn Wartyback (*Obliquaria reflexa*), Fragile Papershell (*Leptodea fragilis*), Deertoe (*Truncilla truncata*), and Plain Pocketbook (*Lampsilis cardium*). The tests quantified host suitability as percent of attached glochidia that metamorphosed to juveniles (%M).

Feeding trials were also conducted on four Freshwater Drum using live Fatmucket to establish if small size-class fish will feed on live mussels and also to determine the role of gape limitation of Freshwater Drum (Schael et al. 1991) when feeding on freshwater mussels. Gape limitation refers to fact that dimensions of the gape of a predator's mouth limit the maximum size of the prey item that the predator is capable of ingesting.

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Methods

Freshwater Drum were obtained from the aquaculture facility at Langston University, Langston, Oklahoma USA. They were maintained at Neosho National Fish Hatchery, Neosho, Missouri USA, until needed for testing. Upon arrival for testing, the fish were held in recirculating aquaria at ambient laboratory temperatures (20°-23°C) in moderately hard synthetic fresh water (USEPA 2002) for a period ranging from days to weeks prior to infection. During this time the fish were fed approximately 1-2% of their body weight in blood worms (chironimid larvae, Hikari Bio-Pure Brand) daily. Fish were starved from 2 days prior to the host testing until the testing was considered complete. Upon completion of each experiment, the length (mm) and mass (g) of each fish was recorded. The size of fish varied from 132mm – 228mm. The drum were used for a single host test and then anesthetized and terminated by spinal cord severance.

All glochidia used in these studies were obtained from MSU mussel lab brood stock, Kansas City Zoo aquaculture facility, or by collecting brooding females from the wild (Table 1). Brood stock mussels were maintained at 10°C in river water (RW), collected from the James River, Greene County, Missouri, USA. The RW had an average hardness of 140 -160 mg/L $CaCO_3$. The wild caught mussels were maintained in SFW at ambient laboratory temperatures. The mussels were held unfed for a period of days to months before testing. One to three brooding female mussels were used for each experiment. Both fish and mussels were held on a 12:12 hour light/dark photoperiod.

A host study was conducted between 2013 and 2015 at Missouri State University, Springfield, Missouri, USA involving 9 species of freshwater mussel and Freshwater Drum. Fatmucket, Pinkmucket, Black Sandshell, Neosho Mucket, Fragile Papershell,

Deertoe, and Plain Pocketbook glochidia were obtained by using a needle and syringe to perforate the marsupial gill and glochidia were flushed with RW into a beaker. Conglutinates of Round Pigtoe were collected after they were released by the mussel. The glochidia were freed from the conglutinate by spraying them with RW through a 400 μm nylon screen. Threehorn Wartyback conglutinates were manipulated from the marsupium by using a dental pick to perforate and lift conglutinates from the gill. The glochidia were freed from conglutinates by pressing them through a 400 μm screen with a small paintbrush, while spraying them with RW.

Glochidia were then suspended in a known volume of RW which was subsampled for counting. Uniform suspension was achieved through agitation by a large rubber-bulb pipette. Each 200 μm sample was placed as a drop on a plastic Petri plate. The glochidia in each drop were counted and classified as either open or closed using a stereomicroscope at 10.5X or 40X. Viability was tested by the closing response to salt. One drop of saturated salt solution (NaCl + SFW) was added to each 200 μm sample to induce closing of the glochidia valves (LeFevre and Curtis 1912). Glochidia that were open initially and that closed in response to the addition of the salt were considered viable. These sample counts were used to estimate the total number of viable glochidia. The mussels used in this experiment had a glochidia viability that ranged from 55-97% viable.

Fish were infected with glochidia by placing them collectively in a bath containing 1L of RW per fish. Glochidia were added for a total concentration of 4000 viable glochidia per liter RW. Homogeneity of the suspension was achieved by stirring and vigorous agitation with a large rubber-bulb pipette. After 15 minutes, fish were

netted out and placed into a rinse bath to allow any unattached glochidia to drop from the fish. After rinsing for 10 minutes, fish were netted and placed into individual tanks.

Inoculated fish were housed in individual 9L tanks in an AHAB recirculating aquarium system (Aquatic Habitats, Inc. Apopka, FL). The water was conditioned by mechanical, biological, and carbon filtration and sterilized by ultraviolet radiation before recirculation. Water temperature was monitored daily and averaged 23°C. Each 9L tank received water continuously from a manifold and the overflow was passed through a filter cup with a nylon mesh screen, containing a mesh size that corresponded to the size of the glochidia being tested. The water flow rate through each tank was approximately 0.5L/min. The first day after an inoculation and every other day thereafter (unless otherwise noted), the rate of water flow through the tank was increased to 2L/min for 10 minutes to flush any particles into the filter cup. Afterwards, the screen was rinsed into a glass dish and examined with a stereomicroscope at 10.5X or 40X to distinguish glochidia from juvenile mussels. Collections with large amounts of glochidia and juveniles were transferred to a Bogorov plankton counting tray. Juveniles were distinguished from glochidia by foot movement. Glochidia and juveniles from each collection were counted to determine the number recovered from each fish. Infections were considered to be over after two consecutive counts with no juveniles and no glochidia present. At that time the fish were sedated and terminated by spinal cord transection. Their gills were dissected to examine for any remaining encystment.

The number of glochidia that attached to each fish was approximated as the sum of the sloughed glochidia and metamorphosed juveniles that were recovered. The percent metamorphosis (%M) for each fish was calculated by dividing the number of juveniles by

the sum of glochidia and juveniles recovered from that fish. A fish was considered a possible host if larval metamorphosis to the juvenile stage was observed; however for a fish to be confirmed as a true ecological host for a mussel species, there should also be observed glochidial infestation of that fish species under natural conditions (Neves 1990).

Eight individual Freshwater Drum from Langston University that ranged in size from a total length of 122mm – 390 mm were offered juvenile Fatmucket of various sizes in an attempt to determine size limitation of ingestion in two separate trials. In the first set of trials, five fish were held individually in 9L tanks in an AHAB aquarium system at 23° C. The fish that were selected for the experiment had been previously starved for a period of 3 – 7 weeks during host trials, and subsequently fed bloodworms for 1-2 weeks prior to the feeding experiment. The feeding of bloodworms was discontinued and the fish were then offered de-shelled Asiatic Clam (*Corbicula fluminea*) and de-shelled Fatmucket (*Lampsilis siliquoidea*) as a primer for feeding on bivalves for a period of one week before being offered live bivalves. Three fish that refused de-shelled bivalves were not tested further. The remaining five fish were tested with live Fatmucket. Two of these fish refused to eat live Fatmucket and were not tested further. Mussel valve length, height and width were recorded before being offered to the fish. One live mussel was introduced per fish each day during the light portion of the 12:12 light cycle, and not removed for at least 24 hours, ensuring that the fish would have an opportunity to feed during the dark portion of the 12:12 cycle. The size of mussel offered to each fish was between 4.4mm – 9.0mm in valve length. Mussel introductions were done each day for the duration of the experiment. A second set of trials with three Freshwater Drum was conducted at Neosho National Fish Hatchery in a flow through circular tank with spring-fed hatchery discharge

water at ambient temperature. The fish were initially fed live fingerling Rainbow Trout (*Oncorhynchus mykiss*) for approximately 3 weeks and then starved for 1 week prior to the feeding experiment. After the starvation period, each fish was offered a primer of deshelled Fatmucket for one week and then offered one live Fatmucket per event during the course of the feeding experiment. The size of mussel offered to each fish was between 4.5mm – 12.5mm valve length. If not eaten after 48 hours, mussels were removed and replaced as described above. This test group was also on 12:12 light cycle. The size of the mussel offered to each fish was randomly selected and initially varied from day to day.

Towards the end of each experiment the size of mussels offered was focused on establishing what size of mussel was too large for individual fish to ingest. Mussels were offered to the fish for up to 48 hours, immediately followed by the introduction of a different sized mussel.

Prior approval for this project was obtained from the Missouri State University IACUC (February 14, 2011; approval #10025).

Results

The results of the host tests indicated that Freshwater Drum was not a suitable host for 5 of the nine species tested. Fatmucket, Neosho Mucket, Black Sandshell, Round Pigtoe, and Plain Pocketbook produced no juveniles, and only showed slight differences in the length of the time of encystment before sloughing. The results are summarized as follows: six Freshwater Drum were inoculated with 70% viable Fatmucket glochidia. Sloughing of Fatmucket glochidia was observed for 16 days with 50% of sloughs recovered by the second day and 90% recovered by day four (Figure 1).

Six Freshwater Drum were inoculated with 85% viable Black Sandshell glochidia. Sloughing of Black Sandshell glochidia was observed for 16 days with 50% of sloughs recovered on the first day and 90% recovered by day three (Figure 2). Nine Freshwater Drum were inoculated with 90% viable Neosho Mucket glochidia. Sloughing of Neosho Mucket glochidia was observed for 10 days with 90% recovery by day two (Figure 3). Two Freshwater Drum were inoculated with 73% viable Round Pigtoe glochidia. Sloughing of Round Pigtoe glochidia was observed for 15 days with 50% of sloughs collected on day one and 90% by day two (Figure 4). Six Freshwater Drum were inoculated with 97% viable Plain Pocketbook glochidia. Sloughing of Plain Pocketbook glochidia was observed for 15 days with 50% of sloughs collected by the second day and 90% by day four (Figure 5).

The host tests resulted in successful juvenile transformation for four of the nine mussel species tested. These were Pink Mucket, Threehorn Wartyback, Fragile Papershell, and Deertoe. The latter two are considered to be drum specialists (Barnhart et al., 2008; Sietman et al., 2018). Six Freshwater Drum were inoculated with 78% viable Pink Mucket glochidia. Five of the six fish produced viable juveniles. Sloughing of glochidia and juvenile mussels was observed for 19 days with 50% of sloughs collected by day 2 of the trial and 90% collected by day six (Figure 6). The length of juvenile sloughing was observed for 9 days. The %M for the six fish was collectively 6.5%. Individual fish had a %M ranging from 0% to 47.3% (Figure 7).

Six Freshwater Drum were inoculated with 55% viable Threehorn Wartyback glochidia. Three of the six fish produced viable juveniles. Sloughing of glochidia and juvenile mussels was observed for 25 days with 50% of sloughs collected by day 7 of the

trial and 90% by day 14 (Figure 8). The length of juvenile sloughing was observed for 14 days. The %M for the six fish was collectively 11.7%. Individual fish had a %M ranging from 0% to 46.1% (Figure 9).

Six Freshwater Drum were inoculated with 93% viable Fragile Papershell glochidia. All of the six fish produced viable juveniles. Sloughing of glochidia and juvenile mussels was observed for 44 days with 50% of sloughs collected by day 33 of the trial and 90% by day 37 (Figure 10). The length of juvenile sloughing was observed for 16 days. The %M for the six fish was collectively 98.1%. Individual fish had a %M ranging from 95.3% to 98.8% (Figure 11).

Six Freshwater Drum were inoculated with 55% viable Deertoe glochidia. All of the six fish produced viable juveniles. Sloughing of glochidia and juvenile mussels was observed for 30 days with 50% of sloughs collected by day 19 and 90% by day 21 of the trial (Figure 12). The length of juvenile sloughing was observed for 14 days. The %M for the six fish was collectively 88.9%. Individual fish had a %M ranging from 78.2% to 97.3% (Figure 13).

The experiment started with eight Freshwater Drum of two size classes. Three fish refused to eat the de-shelled mussels and were dismissed from the experiment. Two of the fish that ate de-shelled mussel would not eat live mussels and were also dismissed from the experiment. The other three fish ate live Fatmucket of varying sizes. Fish that did not eat mussels were only measured for length. Fish that ate mussels were measured for length and mouth gape. The size of the mussel at each feeding event was recorded for the length of the study, as well as whether the mussel was ingested or refused. A summary of the length and gape measurements of fish and the spectrum of shell sizes

ingested is provided (Table 2). The feeding events were only witnessed by researchers on three occasions, as most of these events happened during the dark portion of the 12:12 light cycle. On all three occasions the fish ingested the live mussel whole. On one occasion the fish attempted to ingest the mussel several times before successfully eating the live mussel whole. No fresh fragmented shell material was found in the tank or in the filter, except for particulate valve material intermixed with fish feces that was occasionally found in the filter. Each fish that ate mussels accepted mussels only below a certain size.

Discussion

Host trials for Fatmucket, Neosho Mucket, Plain Pocketbook Black Sandshell, and Round Pigtoe, were unsuccessful in producing juvenile mussels. The majority of the glochidia of these mussels were sloughed within the first three days of the trials. These drum were reared in captivity, excluding the possibility of acquired immunity and indicating that the fish were innately immune to the glochidia of those species. Fatmucket, Neosho Mucket, Plain Pocketbook and Black Sandshell, all have mantle lures that are used to attract their piscivorous fish hosts, and they utilize primarily predatory host fish in the families Centrarchidae and Percidae. Previous laboratory trials have indicated potential hosts of Fatmucket including 8 species of Centrarchidae, 2 species of Cyprinidae, 3 species of Percidae, and 1 species of Moronidae (Watters 1994). Reported hosts for Black Sandshell include 10 species of Centrarchidae, 3 species of Cyprinidae, 3 species of Percidae, and 1 species of Moronidae (Steg and Neves 1997; Watters 1999; Khym and Layzer 2000). Hosts for Neosho Mucket include three species of *Micropterus*

(Centrarchidae) (Barnhart and Roberts 1997). Plain Pocketbook hosts include 5 species of Centrarchidae, three species of Percidae, and the Banded Killifish (*Fundulus diaphanous*) (Watters 1997). Unlike the *Lampsilis* and *Ligumia* mussel species, Round Pigtoe utilizes small conglutinates that attract non-predatory hosts and lack a mantle lure. Reported potential hosts of Round Pigtoe include six species of Cyprinidae and one species of Centrarchidae (Hove et al. 1995).

Host trials for Pink Mucket, Threehorn Wartyback, Fragile Papershell, and Deertoe produced significant numbers of metamorphosed juveniles. The host trials for Threehorn Wartyback resulted in the successful transformation of 121 juvenile mussels on three of the six Freshwater Drum that were tested. The %M of the three fish that produced juvenile mussels ranged from 46% to less than 1% with one fish producing 93% of the total juveniles transformed. The primary host for Threehorn Wartyback is unknown, so this mussel was explored further by testing with other fish species (See Chapter 2).

The success of Pink Mucket (*Lampsilis abrupta*) on drum was a surprising result, because the other 3 *Lampsilis* species tested did not produce juveniles. Pink Mucket trials resulted in 442 juvenile mussels from five of the six fish that were tested. However, %M varied greatly among individual fish, with 95% of juveniles produced by a single fish. Four other fish had a much lower %M with each fish producing less than 10 juvenile mussels and one fish not producing any juveniles. The host trial for Pink Mucket was noticeably different from drum specialists by having a shorter encystment period of eighteen days and a shorter juvenile drop window of nine days. Previously known hosts of Pink Mucket include four species of Centrarchidae and two species of Percidae as

potential hosts. These are Smallmouth Bass (*Micropterus dolomieu*), Spotted Bass (*Micropterus punctulatus*), Largemouth Bass (*Micropterus salmoides*), White Crappie (*Pomoxis annularis*), Sauger (*Sander canadensis*), and Walleye (*Sander vitreus*) (Barnhart et al. 1997). Like the other *Lampsilis* species and *Ligumia recta*, Pink Mucket has a prominent mantle lure that attracts these predatory hosts.

Freshwater Drum is the primary host of Fragile Papershell and Deertoe (Cummings 1993; Barnhart et al. 2008; Sietman et al. 2018). Wilson (1916) recorded the Sauger (*Stizostedion canadense*) and Freshwater Drum as known hosts for Deertoe, but did not report if they were observed in natural infection or in lab trials (Fuller 1974). These species, and the majority of mussel species that have an established relationship with Freshwater Drum do not have obvious mantle lures but may use a visual display that involves 'flashing' the marsupia by abruptly retracting the mantle margin (Sietman et al. 2018). These displays and associated behaviors may function as host-attracting lures similar to other species of Lampsilini.

The Fragile Papershell mussel has been reported to utilize Freshwater Drum since malacologists first began the investigation of the host-parasite relationship between fish and mussels a century ago. Wilson first observed natural infestation of Fragile Papershell on Freshwater Drum in 1916. Research on Fragile Papershell has not been successful in establishing a host other than Freshwater Drum (Howard and Anson 1922; Fuller 1974; Barnhart et al. 1998; Sietman et al. 2018). The results of this study are typical of the drum specialist relationship, which exhibits two basic characteristics. The first characteristic is that the %M was very high. %M for each individual fish was over 95% in a cohort that produced 8097 juvenile mussels with only 155 non-transformed sloughed glochidia. The

second characteristic is a long length of encystment. The mean length of encystment in this experiment was 44 days with the first appearance of juveniles on day 27. The time of encystment on Freshwater Drum varied greatly for mussel species that produced juvenile mussels, with drum specialist species having a much longer encystment time than species that use Freshwater Drum as an intermediate host. This may be due to the fact that drum specialists often have glochidia that grow while encysted on the host, whereas the glochidia of generalist species typically do not noticeably grow during encystment (Barnhart et al. 1998).

Deertoe also have the characteristics of a drum specialist. This study produced 15,388 juvenile mussels with 1,913 non-transformed sloughed glochidia for a %M of 88.94% for the cohort. The length of encystment for this cohort was 30 days with the first juveniles appearing on day 16. Deertoe glochidia is also relatively small, from 60-75 microns in length (Lefevre and Curtis 1910) and grow during encapsulation (Barnhart et al. 2008). The valve is solid at adulthood, but remains small with an overall length of 83mm (Williams et al. 2008). The glochidia for Fragile Papershell also notably grew in size throughout the course of the experiment (Barnhart et al. 2008), although no measurements were taken.

Scaleshell (*Leptodea leptodon*) is a close relative of Fragile Papershell and is also considered to be a drum specialist. Freshwater Drum is the only known host of Scaleshell, which is classified by the U.S. Fish and Wildlife Service as federally endangered. Scaleshell also demonstrates the characteristics of high %M and long length of encystment with glochidial growth associated with Fragile Papershell (Barnhart et al. 1998; 2008). Although Fragile Papershell and Scaleshell have an overall length that can

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exceed 120mm at adulthood, they have thin and brittle valves and small glochidia (Williams et al. 2008). These characteristics can be considered conducive for a self-sacrifice technique used to introduce their glochidia to the Freshwater Drum. Upon consuming mussels from the genus *Leptodea*, Freshwater Drum pharyngeal teeth could easily masticate valve material and rupture the marsupial gills, exposing the fish to the mussel's glochidia. The small size of the mussel's glochidia could serve as an adaptation to maximize infestation of fish gills and to increase the amount of glochidia a gravid female is capable of brooding.

Further investigation into the potential of Freshwater Drum to serve as a host to native mussels is needed to fully realize the role of this species in the natural life cycle of mussels and to be able to fully utilize the species in laboratory propagation techniques. Further laboratory host trials are needed to determine the frequency and specificity of host-parasite relationships between Freshwater Drum and other mussel species. The need to identify natural infestations of larval mussels on Freshwater Drum is also very important in determining if the host-parasite relationship is natural or only exists under laboratory conditions.

In the feeding experiment, small Freshwater Drum exhibited a varied interest level in freshwater mussels as a food source. Three of the eight fish tested ingested whole mussels of different sizes on multiple occasions. Feeding Freshwater Drum in captivity can be challenging, depending on the disposition of the fish (personal observation). Some fish refused to eat any food item offered to them from chironomid larvae to annelid worms to de-shelled Asiatic Clam, while other fish would eat virtually any food item that was introduced. Live mussels were offered to the fish during the light portion of the

12:12 light cycle; but were not removed for at least 24 hours, ensuring that the fish would have an opportunity to feed during the dark portion of the 12:12 cycle.

The small number and size range of fish tested in the present study does not provide much basis for inferring the role of gape limitation in drum feeding on native mussels. However, it is interesting that the smallest fish that ate mussels ingested smaller mussels than the larger fish. In each case, the largest mussel ingested had a shell height of approximately ½ of the maximum horizontal gape (Table 2). Schael et al. (1991) documented that Freshwater Drum have one of the largest gapes found among larval fish and showed a positive correlation between gape and prey length. Gape limitation of Freshwater Drum would not be a factor for the five fish that did not eat mussels, considering they were offered a variety of mussel sizes with some being too small for gape limitation to affect.

The behavior of eating bivalves may develop as fish become larger, and involve both morphological and learning components. There are suggestions that the diet of Freshwater Drum does not shift from arthropods to mollusks until around a total length of 384mm (Davis-Faust 2012). This could reflect the development of the fish's pharyngeal teeth. I observed that the drum apparently ingested the mussels whole, or at least did not egest broken shell fragments. The pharyngeal teeth of small drum may not be adapted for crushing. The pharyngeal teeth, which are located in the throat, are continually being replaced and go through several stages of development as the fish ages. The first pharyngeal teeth of young Freshwater Drum are referred to as cardiform teeth and are small, close set, and pointed. The cardiform teeth are replaced as the fish ages by more elongate, pointed villiform teeth. The blunt adult pharyngeal teeth are referred to as

molariform teeth and are the largest and most durable of the developmental stages. Most Freshwater Drum larger than 265mm in total length exhibit molariform teeth (French and Bur 1992).

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TABLES

Table 1. Chapter 1 Mussel Origin Data.

Mussel Origin			
Location of Collection	Genus/species	Date	n
Silver Fork River, Callaway Co., MO	*Lampsilis siliquoidea*	12-8-2012	3
Kansas City Zoo, Kansas City, KS	*Lampsilis abrupta*	6-10-2013	1
Kansas City Zoo, Kansas City, KS	*Ligumia recta*	6-10-2013	3
Spring River, Cherokee Co., KS	*Lampsilis rafinesqueana*	7-11-2013	3
Spring River, Cherokee Co., KS	*Pleurobema sintoxia*	7-11-2013	1
Meramec River, St. Louis Co., MO	*Obliquaria reflexa*	8-1-2013	3
Sac River, Cedar Co., MO	*Leptodea fragilis*	2-5-2014	1
Sac River, Cedar Co., MO	*Lampsilis cardium*	3-20-2014	1
Sac River, Cedar County, MO	*Obliquaria reflexa*	7-23-2014	3
Genoa National Fish Hatchery	*Truncilla truncata*	6-10-2014	3

Table 2. Fish Feeding Summary.

			Feeding Data		
Fish #	Total Length (mm)	Standard Length (mm)	Mouth Gape vertical x horizontal (mm)	Largest Mussel Consumed length x height x width (mm)	Smallest Mussel Consumed length x height x width (mm)
1	362	318	30.2 x 22.0	30.9 x 11.3 x 10.0	12.9 x 7.4 x 4.1
2	390	332	32.5 x 22.7	29.9 x 11.0 x 9.7	10.2 x 6.2 x 2.9
3	190	141	13.3 x 11.5	9.0 x 5.9 x 3.0	4.4 x 2.8 x 1.7
4*	208	161	N/A	N/A	N/A
5**	177	131	N/A	N/A	N/A
6*	352	313	N/A	N/A	N/A
7*	165	118	N/A	N/A	N/A
8**	161	123	N/A	N/A	N/A

*ate primer, did not eat live mussels **did not eat primer or live mussels

FIGURES
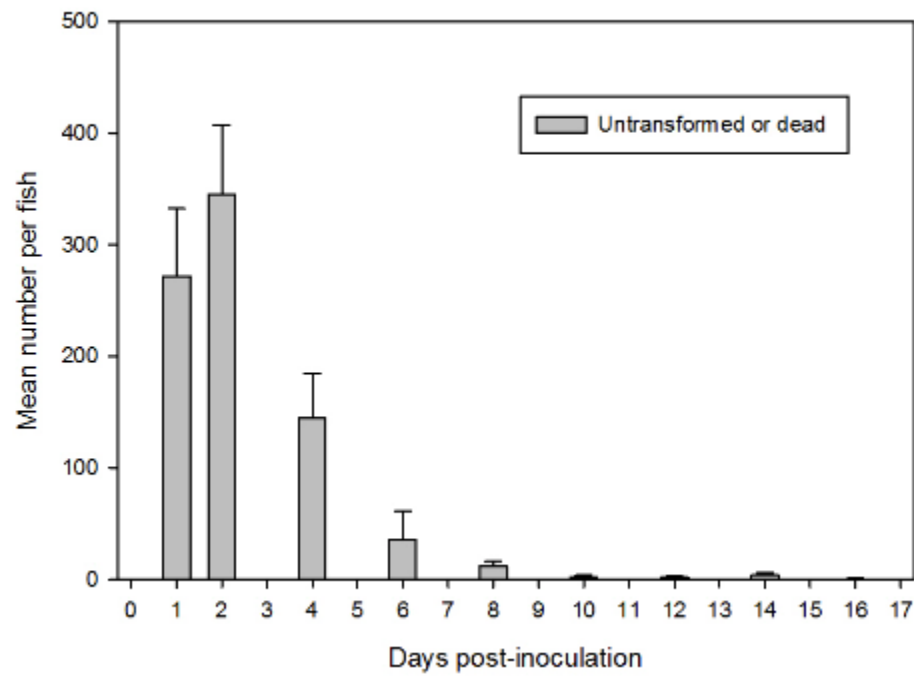
Figure 1. Recovery of Fat Mucket sloughs. Mean and standard deviation, n=6 fish.
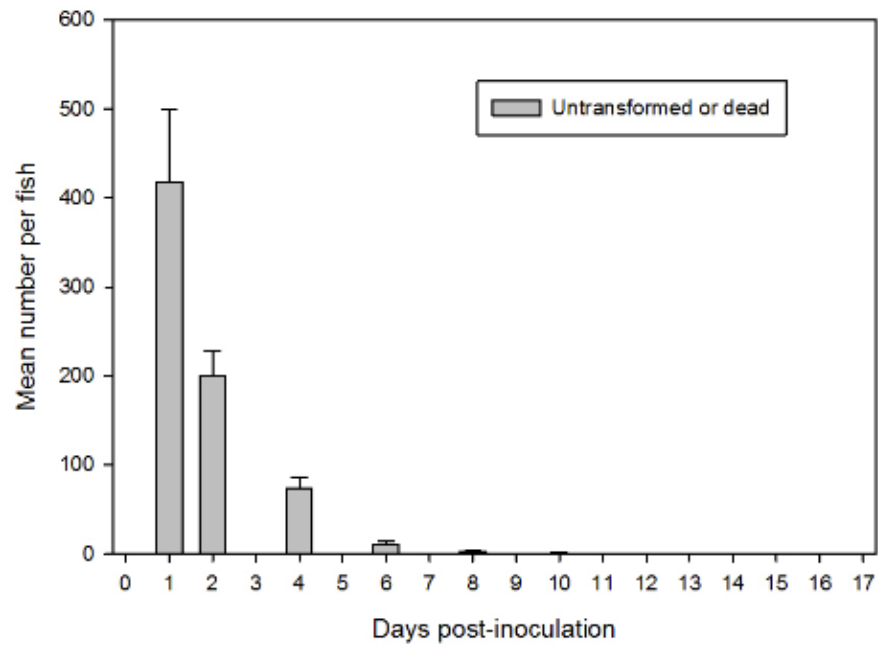
Figure 2. Recovery of Black Sandshell sloughs. Mean and standard deviation, n=6 fish.
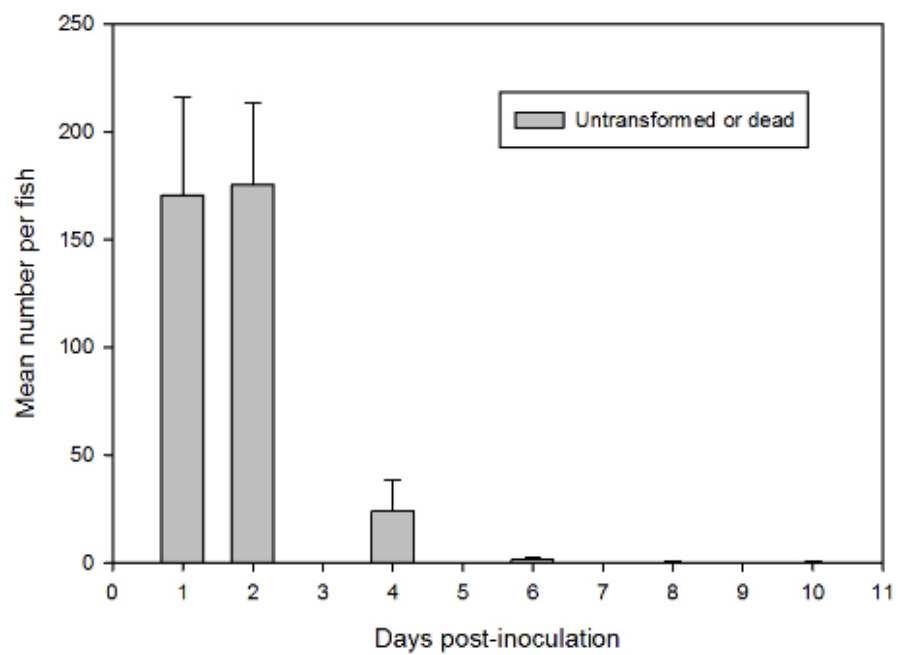

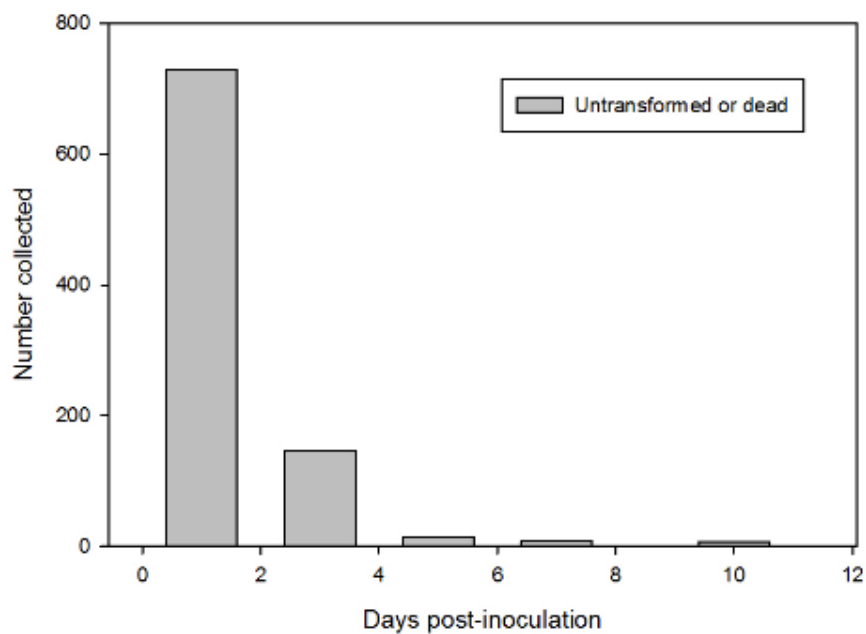Figure 3. Recovery of Neosho Mucket sloughs. Mean and standard deviation, n=9 fish

Figure 4. Recovery of Round Pigtoe sloughs. Mean and standard deviation, n=2 fish.
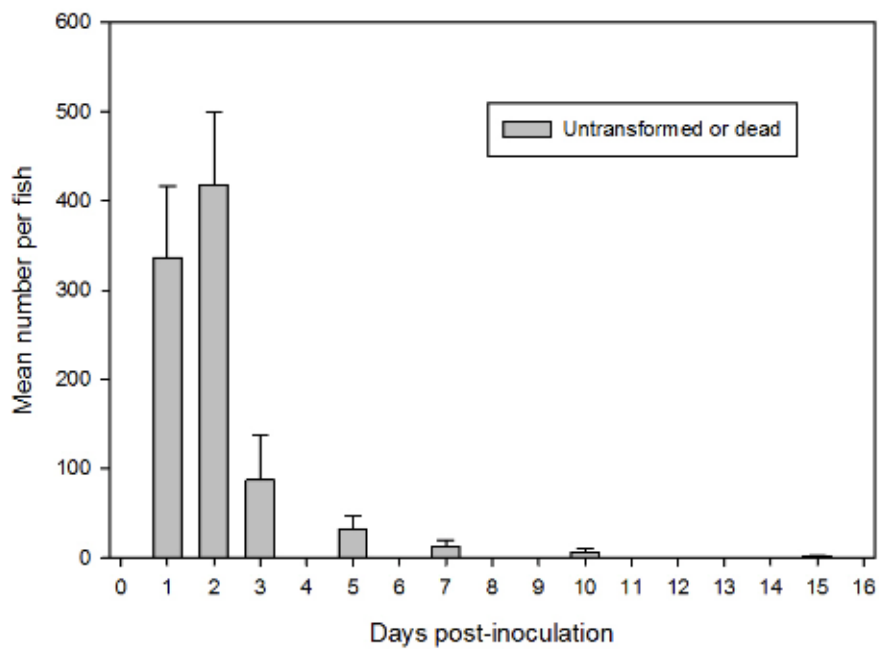

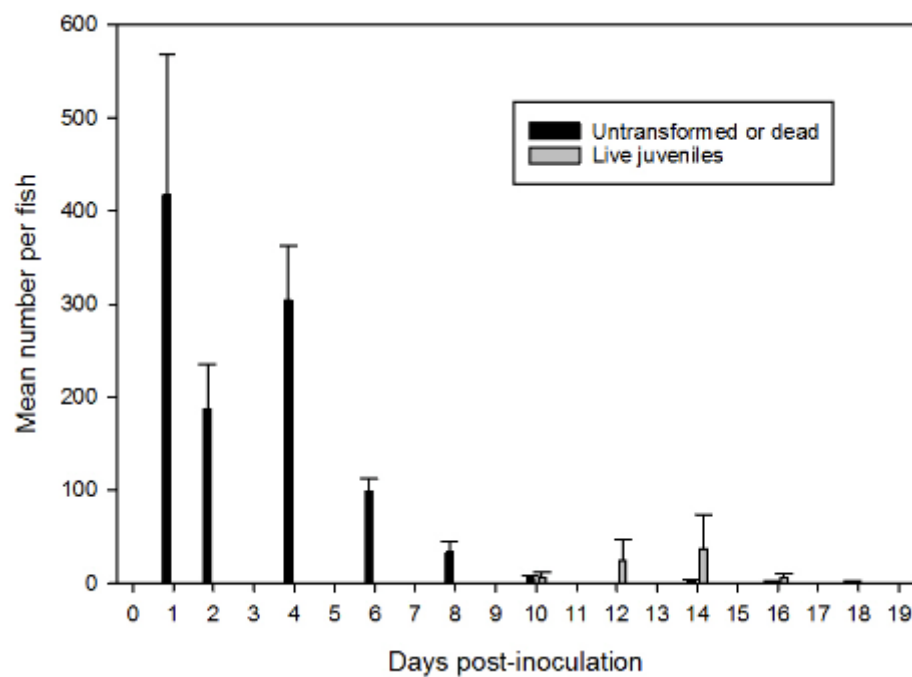Figure 5. Recovery of Plain Pocketbook sloughs. Mean and standard deviation, n=6 fish.

Figure 6. Recovery of Pink Mucket sloughs and metamorphosed juveniles. Mean and standard deviation, n=6 fish.
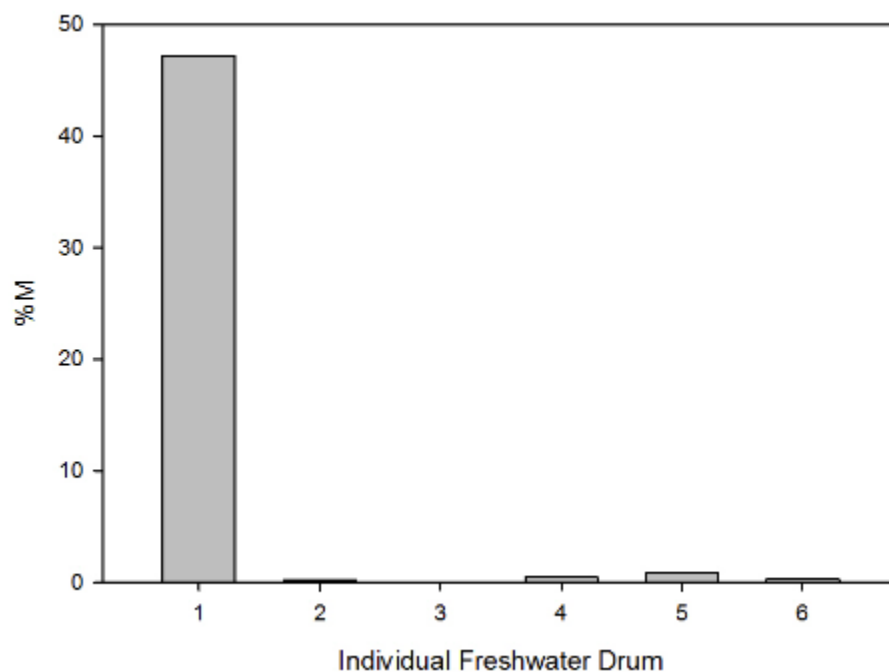

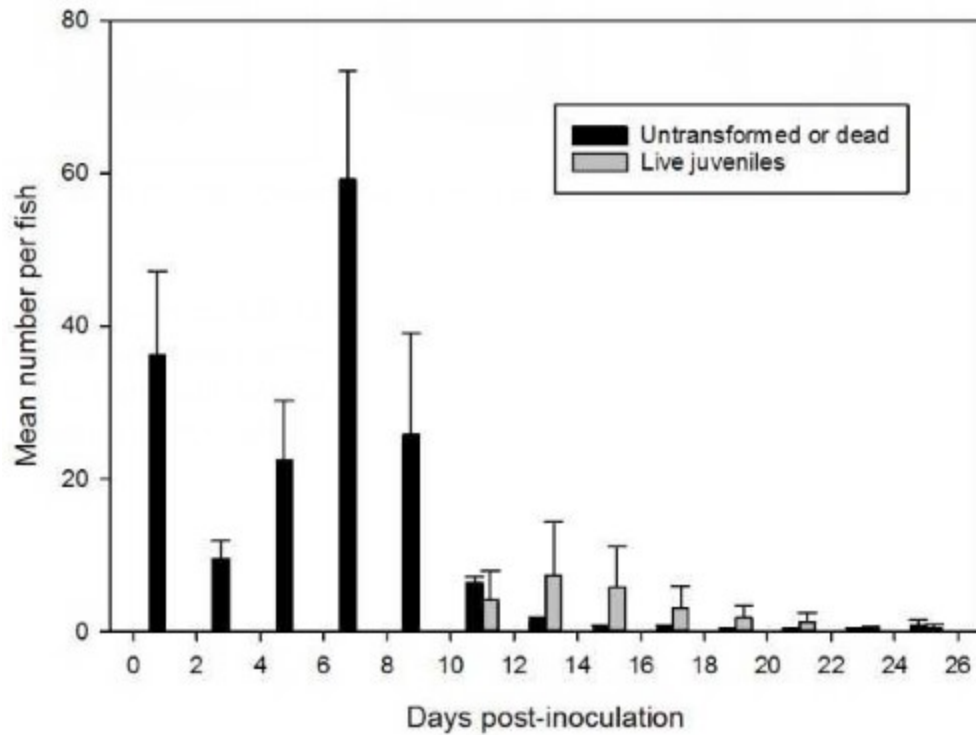Figure 7. Pink Mucket percent metamorphosis on individual Freshwater Drum.

Figure 8. Recovery of Threehorn Wartyback sloughs and juveniles. Mean and standard deviation, n=6 fish.
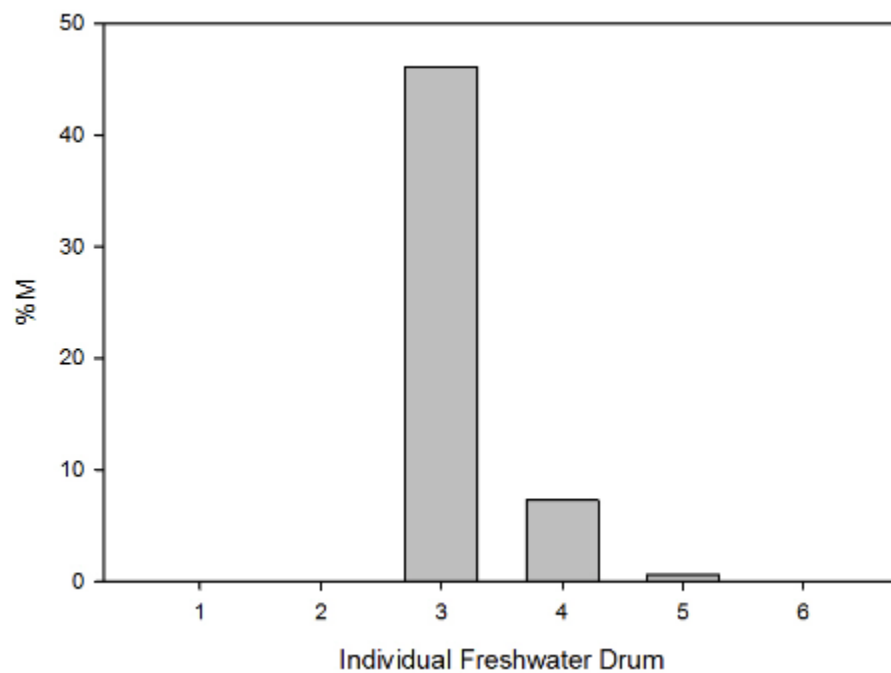

Figure 9. Threehorn Wartyback percent metamorphosis on individual Freshwater Drum.
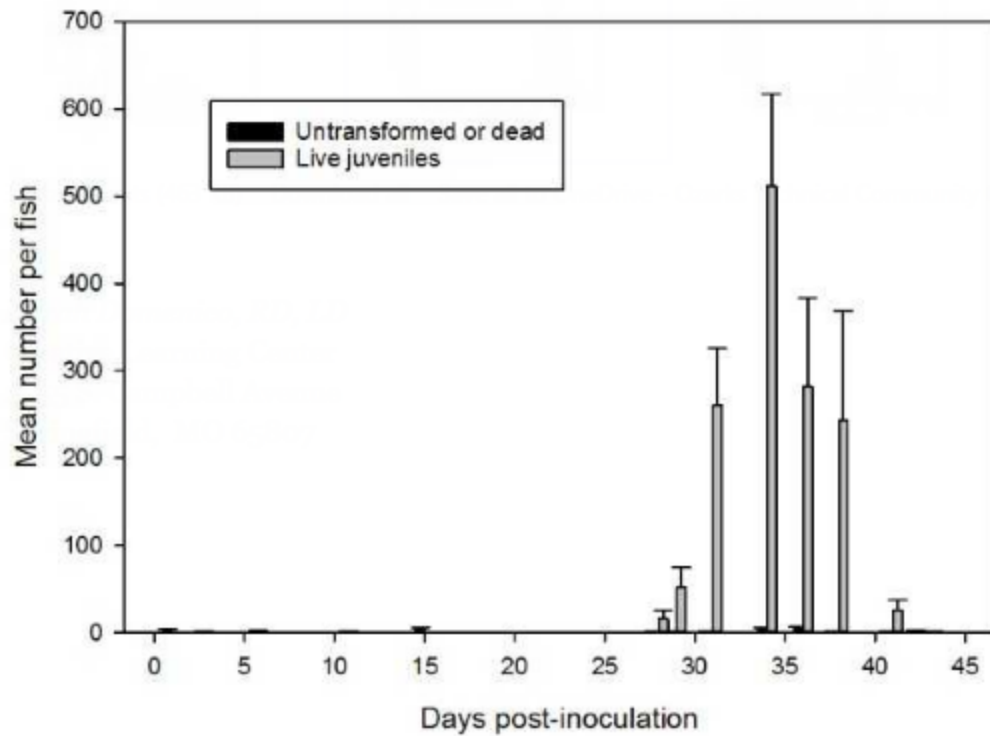
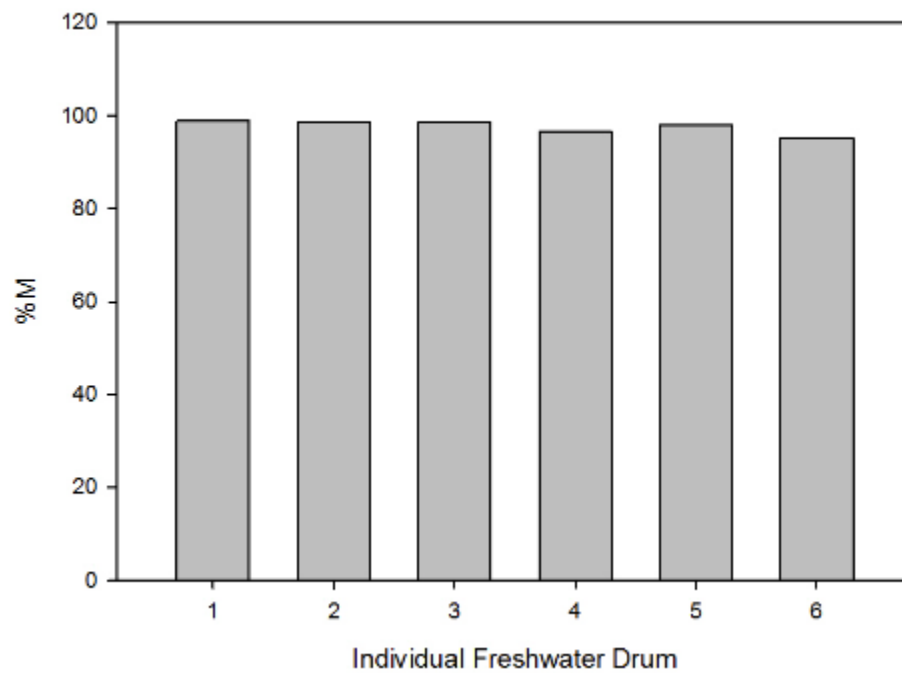Figure 10. Recovery of Fragile Papershell sloughs and juveniles. Mean and standard deviation, n=6 fish.

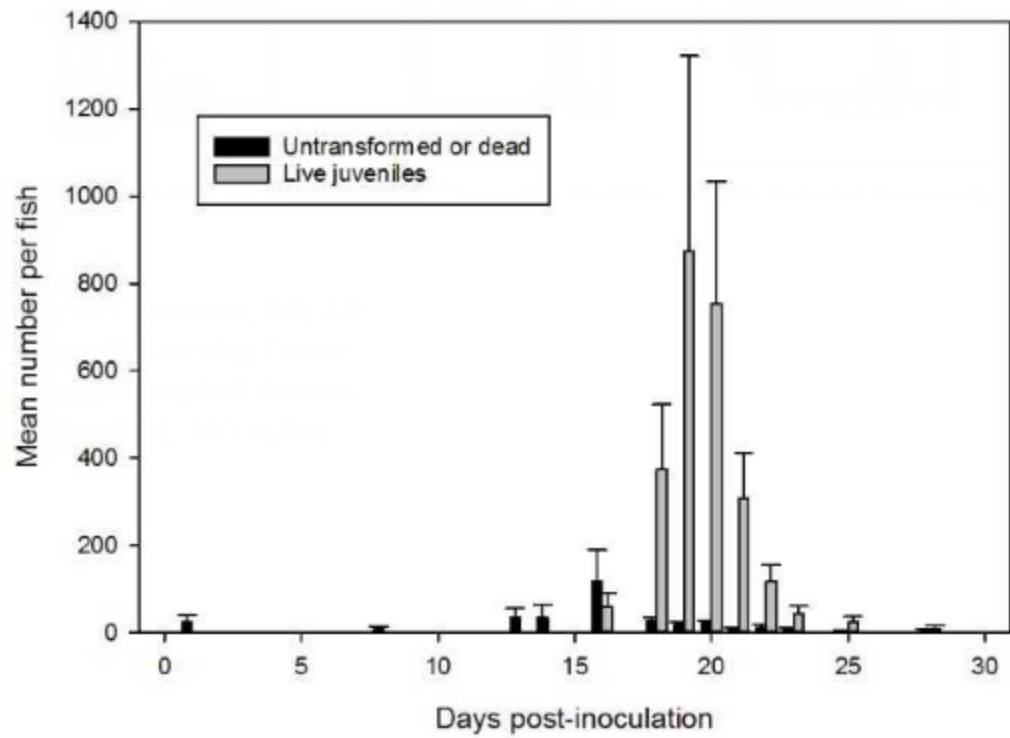Figure 11. Fragile Papershell percent metamorphosis on individual Freshwater Drum.

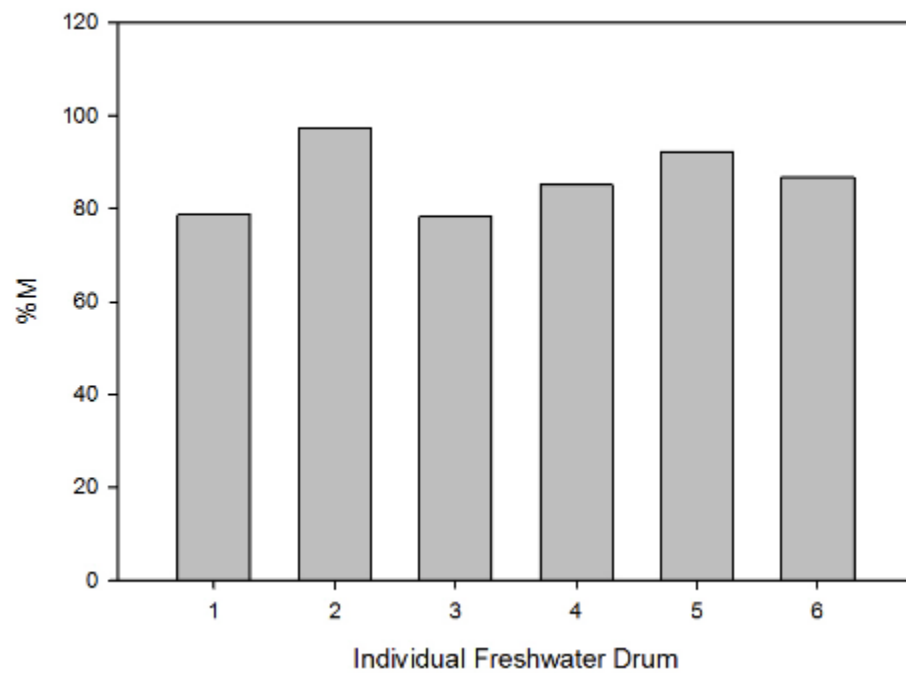Figure 12. Recovery of Deertoe sloughs and juveniles. Mean and standard deviation, n=6 fish.

Figure 13. Deertoe percent metamorphosis on individual Freshwater Drum.

CHAPTER 2: HOST TESTS WITH *OBLIQUARIA REFLEXA*

Introduction

Threehorn Wartyback (*Obliquaria reflexa*) is a member of the freshwater bivalve Order Unionida, Family Unionidae, and Tribe Lampsilini (Campbell et al. 2005; Lopez-Lima et al. 2016). Among the species in this tribe, Threehorn Wartyback is relatively medium sized (to 80mm), slow growing, moderately inflated in shape, and very thick-shelled. All specimens have a single medial row of knobs radiating from the umbo to the ventral margin. The knobs alternate in position from left to right valve (Figure 14). Shell sculpture and color vary greatly, even within a population. The mussel is dioecious, but the shell lacks sexual dimorphism (Watters et al. 2009).

Threehorn Wartyback is geographically widespread and common throughout the large rivers of the Midwestern U.S. Its range includes most of the Mississippi River drainage from Western Pennsylvania to Eastern Kansas and from Lake Erie to the Mobile Basin on the Gulf Coast (Cummings and Mayer 1992). Threehorn Wartyback can be considered somewhat of a habitat generalist, occupying a wide range of depths in a variety of habitats. They can be found at depths of less than 1 meter to over 7 meters, in substrate ranging from sand and mud to cobble. They are known to tolerate slack water or swift currents (Parmalee and Bogan 1998). This species can be locally abundant in reservoirs and is sometimes known to quickly populate newly-formed impoundments (Parmalee and Hughes 1993). From a conservation standpoint, the species is currently considered stable throughout most of its range (NatureServe 2015). Female mussels brood their young in specialized regions of their gills known as marsupia. Unlike most

Lampsilini, Threehorn Wartyback broods from June to August. Most other Lampsilini spawn in the late summer or fall and brood their larvae until the following spring or summer (Barnhart et al. 2008). In Threehorn Wartyback the marsupia are made up of two to nine water tubes in the middle of each of the outer demibranchs (Haag and Staton 2003) (Figure 15).

The glochidia of Threehorn Wartyback are subrotund and symmetrical with a dorsal margin that is slightly curved outward. The lateral dimensions are approximately 211 x 218 microns (length x height) (Barnhart et al. 2008). Glochidia are released in well-formed, solid, club-shaped conglutinates that are white in color and sink in water (Culp et al. 2011). Conglutinates are aggregates of eggs, formed as molds in the water tubes of the female demibranch (Lefevre and Curtis 1912). Threehorn Wartyback releases a small number of very large, compact, and unusually solid conglutinates (Figure 16) (Barnhart and Baird 2000; Barnhart et al. 2008). The conglutinate is composed completely of glochidia enclosed in cohesive egg membranes and are bound by a membrane that encases the glochidia in somewhat of an elastic matrix (Williams et al. 2008). The female mussel releases these conglutinates into the water column to elicit a predatory response in the host fish. When the host fish ingests and masticates the conglutinate it causes the rupture of the conglutinate and the release of the individual glochidia, which snap shut on fish gills.

The primary host or hosts for Threehorn Wartyback remain unknown. Previous laboratory trials suggested that potential hosts include: Silverjaw Minnow (*Notropis buccatus*), Common Shiner (*Luxilus cornutus*), and Longnose Dace (*Rhinichthys cataractae*) (Watters et al. 1998). A small number of naturally encysted glochidia of

Obliquaria were observed on Goldeye (*Hiodon alosoides*) (Barnhart and Baird 2000). Definitive determination of a fish host entails both glochidial transformation on the suspected host fish, as well as observed glochidial infestation of the same species of fish under natural conditions. Suggestions that Threehorn Wartyback may not require a fish host (Utterback 1916) have not been substantiated. In the present study, I used laboratory host trials to further investigate the host relationships of Threehorn Wartyback.

Methods

The fish used in this host trial comprise nine species from four fish families, including: one member of the family Sciaenidae, Freshwater Drum (*Aplodinotus grunniens*); two members of the family Ictaluridae, Channel Catfish (*Ictalurus punctatus*) and Blue Catfish (*Ictalurus furcatus*); one member of the family Centrarchidae, Bluegill (*Lepomis macrochirus*); and five members of the family Cyprinidae, Cardinal Shiner (*Luxilus cardinalis*), Whitetail Shiner (*Cyprinella galactura*), Striped Shiner (*Luxilus chrysocephalus*), Common Carp (*Cyprinus carpio*), and Bighead Carp (*Hypophthalmichtys nobilis*). The fish species tested were locally abundant and native except for the Common and Bighead Carp, which are invasive species. Each species is likely to co-occur with Threehorn Wartyback, although they have varied probabilities of naturally encountering conglutinates in the wild because of differences in feeding habits.

Fish species were obtained from the wild, as well as from aquaculture facilities (Table 3). Upon arrival at MSU Freshwater Mussel Lab for testing, fish were held in recirculating aquaria at ambient laboratory temperatures (20ºC – 23C) in moderately hard synthetic freshwater (USEPA, 2002) for a period ranging from days to weeks prior to

infection. During this time the fish were fed approximately 1-2% of their body weight in blood worms (chironomid larvae, Hikari Bio-Pure Brand) daily. Fish were starved from 2 days prior to testing until the testing was considered complete.

All Threehorn Wartyback glochidia used in these studies were obtained by collecting brooding females from the wild (Table 4). The wild caught mussels were maintained in SFW at ambient laboratory temperatures. The mussels were held unfed for a period of days to weeks before testing. One to three brooding female mussels were used for each experiment. Both fish and mussels were controlled on a 12:12 hour light/dark photoperiod.

Threehorn Wartyback conglutinates were manipulated from the marsupium by using a dental pick to perforate the marsupium and lift conglutinates from the water tube. The glochidia were freed from conglutinates by pressing them through a 400 μm screen with a small paintbrush, while spraying them with river water obtained from James River in Greene County, Missouri USA (RW). River water was used because SFW sometimes caused glochidia to close prematurely. Freed glochidia were suspended in a known volume of RW which was subsampled for counting. Uniform suspension was achieved through agitation by a large rubber-bulb pipette. Ten 200 μm sub-samples of the suspension were placed as drops on a plastic Petri plate. The glochidia in each drop were counted and classified as either open or closed using a stereomicroscope at 10.5X. One drop of saturated salt solution salt (NaCl + SFW) was added to each 200 μm sample to induce a closing effect of the glochidia valves (Lefevre and Curtis 1912). Glochidia that were open initially and closed in response to the addition of the salt were considered viable. These sample counts were used to estimate the total number of viable glochidia.

The mussels use in this experiment showed a significant range of viable glochidia with levels that varied from 44%-92% viable. This varying level of viability could possibly be due in part to the methods of freeing the glochidia from conglutinates or due to the varying maturity of conglutinates.

Fish were infected with glochidia by placing them collectively in a bath containing 1L RW/ fish. Glochidia were added for a total concentration of 4000 viable glochidia/liter RW. Homogeneity of the suspension was achieved by stirring and vigorous agitation with a large rubber-bulb pipette. After 15 minutes, fish were netted out and placed into a rinse bath to allow any unattached glochidia to drop from the fish. After rinsing for 10 minutes, fish were netted and placed into individual tanks.

Inoculated fish were housed in individual 9L tanks in an AHAB recirculating aquarium system (Aquatic Habitats, Inc. Apopka, FL). The water was conditioned by mechanical, biological, and carbon filtration and sterilized by ultraviolet radiation before recirculation. Water temperature was monitored daily and averaged 23°C. Each tank received water continuously from a manifold and the overflow was passed through a filter cup with a nylon mesh screen, containing a mesh size that corresponded to the size of the glochidia being tested. The water flow rate through each tank was 0.5L/min. The first and second day after an inoculation and every other day thereafter (unless otherwise noted), the rate of water flow through the tank was increased to 2L/min for ten minutes to flush any particles into the filter cup. Afterwards, the screen was rinsed into a glass dish and examined with a stereomicroscope at 10.5X or 40X to distinguish glochidia from juvenile mussels. Collections with large amounts of glochidia and juveniles were transferred to a Bogorov plankton counting tray. Juveniles were distinguished from

glochidia by foot movement. Glochidia and juveniles from each collection were counted to determine the number recovered from each fish. Infections were considered to be over after two consecutive counts with no juveniles and no glochidia present. At that time the fish were sedated and terminated by spinal cord severance and their gills were dissected to examine for any remaining encystment. The number of glochidia that attached to each fish was approximated as the sum of the sloughed glochidia and metamorphosed juveniles that were recovered. The percent metamorphosis (%M) for each fish was calculated by dividing the number of juveniles by the sum of glochidia and juveniles recovered from that fish.

A 37.9L gallon aquarium filled with SFW was used to house an individual Freshwater Drum for observation of interactions with Threehorn Wartyback conglutinates. Conglutinates were obtained from gravid mussels in the manner described above and introduced into the tank by pipette from the surface of the tank. Conglutinates were presented to a single fish during three separate trials by pipette, letting the conglutinate sink from the top of the tank in an attempt to be visually conspicuous to the fish. These trials were conducted approximately 20 minutes apart on an individual fish on the same day. Freshwater Drum feeding behavior was observed and recorded using a video camera. After the conclusion of the experiment, the water from the tank was filtered through a 100 micron screen to determine if any glochidia were freed from conglutinates during feeding. The Freshwater Drum that was used in this experiment was selected because of its history of feeding on multiple types of food items such as annelid worms, small insects, and mussels that had been introduced in the lab.

Prior approval for this project was obtained from the Missouri State University IACUC (February 14, 2011; approval #10025).

Results

The only metamorphosis of Threehorn Wartyback glochidia that was observed occurred in the host trial with Freshwater Drum, Three of the six Freshwater Drum that were investigated produced juvenile mussels (Figure 17). The Freshwater Drum were inoculated with 55% viable glochidia with a collective %M of 8.5%, sloughing glochidia/juveniles for 24 days (Figure 8). Individual fish had %M varying from 0% to 44.61% (Figure 9). In trials with all other fish species, glochidia attached initially but were unsuccessful in metamorphosis. There was little difference in the length of time for sloughing, with all non-host fish species sloughing 50% of glochidia within the first two days post-inoculation (Figures 18 – 25). A summary of sloughs/glochidia/trials is available in Table 5.

The first attempt at feeding conglutinate to Freshwater Drum was unsuccessful due to the apparent failure of the fish to notice the conglutinate. On the second feeding attempt the fish ingested and masticated the conglutinate for approximately 6 seconds before rejecting it as a food item and expelling it back into the tank. The conglutinate appeared to fragment slightly at one end upon being expelled from the fish, but retained its overall structural integrity and shape. The third feeding attempt resulted in the fish ingesting and masticating 2 different conglutinates at separate times, each for approximately two seconds before rejecting them as food items and expelling them back into the tank. After the experiment, the water was strained through a 100 micron screen to determine if individual glochidia were freed from the conglutinate during the feeding

attempt. Approximately thirteen glochidia were found free from conglutinate. The Drum was later sedated and terminated by spinal cord transection and its gills showed no sign of glochidial infestation.

Discussion

Only a few other studies have reported host tests for Threehorn Wartyback. Watters et al. (1998) recorded metamorphosis on Silverjaw Minnow (*Ericymba buccata*), Common Shiner (*Luxilus chrysocephalus*), and Longnose Dace (*Rhynichthys cataractae*). However, the tests recovered only 1-3 juveniles from each species, so that their status as significant hosts remains questionable. Barnhart and Baird (2000) collected 690 fish of 32 species from the Gasconade and Meramec Rivers in Missouri on June 9-10 1999. The fish were sacrificed and the gill examined for attached glochidia. One individual Goldeneye (*Hiodon alosoides*) was carrying 69 attached glochidia that were reported as Threehorn Wartyback. However the dimensions that were reported for these glochidia (134 x 91 microns) are not consistent with that identification.

These host trials confirm that Freshwater Drum can host Threehorn Wartyback, but making an allowance for the high degree of variation in %M between individual drum they are not likely the primary host. The variation in %M between fish may be some underlying genetic factor or immunological susceptibility within certain individuals but would have nothing to do with an acquired immunity to the glochidia of Threehorn Wartyback, considering these were not wild-caught fish. Besides Freshwater Drum, all of the other fish species tested showed a similar pattern of producing no juvenile mussels

and sloughing at least half of the glochidia in the first two days post-inoculation, which is an expected pattern for attachment of glochidia to non-host species.

The conglutinate of Threehorn Wartyback has been described as a remarkably tough and cohesive conglutinate from which the glochidia can be dislodged only with difficulty (Lefevre and Curtis 1912). The structure of conglutinate is composed almost exclusively of membrane and glochidia and contains no structural eggs. Both the persistence of the egg membranes and the degree of contact among eggs affect the durability of the conglutinate (Barnhart et al. 2008). To free the glochidia from conglutinate, the conglutinate had to be broken up manually by pressing through a screen. This undoubtedly reduced the viability of the glochidia by triggering the closing of valves in some larvae and also by damaging some larvae to the degree that they become non-viable.

Conglutinate structure in Threehorn Wartyback may act as a mechanism for the infection of multiple fish by a single conglutinate. In the conglutinate feeding experiment with Freshwater Drum a single conglutinate was masticated and partially ruptured; however, the overall integrity of the conglutinate remained intact. This observation leads to the assumption that it could be masticated by an additional fish or even multiple fish, ultimately acting as a tool to infest more than one fish with larvae. The purpose and functionality of Threehorn Wartyback conglutinate may remain unknown until a primary host is determined.

The dates of observation of gravid Threehorn Wartyback varied from year to year. In 2013 and 2014, mussels were monitored in Sac River, Cedar County, Missouri USA from early June until Late August with gravid mussels found only in late July and early

August. In 2016, mussels were monitored from early June until late August with gravid mussels found from late June to mid-July. No gravid mussels were found after mid-July. Finding gravid mussels was a challenge in itself, with approximately less than 10% of Threehorn Wartyback found during the monitoring period being visibly gravid. Due to the lack of sexual dimorphism of this species, it was impossible to determine sex of the individuals in the field.

The problematic nature of working with Threehorn Wartyback in the laboratory likely contributes to its unknown host status. The challenge of locating gravid mussels that have a high level of viable glochidia, even in areas of local abundance; as well as the task of separating glochidia from conglutinate matrix were two aspects of this host trial that were much more difficult than would be expected of most species of freshwater mussel. Multiple mussels that appeared to be gravid were collected only to find that their glochidia were unresponsive or unable to attach to fish. This difficulty was also noted by Barnhart and Baird (2000) during their work with Threehorn Wartyback. Continued host trials are needed for this species, as well as surveys of reproductive timing and methods for a more successful mechanism for separating glochidia from conglutinate matrix.

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Table 3. Fish Origin Data.

Fish Origin			
Location of Collection	Fish Species	Collection Date	n
Langston University, Langston, OK	*A. grunniens*	6-1-2013	58
Langston University, Langston, OK	*A. grunniens*	6-5-2014	12
Osage Catfisheries, Osage Beach, MO	*I. punctatus*	7-17-2014	8
Osage Catfisheries, Osage Beach, MO	*L. macrochirus*	7-17-2014	10
Osage Catfisheries, Osage Beach, MO	*C. carpio*	7-17-2014	9
Osage Catfisheries, Osage Beach, MO	*H. nobilis*	7-17-2014	13
Osage Catfisheries, Osage Beach, MO	*I. furcatus*	7-17-2014	10
James River, Greene County, MO	*L. chrysocephalus*	7-31-2014	2
James River, Greene County, MO	*C. galactura*	7-31-2014	1
Spring River, Lawrence County, MO	*L. cardinalis*	8-4-2014	10

Table 4. Chapter 2 Mussel Origin Data.

	Mussel Origin		
Location of Collection	Mussel Species	Collection Date	n
Sac River, Cedar Co., MO	*Obliquaria reflexa*	7-23-2014	3
Meramec River, St. Louis Co., MO	*Obliquaria reflexa*	8-1-2013	3
Pomme de Terre River, Hickory Co., MO	*Obliquaria reflexa*	7-9-2014	3
Meramec River, St. Louis Co., MO	*Obliquaria reflexa*	7-28-2014	2

Table 5. Summary of Chapter 2 Host Tests.

	Chapter 2 Host Test Summary			
Fish Species	Trials	Recovered Glochidia	Recovered Juveniles	%M
Ictalurus furcatus	6	1589	0	0
Ictalurus punctatus	5	637	0	0
Lepomis macrochirus	5	268	0	0
Hypophthalmichthys nobilis	2	27	0	0
Cyprinus carpio	5	225	0	0
Luxilus chrysocephalus	2	226	0	0
Cyprinella galactura	1	103	0	0
Luxilus cardinalis	8	791	0	0
Aplodinotus grunniens	6	1034	121	11.7

FIGURES

Figure 14. Adult Threehorn Wartyback, *Obliquaria reflexa*.

Figure 15. Right marsupial demibranch of Threehorn Wartyback. In this specimen, four water tubes contain conglutinates. M. C. Barnhart photograph.

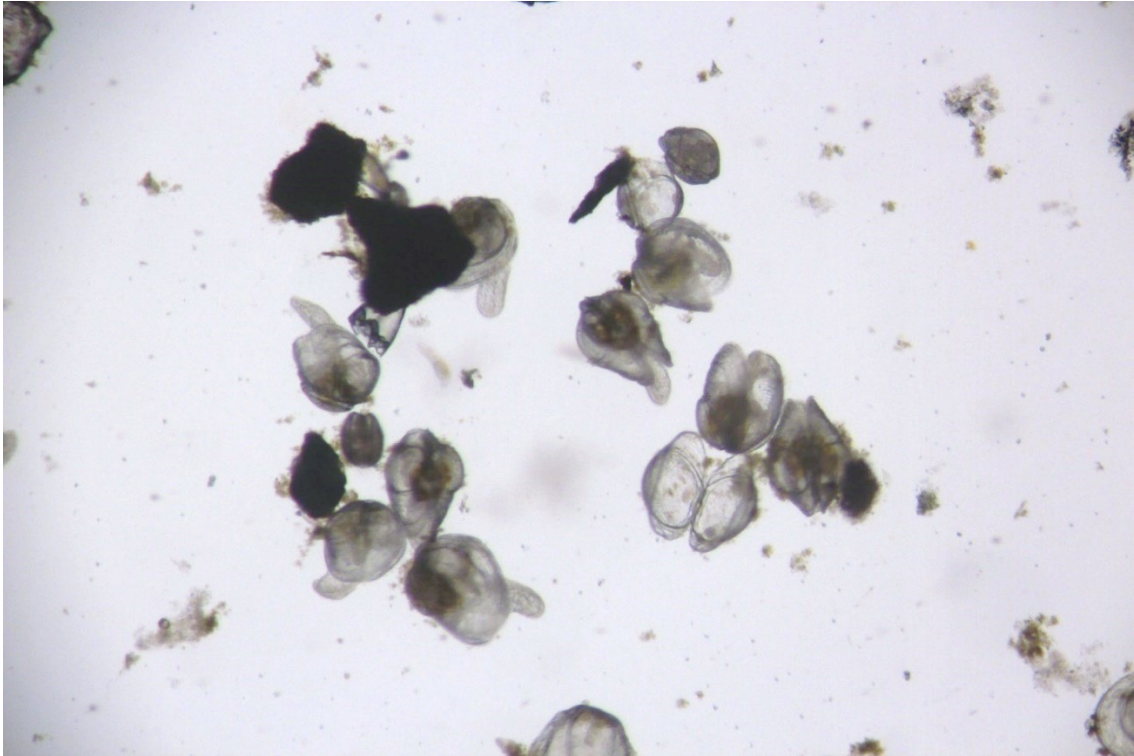Figure 16. Conglutinates of Threehorn Wartyback. M. C. Barnhart photograph.

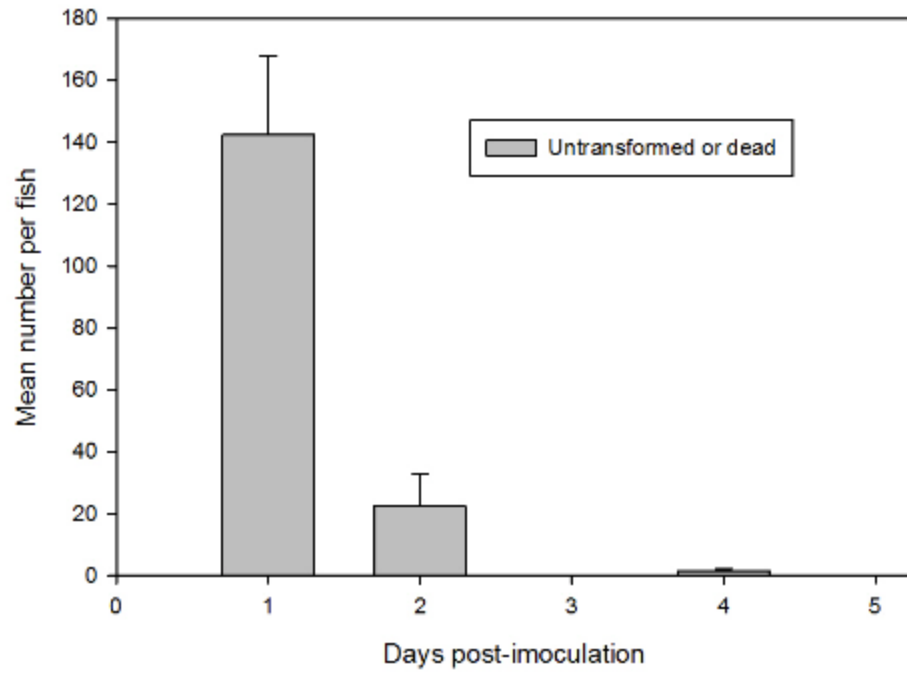Figure 17. Newly metamorphosed Threehorn Wartyback juvenile mussels.

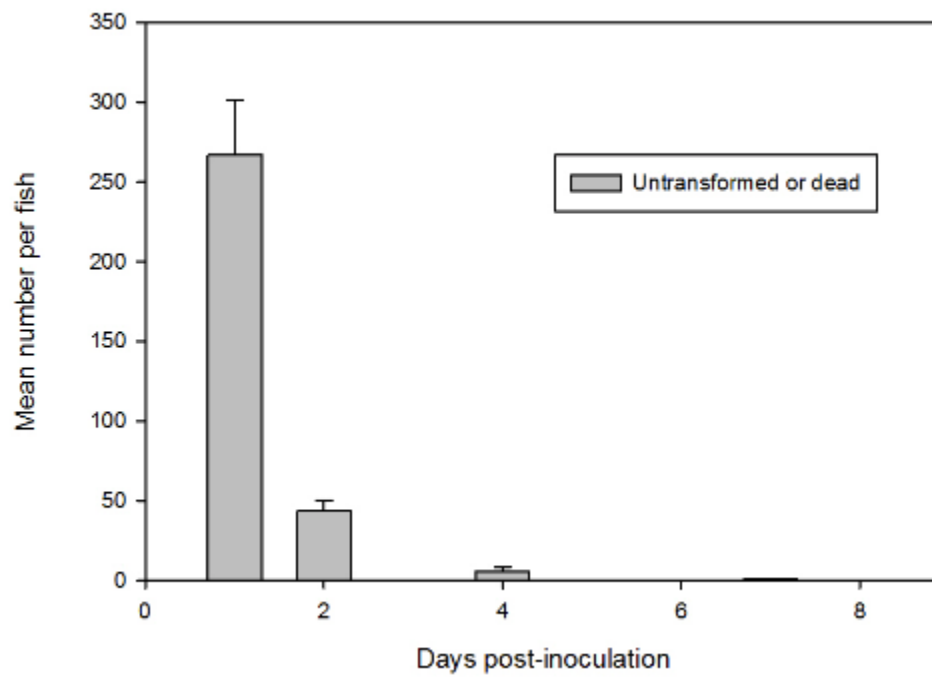Figure 18. Recovery of Channel Catfish sloughs. Mean and standard deviation, n=5 fish.

Figure 19. Recovery of Blue Catfish sloughs. Mean and standard deviation, n=5 fish.
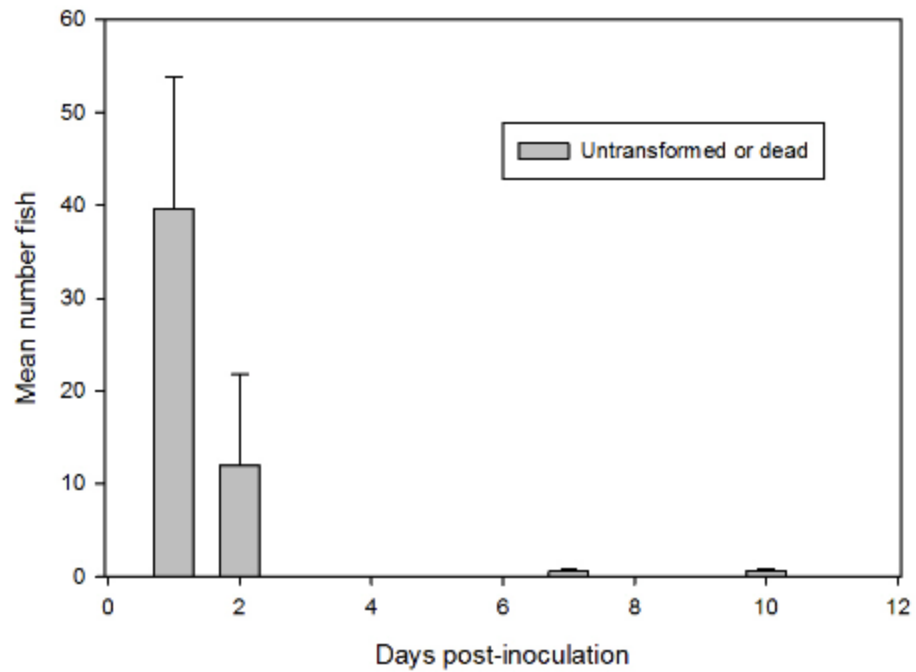
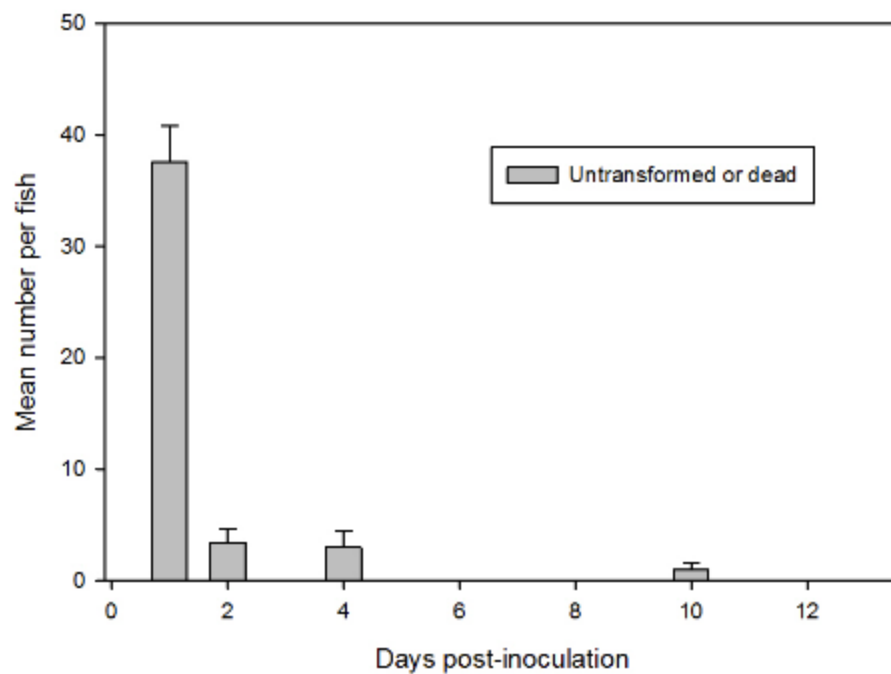Figure 20. Recovery of Bluegill sloughs. Mean and standard deviation, n=5 fish.
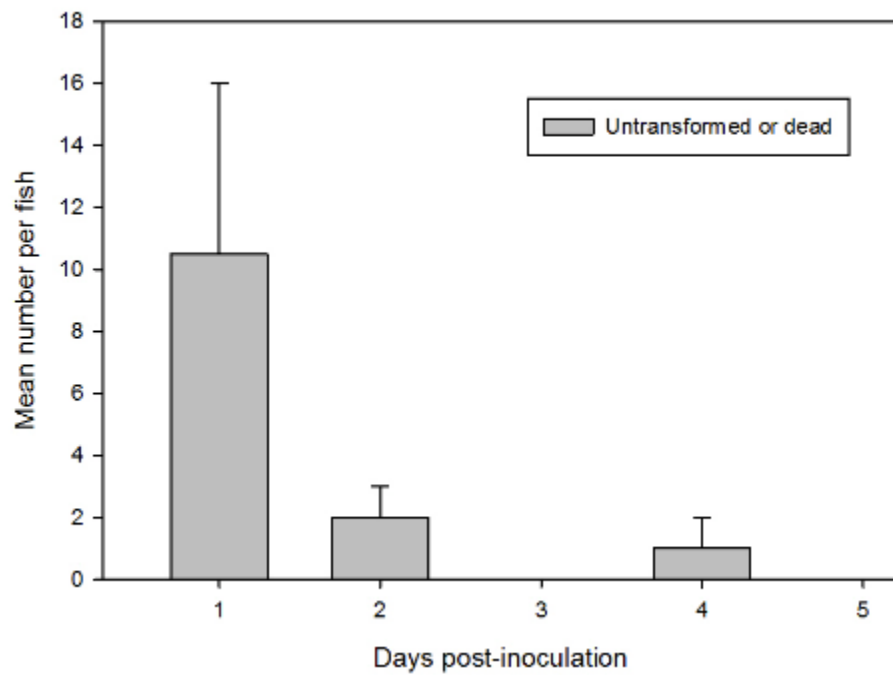
Figure 21. Recovery of Bighead Carp sloughs. Mean and standard deviation, n=2 fish.

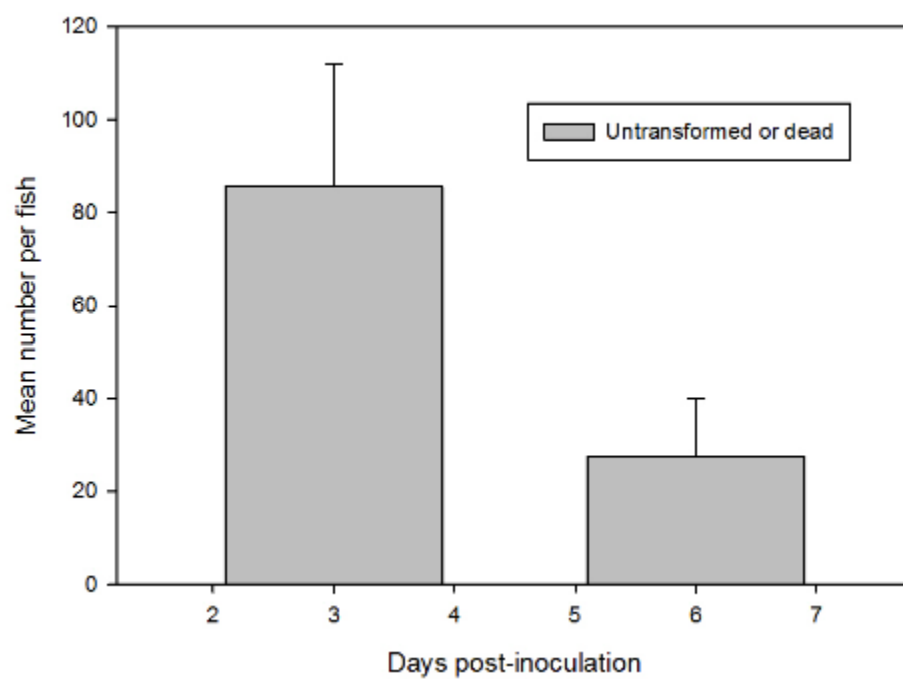Figure 22. Recovery of Common Carp sloughs. Mean and standard deviation, n=2 fish.

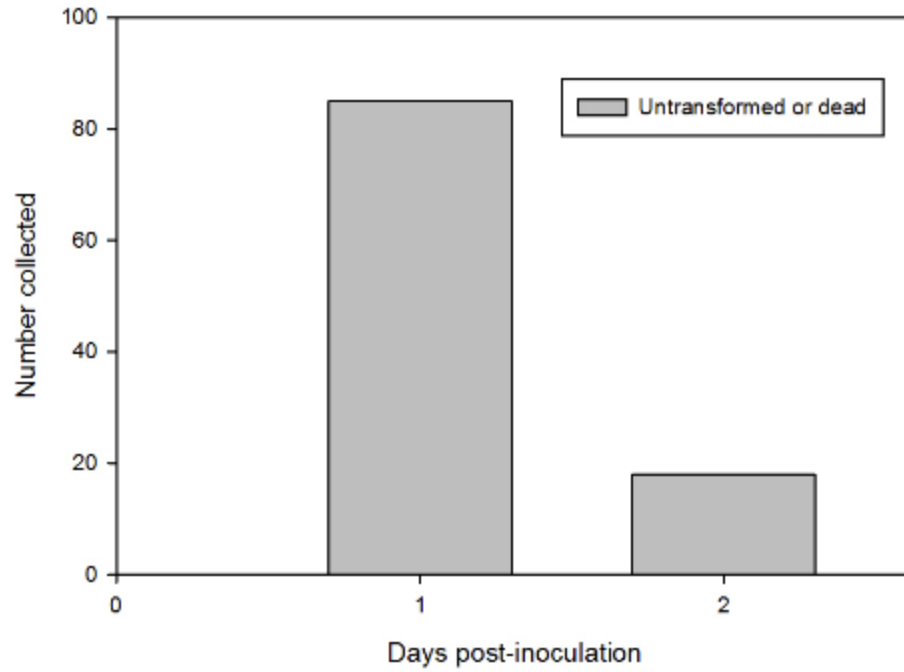Figure 23. Recovery of Striped Shiner sloughs. Mean and standard deviation, n=2 fish.

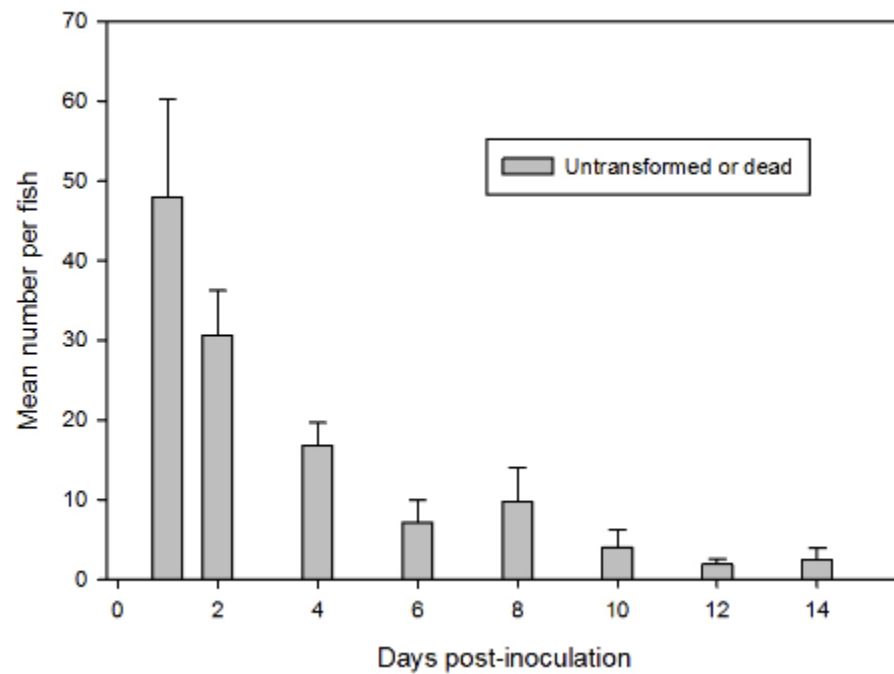Figure 24. Recovery of Whitetail Shiner sloughs. n=1 fish.

Figure 25. Recovery of Cardinal Shiner sloughs. Mean and standard deviation, n=9 fish.