INVESTIGATIONS OF EARLY LIFE HISTORY AND PROPAGATION TECHNIQUES FOR FRESHWATER MUSSELS IN THE ALTAMAHA RIVER BASIN, GA

by

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(Under the Direction of ROBERT B. BRINGOLF)

ABSTRACT

North America has the highest diversity of freshwater mussels (Family: Unionidae) which is currently being threatened by extinction. To properly manage mussel populations knowledge of early life histories is necessary. I identified host fish for seven mussel species residing in the Altamaha River, GA and described glochidia morphology and valve ultrastructure. However, host fish are often unknown or unavailable so it is prudent to evaluate other options for mussel conservation and propagation. The immune system of fish is theorized to determine whether or not mussel glochidia can attach and successfully metamorphose into juveniles. I hypothesized that suppressing the immune system of non-host fish will increase metamorphosis rates. I compared metamorphosis success from fish that were treated with an immunosuppressant drug, Dexamethasone, to fish that were not treated. This approach is promising but requires additional refinement if it will become a viable approach for mussel propagation.

INDEX WORDS: Altamaha River, freshwater mussel, host fish, glochidia morphology, immunosuppression, dexamethasone

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DEDICATION

I would like to dedicate my thesis to my husband Andrew, my two daughters Amara and Rhiannon, and to my mother Kathleen.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Freshwater Mussels

North America has the highest diversity of freshwater mussels (Family: Unionidae) with 297 species, 35 of which are thought to be extinct and 70 currently listed either as threatened or endangered under the Endangered Species Act (USFWS 2007). The southeastern U.S. is especially rich in mussel fauna; 91% of the species in North America is located within this region (Neves et al. 1997; Turgeon 1988). Georgia is home to approximately 127 freshwater mussel species and mussels comprise 21% of the total federally listed animals in the state. Additionally, *Elliptio spinosa* was proposed for formal listing under the ESA in October 2010 and will increase that percentage when formally listed.

Mussels are long-lived, sessile filter feeders, which makes them an integral component of aquatic ecosystems, as well as useful indicators of ecosystem health (Strayer 1999). Freshwater mussels typically comprise the majority of biomass of the zoobenthos (Dame 1996). Mussel beds have been shown to filter 10-100% of the water column in a day (Strayer et al. 1999). The efficiency of filtration often leads to bioaccumulation of heavy metals and other pollutants from the water, which can be detrimental to mussels by altering growth patterns, filtration rates, and reproductive behaviors (Naimo 1995). Contaminants and other factors such as sedimentation, pollution, urbanization, and habitat fragmentation have indeed been detrimental to mussels, resulting in an unparalleled 70% of all mussel species listed as imperiled at the state or

federal level (Williams et al. 1993). Another factor that may play a key role in the decline of freshwater mussels is the loss of host fish; mussel larvae (glochidia) are obligate parasites on fish. Success of mussel populations is dependent upon healthy host fish populations and if host species are reduced or eliminated mussel populations may suffer as well.

Little is known about the mechanism(s) that determines which fish species are suitable hosts for mussel glochidia but many investigators have speculated that the host fish immune response dictates whether or not glochidia can successfully attach and metamorphose. Once glochidia attach to gills or fins of the host fish, the fish's epithelial tissue encapsulates the glochidia (Arey 1921; Kat 1984; Rogers-Lowery et al. 2007). If the fish is a suitable host species the glochidia will survive the encapsulation; however, if the fish is not a suitable host the glochidia are sloughed within a few days (Arey 1932a; Arey 1932b; Barnhart et al. 2008; Kat 1984; Rogers-Lowery et al. 2007). Glochidia encapsulation was further investigated by comparisons of a host and non-host fish (Rogers-Lowery and Dimock 2006). If a glochidium attaches on a suitable host, the epithelial tissue of the fish forms a cyst around the glochidia, enabling metamorphosis to the juvenile stage. However, if the fish is not a suitable host, an irregular shaped cyst is formed around the glochidium and it is sloughed within the next few days (Rogers-Lowery and Dimock 2006). Little is known of the determining factor of survival. The complex and sometimes mysterious life history strategy of mussels makes mussels susceptible to habitat degradation such as sedimentation and poor water quality which directly affects the mussels as well as host fish.

Altamaha River Mussels

The Altamaha River in Georgia has one of the largest river basins, approximately 36,976 km^2, along the Atlantic Coast. The Altamaha River's major tributaries consist of the Ocmulgee, Ohoopee, and Oconee rivers. From the Altamaha River's origins at the confluence of the Ocmulgee and Oconee rivers, it flows 220 km to Darien, Georgia where it then enters the Atlantic Ocean. The Altamaha River is known for its high number (seven) of endemic mussels and many surveys have been conducted to assess changes in these unique mussel communities (Dinkins 2004; Keferl 1981; O'Brien 2002b; Skelton 2002). Data from these surveys indicates that three of the seven mussel species endemic to this basin are thought to be declining substantially: *Alasmidonta arcula* (Lea 1838), *Pyganodon gibbosa* (Say 1824), and *E. spinosa* (Wisniewski 2005). Wisniewski (2005) reported that site occupancy of *A. arcul*a decreased from 22% (pre-2000) to 16% (post-2000) and site occupancy of *P. gibbosa* decreased from 17% (pre-2000) to 6% (post-2000). Site occupancy of *E. spinosa* post-2000 was low and relatively unchanged from pre-2000 levels (10% and 12%, respectively); however, searches for *E. spinosa* have produced little success when compared to historical information. For example, in 1967, 60 *E. spinosa* were found at a single location, whereas from 1990-present, the most individuals researchers have found at any site was nine (USFWS Federal Register 50 (October 2010) CFR Part 17). Habitat alteration caused by sediment loads from poor land use practices is one likely reason for the declines of these species. Increased sedimentation can cause mussel beds to be buried and consequently reduce respiration, feeding, and growth rates of mussels (Brim-Box and Mossa 1999). Sedimentation can also lead to unstable sediments and increased turbidity, thereby providing unsuitable habitat for both mussels and possibly host fish (USFWS 2010 Federal Register 50 CFR Part 17). For proper management and potential recovery of the declining

endemic mussels and other mussel species, it is imperative to know what specific factors are adversely affecting these populations (Wisniewski 2005).

Knowledge of early life history is a necessary component in management of all mussel populations, including those endemic to the Altamaha River Basin (Georgia Wildlife Action Plan 2005). Unfortunately, little is known about several key aspects of early life history of Georgia mussels. For instance, host fish information is available for only 49 of the 127 species of freshwater mussels historically found in Georgia (Personal communication, Jason Wisniewski, Georgia Department of Natural Resources). Identification of host fish species allows managers to determine if there is a large enough fish population in the basin to sustain the mussel populations, information that is critical for management plans regarding mussel recovery. Additionally, identification of suitable host fish species is the first step in advancing mussel propagation programs. Stocking of propagated mussels may be useful in supplementing imperiled populations and has been successful in other river basins across the U.S. (Genoa National Fish Hatchery, Genoa, Wisconsin and the Virginia Department of Game and Inland Fisheries at the Aquatic Wildlife Conservation Center, Marion, Virginia). Additionally, propagation could be used to produce mussels for toxicity testing with contaminants found in the Altamaha River basin. Toxicity testing would help managers identify any specific chemical or physiochemical stressors in the water or sediment that may be impacting mussel growth, reproduction or survival.

Life history information is limited for the mussels of the Altamaha River; only eight out of the 18+ mussel species have confirmed primary host fish. A fish is considered primary host when a high number of glochidia are able to metamorphose into juveniles. Furthermore, only two (*E. hopetonensis* (Lea 1838) and *L. dolabraeformis* (Lea 1838)) of the seven endemic mussel

species have a known primary host (O'Brien 2002a). In addition to the limited knowledge of host fishes for Altamaha endemic mussels, glochidia morphology descriptions are lacking for most Altamaha River species. Glochidia morphology has only been described for three endemic species: *E. hopetonensis*, *E. shepardiana,* and *E. dariensis* (O'Brien et al. 2003). Glochidia often have conspicuous physical characteristics (i.e. hooks, grooves, shape) that may assist biologists with positive identification of the brooding adult female mussels. As adults, shell morphology can vary greatly within a species (O'Brien et al. 2003; Sayenko et al. 2005), even within the same river system (Hornbach 2010). Alternatively, some adult mussel species may have few distinguishing features and may be commonly misidentified, resulting in inaccurate survey data which could mislead resource managers and delay appropriate management decisions. Thus, glochidia morphology descriptions may provide another line of evidence for identification of brooding female mussels. Glochidia morphology may also be useful for identifying glochidia attached to fish collected in the wild, thereby providing some host information. Glochidia morphology may also provide key insights into phylogenetic relationships among mussel groups. Knowledge of glochidia characteristics is vital to the development of proper management strategies for the endemic Altamaha mussel species and beyond.

Mussel Propagation

In recent years, university, state, and federal agencies have made great strides in mussel conservation by advancing propagation techniques. Use of ponds and pondwater as well as tank designs that include air-driven water-recirculation, automatic feeding, and controlled water chemistry and flow have been used successfully for rearing juvenile and adult mussels in culture settings (Henley et al. 2001). In 2009, the Virginia Department of Game and Inland Fisheries at

the Aquatic Wildlife Conservation Center (Marion, Virginia), successfully propagated seven imperiled species of freshwater mussels, totaling ~860,500 juveniles (http://www.dgif.virginia.gov/awcc/freshwater-mussel-restoration/). Since 2000, the Genoa National Fish Hatchery (Genoa, WI) has released over 2 million juveniles of seven mussel species (http://www.fws.gov/midwest/genoa/mussel_recovery.html). Advancements such as the "Mucket Bucket" system (Barnhart 2006), have been valuable for improving survival and growth of juvenile mussels in laboratory settings. The "Mucket Bucket" system is beneficial because it is relatively inexpensive, compact, allows for replication and provides for keeping groups of juveniles isolated from one another for a variety of purposes.

Techniques have greatly advanced in the propagation of mussels; however, there are currently few options for the propagation of mussel species with unknown host fish. The use of in vitro techniques with culture media, a method in which the glochidia are grown into juveniles in a petri dish, has been successful with generalist mussel species- species whose glochidia can metamorphose on a variety of fish species. To date, host-specialist species (metamorphoses on one or two species of fish) have not successfully metamorphosed on culture media (McGregor and Alexander 2008). Therefore it is prudent and imperative to explore other avenues of inducing metamorphosis of juveniles when host fish are not known or are unavailable.

It is theorized that the fish's immune system determines if it is a suitable host fish for glochidia of a particular mussel species. With non-host fish, if the fish immune system is responsible for preventing glochidia from attaching and metamorphosing, then the use of immunosuppressant drugs may be a viable method of inducing juvenile metamorphosis on non-host fish species. Indeed, research has shown that the fish immune system is critical for controlling parasite infections and immune suppression results in greater parasite body burden.

For example, Atlantic salmon (*Salmo salar*) treated for 48 hr with 240µg/L of dexamethasone, a synthetic corticosteroid immunosuppressant drug, were significantly compromised in their ability to combat ectoparasites (Nielsen and Buchmann 2003). Because glochidia are ectoparasites on fish it is plausible that dexamethasone or other immunosuppressant drugs may provide a way to induce glochidia attachment and metamorphosis on non-host fish.

To date, only one study has investigated the use of immunosuppressant treatments to enhance successful juvenile transformations for freshwater mussels. Kirk and Layzer (1997) showed promising results when they used intraperitoneal implants of cortisol to facilitate juvenile transformation on known non-host fish. Their experimental approach consisted of two mussel species, *Venustaconcha sima* and *Villosa taeniata*, and a confirmed host and non-host species of fish for each mussel, as well as two additional fish species that were previously untested hosts. Juvenile transformation was documented on cortisol-treated non-host fish for both species of mussels. However, relatively few juveniles (maximum of 17 per fish) were produced in each experiment and the results varied between each set of experiments. Kirk and Layzer (1997) hypothesized a possible seasonal susceptibility to glochidia, often coinciding with spawning seasons. They concluded that use of immunosuppression to induce juvenile transformation has merit, especially when involving threatened and endangered mussels, but is in need of modification before any large scale attempts are made (Kirk and Layzer 1997). Further investigation into use of immunosuppression of non-host fish could prove to be pivotal in the recovery of imperiled mussel species, such as those in the Altamaha River. Immunosuppression of non-host fish may provide researchers and managers with an alternative to traditional propagation methods in the absence of a known host and may afford additional time to find the natural host fish before these mussel species experience further population declines.

Summary

Successful management of endemic Altamaha mussel species depends on knowledge of their early life history. The second chapter of this thesis provides some of this valuable information and includes host fish information for seven species of freshwater mussels of the Altamaha Basin, three of which had no previous known host: *E. shepardiana, L. splendida,* and *A. arcula*. New details of glochidia morphology for seven Altamaha mussel species, including five endemics are also discussed.

Alternatives to traditional propagation methods are needed for mussel species with no known host fish. If host fish suitability is determined by the immune response, immunosuppression of non-host fish may facilitate glochidia attachment and metamorphosis and thus provide a valuable option for culture of juvenile mussels. The third chapter of this thesis describes an investigation of treatment of non-host fish with the immunosuppressant dexamethasone for enhancing glochidia metamorphosis success. I attempted to determine the optimal exposure route and dose as well as determine if dexamethasone had any toxic effects on glochidia. Ultimately, immersions of fish in dexamethasone led to production of only a few juvenile mussels. However, some interesting trends were identified and further investigations into mechanisms of host suitability are recommended. I also determined that dexamethasone did not decrease glochidia viability at concentrations up 4000 µg/L (highest I tested). The highest concentration used on fish in my study was 1000 µg/L, which means higher concentrations could be tested for their effects on metamorphosis as well. Despite the limited success with dexamethasone treatments in my study, the use of immunosuppression to induce juvenile metamorphosis on non-host fish should not be ruled out and could eventually prove to be a

valuable tool for restoration of freshwater mussels, particularly for threatened and endangered populations for which host fish have not been identified.

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CHAPTER 2

EARLY LIFE HISTORIES OF FRESHWATER MUSSELS FROM THE ALTAMAHA RIVER BASIN[1]

[1] Johnson, Jennifer, Jason Wisniewski, and Robert Bringolf. To be submitted to Southeastern Naturalist.

Introduction

Freshwater mussels (Family: Unionidae) are considered one of the most imperiled taxa worldwide (Neves et al. 1997; Williams et al. 1993). Factors thought to contribute to the decline of mussels in general include sedimentation, pollution, urbanization, and habitat fragmentation (Williams et al. 1993). Another factor that may play a key role in the decline of freshwater mussels is the loss of host fish; mussel larvae (glochidia) are obligate parasites on fish. If host fish species are reduced or eliminated mussel populations may suffer as well.

North America has the highest diversity of freshwater mussels with 297 species and 35 species are thought to be extinct and 70 species are currently listed either as threatened or endangered under the Endangered Species Act (USFWS 2007). Specifically, 91% of North American mussel diversity is located within the southeastern portion of the United States (Neves et al. 1997; Turgeon 1988). Georgia contains approximately 127 freshwater mussel species and mussels comprise 21% of the total federally listed animals in the state. Additionally, the endemic Altamaha spinymussel (*Elliptio spinosa* Lea, 1836) is a candidate for listing and will increase that percentage when formally listed. *Elliptio spinosa* was proposed for formal listing under the ESA in October 2010.

In addition to *E. spinosa*, there are 18+ species of mussels that reside in the Altamaha River basin, which is located completely within the state of Georgia (Figure 1.1). Encompassing 36,976 km², it is among the largest basins along the eastern seaboard (Wisniewski 2005). The Altamaha River officially begins at the confluence of the Ocmulgee and Oconee Rivers and is unique in that it holds seven out of the eight Georgia mussel endemics including *Alasmidonta arcula* (Lea, 1838), *E. dariensis* (Lea, 1842), *E. hopetonensis* (Lea, 1838), *E. spinosa*, *E.*

shepardiana (Lea, 1834), *Lampsilis dolabraeformis* (Lea, 1838), and *Pyganodon gibbosa* (Say, 1824) (Wisniewski 2005).

Numerous studies of mussel populations, habitat, and host fish identification have been conducted with the overall goal of proper management and restoration of these unique animals (O'Dee and Watters 1998; Strayer and Fetterman 1999; Strayer 1981; Strayer 1993; Williams et al. 1993). Surveys in the Altamaha River Basin have given managers invaluable information regarding the status and distribution of the mussels that reside there, particularly the endemic species (Dinkins 2004; Keferl 1981; O'Brien 2002b; Skelton 2002). Data from the Altamaha mussel surveys indicates that three of the seven endemics, the *A. arcula*, *P. gibbosa*, and *E. spinosa*, are declining substantially (Wisniewski 2005). For proper management and potential recovery of the Altamaha endemic species, it is imperative to know what specific factors are adversely affecting these populations (Wisniewski 2005). Knowledge of early life histories is a necessary component in management of all endemic mussels of the Altamaha River Basin (Georgia Wildlife Action Plan 2005). Unfortunately, little is known about several key aspects of early life history of Georgia mussels. For example, host fish have been identified for only 49 out of the 127 species of freshwater mussels historically found in Georgia (Personal communication, Jason Wisniewski, Georgia Department of Natural Resources). Additionally, glochidia morphology descriptions are lacking for most Altamaha River mussel species (O'Brien et al. 2003). Knowledge of host fish and glochidia characteristics is vital for development of a proper management strategy for the endemic Altamaha mussel species.

Host species identification will aid in managing freshwater mussel populations and may provide further insight about why certain mussel populations have declined or flourished in certain areas (i.e. absence or presence of host fish). Furthermore, host identification will allow

managers to determine if there is a large enough host fish population in the basin to sustain the endemic mussel populations and will be the basis for future management decisions. Managers may begin to formulate recovery plans that involve recovery of host fish(es) as well as mussels and suitable physical and physiochemical habitat. Identification of a host fish species is the first step in advancing mussel propagation programs which may be useful for supplementing imperiled endemic populations. Propagation could also be used to produce mussels for toxicity testing to determine sensitivity to contaminants found in the Altamaha Basin. Such testing would aid managers in their efforts to determine key habitat attributes that may be limiting mussel recruitment or survival.

Little is known about host fishes for the three endemic mussel species that are declining; only *A. arcula* has a confirmed host, the exotic mosquitofish (*Gambusia holbrooki*) (O'Brien 2002a). However, the mosquitofish is only a marginal host and it is unlikely that mosquitofish occur in habitats where *A. arcula* are found so no ecologically-relevant hosts have been identified for this mussel species. Fish are considered 'marginal' hosts when only a few glochidia are able to metamorphose into juveniles. Host trials for the other declining Altamaha endemic mussels have produced few promising results. In 2004, researchers at the Tennessee Aquarium Research Institute (Cohutta, GA) attempted to determine host fish for *E. spinosa*. Unfortunately the host trials were unsuccessful, mainly attributed to the lack of mature glochidia (Johnson 2004).

Host fish identification is a vital knowledge component of early life history; however, glochidia morphology may equally be as important. As adults, mussel morphology can vary greatly within a species (O'Brien et al. 2003; Sayenko et al. 2005) and even within the same river system (Hornbach 2010). Alternatively, some mussel species may have few distinguishing

features and misidentification is a persistent problem. Misidentification of adult mussels can lead to inaccurate survey data which could mislead resource managers and delay appropriate management decisions. Glochidia often have conspicuous physical characteristics (i.e. hooks, grooves, shape) that may provide an important tool for positive identification of the brooding adult female mussels. Glochidia morphology comparisons may also provide insights into phylogenetic relationships among mussel groups.

The primary objectives of my study were to identify host fish and describe glochidia morphologies for freshwater mussels of the Altamaha River Basin, Georgia. I examined host fish and glochidia morphologies for seven species of freshwater mussels that reside in the Altamaha River Basin: *A. arcula*, *E. hopetonensis*, *E. spinosa*, *E. shepardiana*, *L. dolabraeformis*, *L. splendida*, and *V. delumbis*.

Methods

Mussel Collection

All mussels were collected from the mainstem of the Altamaha River from 2008 – 2010. Specifically, all species except the *A. arcula* and *E. spinosa* were collected ~2.1 km downstream of Oglethorpe Bluff Landing, ~12 km north of Jesup, Georgia (Figure 1.1). The *A. arcula* and *E. spinosa* were collected ~1.8 km upstream of Upper Wayne Landing off of Arnold River Road, ~17.1 km SSW of Glennville, Georgia (Figure 1.1). Seven species of freshwater mussels were collected in various months from 2008-2010 (Table 1.1). Search methods were similar to previous surveys in the Altamaha River, which included visual, tactile, SCUBA, and snorkel (Dinkins 2004; Meador 2008; O'Brien 2002b; Skelton 2002). Mussels were gently pried open to examine for inflated marsupial gills which indicate brooding glochidia. At least five female

mussels of each species were checked for glochidia and if no mussels were shown to be brooding the inspections ceased. Up to 10 brooding females of each mussel species were transported in coolers (Cope 2003) to the Whitehall Fish Lab at the University of Georgia. To minimize premature expulsion of glochidia, mussels were maintained at 5-10ºC in a Living Stream (Frigid Units Inc., Toledo, Ohio) until glochidia were extracted for morphological characterization and host fish trials.

Glochidia Morphology

Glochidia were obtained from one to four females of each species of mussel by gently flushing the marsupial gills with water from a 5 ml syringe (O'Brien and Brim-Box 1999; O'Dee and Watters 1998; Rogers 2001). Glochidia were fixed in formalin for at least 24 hours then stored in 95% ethanol for future analysis. For morphological measurements, 25 glochidia per female were examined for each species, the exception being *V.delumbis* with 6 glochidia measured (Table 1.1). Digital photographs of glochidia were obtained at magnifications of 16-50X using a stereomicroscope (Leica MZ 7.5) equipped with a digital camera (Leica DCF 290). Glochidia morphological measurements of length (parallel to hinge), height (perpendicular to hinge), and hinge length (O'Brien et al. 2003; Sayenko et al. 2005) were obtained with image analysis software (Leica LAS Suite). Glochidia shape classifications were based on previous descriptions (Hoggarth 1999).

Glochidia ultrastructure (i.e. presence of microstylets, interior/exterior valve sculpture) was determined for *L. dolabraeformis, E. shepardiana, E. spinosa,* and *A. arcula* with the use of a Zeiss 1450EP scanning electron microscope (SEM; Zeiss SMT, Peabody, MA USA). Glochidia for *L. splendida* and *V. delumbis* were not able to be used for this analysis due to the

low quality of preserved specimens. Glochidia samples were mounted on SEM stubs with carbon adhesive tabs (EMS, Hatfield, PA USA) and a SPI module sputter coater (SPI Supplies, Inc. West Chester, PA USA) was used to coat samples with 20 μm of gold. Specimens were then examined under the SEM which ran at 20Kev with a probe size of 450uA.

Host Trials

I obtained fish for host trials from state, federal and private fish hatcheries located within the Southeastern U.S. Additional fish were captured by electroshocking or seined from ponds located in Whitehall Forest and McNutt Creek (Athens, GA), and Cloud Creek in Watson Mill State Park (Comer, GA). All of the site locations are within the Altamaha River Basin and did not contain mussels. It was essential that fish used in the host trials did not have any previous exposure to mussels because fish develop acquired immunity to glochidia following exposure (Dodd et al. 2006; Meyers et al. 1980; Zale and Neves 1982).

Fish species tested as hosts depended on availability and were not the same for each mussel species. Test fish species for *A. arcula* included bluegill (*Lepomis macrochirus*), channel catfish (*Ictalurus punctatus*), fathead minnow (*Pimephales promelas*), koi (*Cyprinus carpio*), lake sturgeon (*Acipenser fulvenscens*), redbreast sunfish (*Lepomis auritus*), robust redhorse (*Moxostoma robustum*), rosyface chub (*Notropis rubescens*), spottail shiner (*Notropis hudsonius*), striped bass (*Morone saxatilis*), and yellow bullhead (*Ameiurus natalis*). Fish evaluated as hosts for *E. hopetonensis* included bluegill, channel catfish, fathead minnow, koi, lake sturgeon, and largemouth bass (*Micropterus salmoides*). Bluegill, bluehead chub (*Nocomis leptocephalus*), brown bullhead (*Ameiurus nebulosus*), fathead minnow, golden shiner (*Notemigonus crysoleucas*), lake sturgeon, northern hogsucker (*Hypentelium nigricans*), redear sunfish (*Lepomis microlophus*), robust redhorse, spottail shiner, yellow bullhead, and yellowfin

shiner (*Notropis lutipinnis*) were fish species evaluated as hosts for *E. shepardiana*. Bluegill, channel catfish, fathead minnow, koi, lake sturgeon, largemouth bass, redbreast sunfish, spotted sucker (Minytrema melanops), striped bass, and white bass (*Morone chrysops*) were tested as hosts for *E. spinosa.* Bluegill, channel catfish, fathead minnow, golden shiner, lake sturgeon, and largemouth bass were evaluated as potential hosts for *L. dolabraeformis*. Largemouth bass were tested as hosts for *L. splendida* and *V. delumbis*.

I removed glochidia from female mussels by gently flushing the marsupial gills with water from a 5 ml syringe and glochidia were collected in a petri dish (O'Brien and Brim-Box 1999; O'Dee and Watters 1998; Rogers 2001; Zale and Neves 1982). *E. spinosa*, *E. hopetonensis*, and *E. shepardiana* released mature glochidia within one month of transport to the lab so glochidia of these species were collected without the use of a syringe. Subsamples of glochidia were tested for viability by standard method, i.e. glochidia were exposed to a saturated sodium chloride solution; viable glochidia quickly closed their valves upon exposure to the sodium chloride solution (O'Dee and Watters 1998; Rogers 2001). Glochidia viability was quantified as a percentage of glochidia that responded to the saturated sodium chloride solution. Potential host fish were separated by species and then exposed to glochidia for one hour in aerated 19-L buckets (O'Dee and Watters 1998). Each bucket contained multiple fish of each species (Table 2). Target concentrations of glochidia were 4,000 per liter of water. The exception to this method was *E.* spinosa because there was a limited amount of viable glochidia available. Rather than inoculating fish to *E. spinosa* glochidia suspended in water, glochidia were pipetted directly on the fish gills which created a 'best case scenario' for glochidia attachment. Concentrations of glochidia were not calculated for *E. spinosa* and therefore I performed a qualitative metamorphosis assessment rather than a quantitative assessment for *E.*

spinosa. Fish were then separated into individual holding tanks (monitoring tanks) within a recirculating aquaculture system (AHAB®, Aquatic Habitats Inc., Apopka, FL). The AHAB unit is a closed system composed of racks of self-cleaning tanks through which water flows and then re-circulates back into a main sump for treatment including removal of solids, biofiltration, clarification with activated carbon and UV sterilization. Water temperatures ranged from 21-25ºC among all host trials. Filter cups (2" pvc pipe with a 153 μm mesh screen) were fitted to the outlet on each monitoring tank to catch dead glochidia and metamorphosed juveniles as they were sloughed from the fish.

Beginning the day after exposure (day 1), I checked filter cups daily for dead glochidia and metamorphosed juveniles for seven days; beyond day 7 the cups were checked every other day. Monitoring was accomplished by gently rinsing the mesh of the filter cup into a petri dish or a Bogorov tray and observing contents under a stereomicroscope. Glochidia and juveniles were counted, photographed, and recorded on a data sheet. Filter cups were checked every two days until there were no glochidia or juveniles observed for at least five consecutive days (Zale and Neves 1982). I placed juvenile mussels in a rearing system (Barnhart 2006) for grow out.

Few previous host trials have established quantifiable criteria for determination of primary and marginal hosts (O'Dee and Watters 1998; Rogers 2001). This is likely because most previous investigations have not determined the success rate for metamorphosis from glochidia to juvenile stage. To my knowledge, only one other study has established quantifiable criteria for primary and secondary (marginal) hosts (O'Brien and Brim-Box 1999), although others have used these terms for qualitative purposes (Haag and Warren Jr 2003). For this study primary and marginal hosts were determined *a priori* by defined metamorphosis success rates (%M). Percentage of successful juvenile metamorphosis was calculated by the following formula: [(# of

juveniles/ (juveniles + glochidia)) x 100]. A fish species with a %M of >30% was considered to be a primary host and a marginal host was defined as %M of 1-30% (O'Brien and Brim-Box 1999). Percentages between 0-0.9 were not considered to be suitable hosts.

Results

Mussel Collection

One brooding *V. delumbis,* two brooding *L.splendida*, and eight brooding *L. dolabraeformis* were collected on October 1, 2008, at a water temperature of 26ºC. Five *L. dolabraeformis,* eight *E. dariensis,* three *E. shepardiana*, and ten *E. hopetonensis* were all brooding and collected on June 18, 2009 at a water temperature of 29ºC. *E. shepardiana* were brooding on June 11, 2010 at a water temperature of 27ºC. Coordinates for the three sampling events were Lat/Long: 31.71383N -81.88427.

One brooding *E. spinosa,* one *V. delumbis*, nine *L. dolabraeformis*, and nine *E. hopetonensis* were collected on May 18, 2009 when water temperature was 20.5ºC. I observed five *A. arcula* and four *L. splendida* brooding on November 12, 2009 at a water temperature of 16ºC. Coordinates for the May 18th and November 12, 2009 sampling dates were Lat/Long: 31.79476N -81.99999W.

Glochidia Morphology & Shell Ultrastructure

Glochidia shell structure varied by species. Shell structure of *Lampsilis dolabraeformis* consisted of a pitted subelliptical valve with a mean ± standard deviation (SD) height of 239µm ± 13), and the ventral edge (flange) was covered with micropoints (Figure 1.2A, 1.3A, 1.4A, Table 1.1). Glochidia of *A. arcula* were pyriform and contained a styliform hook ventrally

located on each pitted valve (Figure 1.2B, 1.3B, 1.4B, 1.5). Mean ± SD height for *A. arcula* glochidia was 309µm ± 35 (Table 1.1). *E. shepardiana* glochidia had a pitted valve structure and a depressed subelliptical shape (Figure 1.2C, 1.3C). The ventral flange extended beyond the gill margin and was covered with numerous microstylets (Figure 1.4C). Mean ± SD height was 259µm ± 16 (Table 1.1). Average height of *E. hopetonensis* was 199µm ± 12 (Table 1.1) with a depressed subelliptical shape. Specimens of *E. hopetonensis* were not preserved for SEM analysis. Similar to the other members of the genus Elliptio, *E. spinosa* was also pitted with a depressed subelliptical shape (Figure 1.2D, 1.3D), the ventral flange was covered with microstylets, but not extended (Figure 1.4D) and had an average height of 208 µm ± 9 (Table 1.1). Eight days after being brought into the lab, *E. spinosa* released subcylindrical white conglutinate packets that contained immature glochidia. Conglutinate length was measured at 18mm and width was 5mm (Figure 1.6). *L. splendida* were subelliptical and were longer than *L.* dolabraeformis with an average height of 268µm ± 7.4 (Table 1.1). Specimens of *V. delumbis* had an average height of 395 ± 36 µm (Table 1.1).

Host Trials

Robust redhorse (*Moxostoma robustum*) was a marginal host (%M = 4.3) for *A. arcula*; 11 other fish species were not suitable hosts (Table 1.2). I identified three marginal hosts for *E. hopetonensis*: bluegill (*Lepomis macrochirus*; %M = 3.7), fathead minnow (*Pimephales promelas*; %M = 3), and largemouth bass (*Micropterus salmoides*; %M = 0.8). Three other species of fish were determined to be non-hosts for *E. hopetonensis* (Table 1.2). Brown bullhead (*Ameiurus nebulosus*) was identified as a primary host (%M = 45.2) for *E. shepardiana*. Marginal hosts for *E. shepardiana* were yellow bullhead (*A. natalis;* %M = 18.9) and bluegill

(%M = 2.3%). A single *E. shepardiana* juvenile was produced on robust redhorse and nine other species were not suitable hosts (Table 1.2). Metamorpohsis rates were high (84.5%, 75%, and 43%) for each of three host trials with *L. dolabraeformis* on largemouth bass, which was identified as a primary host. Bluegill was a marginal host in each of the three trials (%M = 0.6, 1.1, and 2.0, respectively). There was no metamorphosis of *L. dolabraeformis* on four additional fish species (Table 1.2). Largemouth bass was also identified as a primary host for *L. splendida* (% M = 43) and *V. delumbis* (%M = 73). No other fish species were tested with *L. splendida* and *V. delumbis*.

None of the ten fish species tested were suitable hosts for *E. spinosa*, the candidate to be listed under the ESA, and eight fish species sloughed 100% of the attached glochidia within three days after initial glochidia exposure (Table 1.2). However, four *E. spinosa* glochidia remained attached on lake sturgeon (*Acipenser fulvescens*) and five on redbreast (*Lepomis auritus*) until five days after initial exposure.

Discussion

Early life history information from this study will provide managers with some of the essential ecological information necessary when formulating management and recovery plans for mussels of the Altamaha River basin. For example, host identification for the *A. arcula* was listed as a top priority in the 2005 Georgia Wildlife Action Plan and mussel recovery plans must consider host fish information to determine if suitable fish species are present in the basin. Without host fish (and other suitable habitat), population augmentation will likely be unsuccessful in the long-term. I identified primary or marginal host fish for seven species of

freshwater mussels of the Altamaha Basin, three of which had no previously known hosts: *E. shepardiana, L. splendida,* and *A. arcula*.

Many investigators have speculated that loss of host fish is one factor in the decline of freshwater mussels but to the best of my knowledge this is the first report of an established mussel-host fish relationship between two imperiled species (*A. arcula* and robust redhorse). Robust redhorse were historically abundant in the Altamaha Basin but are now state endangered fish. They were thought to be extinct for 122 years until the fish was 'rediscovered' in 1991 in the Altamaha Basin and subsequently identified in other Atlantic Slope drainages north to Virginia (Grabowski and Jennings 2009). Robust redhorse are not likely the sole host for *A. arcula* because of the low metamorphosis rate (~ 4%), but because A. arcula populations are in decline it is possible that this is the only remaining host species in the river. However, I did not test other species of the sucker family (Catastomidae) as potential hosts for *A. arcula* and it is likely that another Catastomid is a primary host. Future host identification efforts for *A. arcula* should concentrate on the Catastomid family.

It is not clear if the *A. arcula* population decline was a result of a loss of host fish or if the mussels and fish declined simultaneously as a result of other stressors such as habitat alteration. Other investigators have previously reported that *A. arcula* metamorphose on *Gambusia holbrooki* (O'Brien 2002a); however, this species is not likely a host because it does not co-occur in the same stream environments as *A. arcula*. For successful conservation, managers should concurrently evaluate both species. Perhaps if robust redhorse populations recover, *A. arcula* will follow suit.

One of the objectives for this study was to identify a host fish for *E. spinosa*. Unfortunately, host(s) for *E. spinosa* remain unknown at this time. The major limiting factor in

host identification was the inability to collect brooding females. In 2009 and 2010, high flows in the Altamaha mainstem limited search efforts in April-June, the period when brooding *E. spinosa* had been collected by other investigators. Despite the high flows, approximately 160 hours of search time were logged and only one brooding female was collected. Future host trials with *E. spinosa* are dependent upon a source of brooding females and it is currently unclear if this is feasible. One option is to collect *E. spinosa* throughout the year and relocate them to a centralized location where the chances of recapture are greater. One option is to attach external sonic tags to the mussels to enhance the chances of recovery. Additionally, *E. spinosa* may be collected throughout the year and returned to a culture facility to determine if they will undergo fertilization and brooding in captivity.

My study provided some of the first detailed descriptions for glochidia of endemic mussels of the Altamaha River basin. Glochidia morphology may be useful for identifying glochidia attached to fish collected in the wild, thereby providing some host information. This approach may be more applicable in regions with fewer species of mussels of a given genus because morphological variation with a genus is often limited. Detailed descriptions of glochidia morphology may provide identification of key taxonomic characteristics that help scientists and resource managers unequivocally identify the adults from which they were obtained.

Dimensions and features of *A. arcula* glochidia were previously undescribed but were consistent with other members of the clade Unioninae (Ortmann 1911). Glochidia in this clade exhibit a styliform hooked shell and tend be larger in size than hookless glochidia (Barnhart et al. 2008; Williams 2008). Measurements and morphology of *E. hopetonensis* and *E. shepardiana* were similar to those previously reported for these species by O'brien (2003). Morphology of viable *E. spinosa* glochidia had not been reported prior to this study. The size and shape are

generally similar to those previously reported for other members of the genus *Elliptio* (O'Brien et al. 2003; Williams 2008). However, the ventral flanges of *E. spinonsa* and *E. shepardiana* were distinctly different in length, which may be helpful when trying to distinguish between the species. Many characters of *L. dolabraeformis* and *L. splendida* glochidia were similar to other species in the genus *Lampsilis*. For example, *L. dolabraeformis* and *L. splendida* were comparable to *L. subangulata* glochidia which had an average height of 261 ± 7 μm (O'Brien and Brim-Box 1999). However, *L. straminea* (Conrad, 1834), *L. ornata* (Conrad, 1835), and *L. teres* (Rafinesque, 1820) all were markedly smaller than both *L. dolabraeformis* and *L. splendida* (Kennedy and Haag 2005). When feasible, future analyses of glochidia morphology should be conducted using a scanning electron microscope which can provide accurate measurements and detailed images of shell ultrastructure that are not feasible with traditional light microscopy techniques.

Although brooding period was not evaluated in this study, there were some differences between previous reports of brooding periods and the times brooding mussels were collected for the present study. For example, I observed brooding *A. arcula* on November 12, 2009, whereas O'brien (2002a) observed brooding *A. arcula* on April 25, 1998. This is curious because I did not observe brooding *A. arcula* in any month other than November. For this reason it does not appear that *A. arcula* is a long-term brooder but may have multiple short-term brooding periods throughout the year. More detailed monthly observations will be necessary to accurately determine the brooding period. Also, in this study, I observed brooding *E. shepardiana* and *E. hopetonensis* in the month of June. O'Brien et al. (2003) reported that brooding *E. hopetonensis* and *E. shepardiana* were collected on April 21, 2006. Though not as contrasting as the *A. arcula* reports, these differences in observed brooding dates highlight the need for systematic

monitoring to accurately determine brooding period(s) for each mussel species. Anecdotal evidence suggests that some species of *Lampsilis* and *Elliptio* exhibit multiple brooding events within a year (Watters and O'Dee 1998). To further our understanding of the reproductive biology of endemic mussels of the Altamaha River, I recommend that future studies include a sampling regime that involves monitoring female mussels monthly throughout the entire year. Another important factor to consider, particularly in light of global climate change, is optimal water temperature for growth, spawning and the subsequent brooding period. Different mussel species within the same genera may have different preferred temperatures at which pivotal reproductive processes occur (O'Brien and Brim-Box 1999). If optimal temperatures are established for key processes and life histories are further described, managers will have additional important information when formulating management plans for mussel recovery.

Future studies should focus resources on investigating reproductive biology and early life history of imperiled species. Efforts to protect and enhance imperiled species will be greatly enhanced by knowledge of brooding periods, optimal brooding temperatures, host fish, and descriptions of glochidia morphology. Knowledge of mussel early life history can help answer questions about why mussel populations are declining (ie. loss of fish host or preferred habitat), which can ultimately lead to the preservation of freshwater mussel biodiversity.

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Tables

Table 1.1. Glochidia (N=25) measurements for freshwater mussels of the Altamaha River, GA

		Mean ± SD (μm)				
Species	Number of females	height	length	hinge	height/length ratio	hinge/length ratio
Alasmidonta arcula	4	309 ± 35	274 ± 13	212 ± 10	1.13 ± 0.11	0.78 ± 0.05
Elliptio hopetonensis	1	199 ± 12	186 ± 9	134 ± 6	1.07 ± 0.05	0.72 ± 0.04
Elliptio shepardiana	4	259 ± 16	217 ± 10	153 ± 7	1.20 ± 0.08	0.71 ± 0.04
Elliptio spinosa	1	208 ± 9	197 ± 7	133 ± 9	1.05 ± 0.05	0.67 ± 0.04
Lampsilis dolabraeformis	4	239 ± 13	207 ± 8	100 ± 8	1.16 ± 0.08	0.49 ± 0.04
Lampsilis splendida	4	268 ± 7	222 ± 10	97 ± 10	1.21 ± 0.07	0.44 ± 0.04
Villosa delumbis	1	395 ± 36	328 ± 14	174 ± 6	1.21 ± 0.11	0.53 ± 0.02

Table 1.2. Summary of fish host trials with mussels that reside in the Altamaha River, GA.

Mussel sp.	Fish sp.	Metamorphosis %	# of Juveniles produced	Metamorphose Period	# Fish used
Alasmidonta arcula	*Moxostoma robustum*	4.25	61	Days 8-13	4
	Acipenser fulvenscens	0	0	-	5
	Ameiurus natalis	0	0	-	1
	Cyprinus carpio	0	0	-	2
	Ictalurus punctatus	0	0	-	5
	Lepomis auritus	0	0	-	1
	Lepomis macrochirus	0	0	-	7
	Morone saxatilis	0	0	-	5
	Notropis hudsonius	0	0	-	1
	Notropis rubescens	0	0	-	4
	Pimephales promelas	0	0	-	4
Elliptio hopetonensis	*Lepomis macrochirus*	3.7	49	Day 7-8	8
	Pimephales promelas	3	55	Day 7	4
	Micropterus salmoides	0.8	16	Day 7	4
	Acipenser fulvenscens	0	0	-	3
	Cyprinus carpio	0	0	-	4
	Ictalurus punctatus	0	0	-	3
Elliptio shepardiana	*Ameiurus nebulosus*	45.2	378	Days 11-17	2
	Ameiurus natalis	18.9	17	Days 11-14	1
	Lepomis macrochirus	2.3	4	Days 11-12	6
	Moxostoma robustum	0	1	Day 17	3
	Acipenser fulvenscens	0	0	-	2
	Hypentelium nigricans	0	0	-	2
	Lepomis microlophus	0	0	-	5
	Nocomis leptocephalus	0	0	-	4
	Notemigonus crysoleucas	0	0	-	1
	Notropis hudsonius	0	0	-	1
	Notropis lutipinnis	0	0	-	15
	Pimephales promelas	0	0	-	5

Table 1.2 (Continued). Summary of fish host trials with mussels that reside in the Altamaha River, GA.

Mussel sp.	Fish sp.	Metamorphosis %	# of Juveniles produced	Metamorphose Period	# Fish used
Elliptio spinosa	*Acipenser fulvenscens*	0	0	-	4
	Cyprinus carpio	0	0	-	4
	Ictalurus punctatus	0	0	-	4
	Lepomis auritus	0	0	-	4
	Lepomis macrochirus	0	0	-	3
	Micropterus salmoides	0	0	-	4
	Minytrema melanops	0	0	-	4
	Morone chrysops	0	0	-	1
	Morone saxatilis	0	0	-	4
	Pimephales promelas	0	0	-	2
Lampsilis dolabraeformis	*Micropterus salmoides*	84.5	1209	Days 19-29	4
		75	1023	Days 11-21	3
		43	117	Days 11-15	5
Lampsilis dolabraeformis	*Lepomis macrochirus*	0.6	1	Day 19	8
		1.1	7	Days 10-12	9
		2	15	Day 11	5
Lampsilis dolabraeformis	*Ictalurus punctatus*	0	0	-	2
		0	0	-	2
Lampsilis dolabraeformis	*Acipenser fulvenscens*	0	0	-	3
	Notemigonus crysoleucas	0	0	-	5
	Pimephales promelas	0	0	-	4
Lampsilis splendida	*Micropterus salmoides*	43	2352	Days 13-24	5
Villosa delumbis	*Micropterus salmoides*	73	4673	Days 12-24	18

Figures

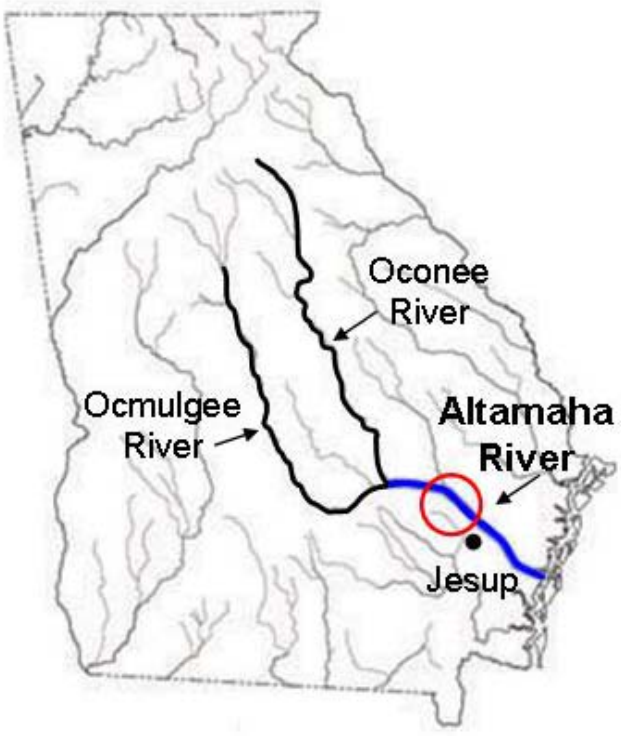

Figure 1.1. Map illustrating the collection area for freshwater mussels from the mainstem Altamaha River, GA, USA. Locations were approximately 12-21 kilometers northeast of Jesup, GA. Original map is from GeorgiaInfo, Ed Jackson.

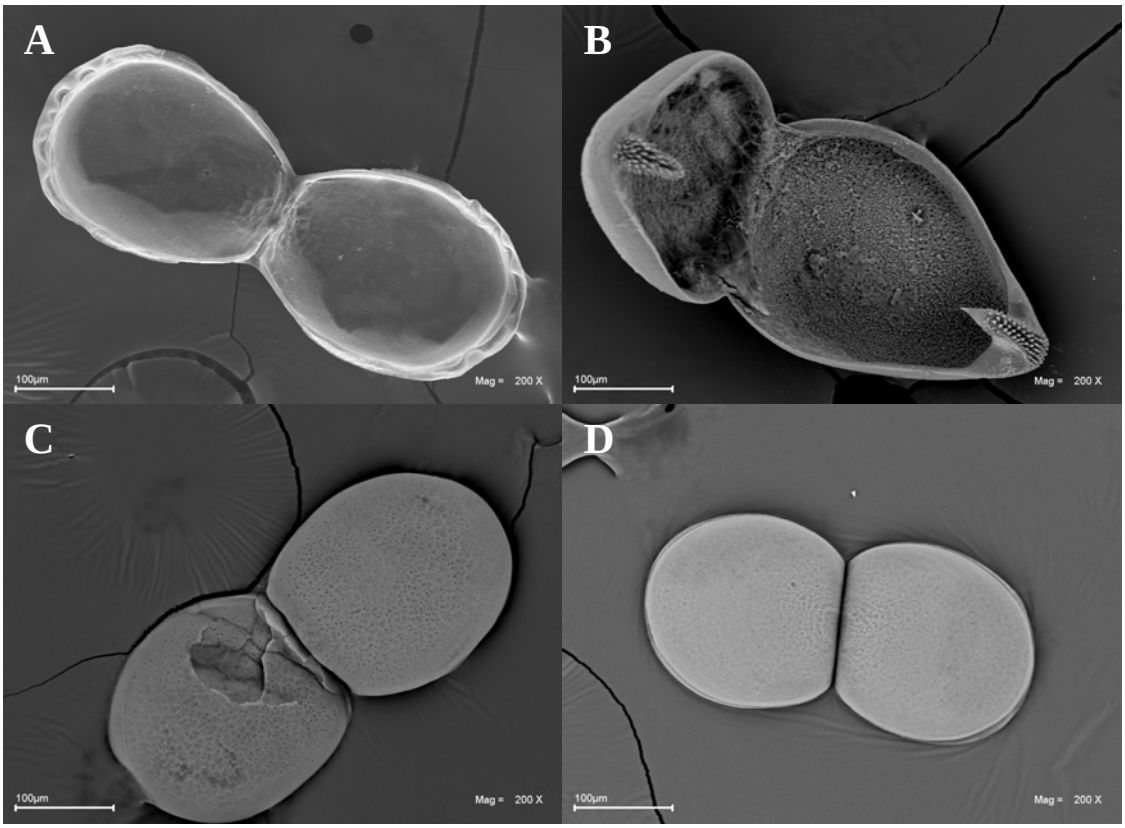

Figure 1.2. Scanning electron micrographs of glochidia from (A) *Lampsilis dolabraeformis* interior, (B) *Alasmidonta arcula* interior, (C) *Elliptio shepardiana* exterior, and (D) *Elliptio spinosa* exterior. All photographs were taken at 200X. Scale bars= 100µm.

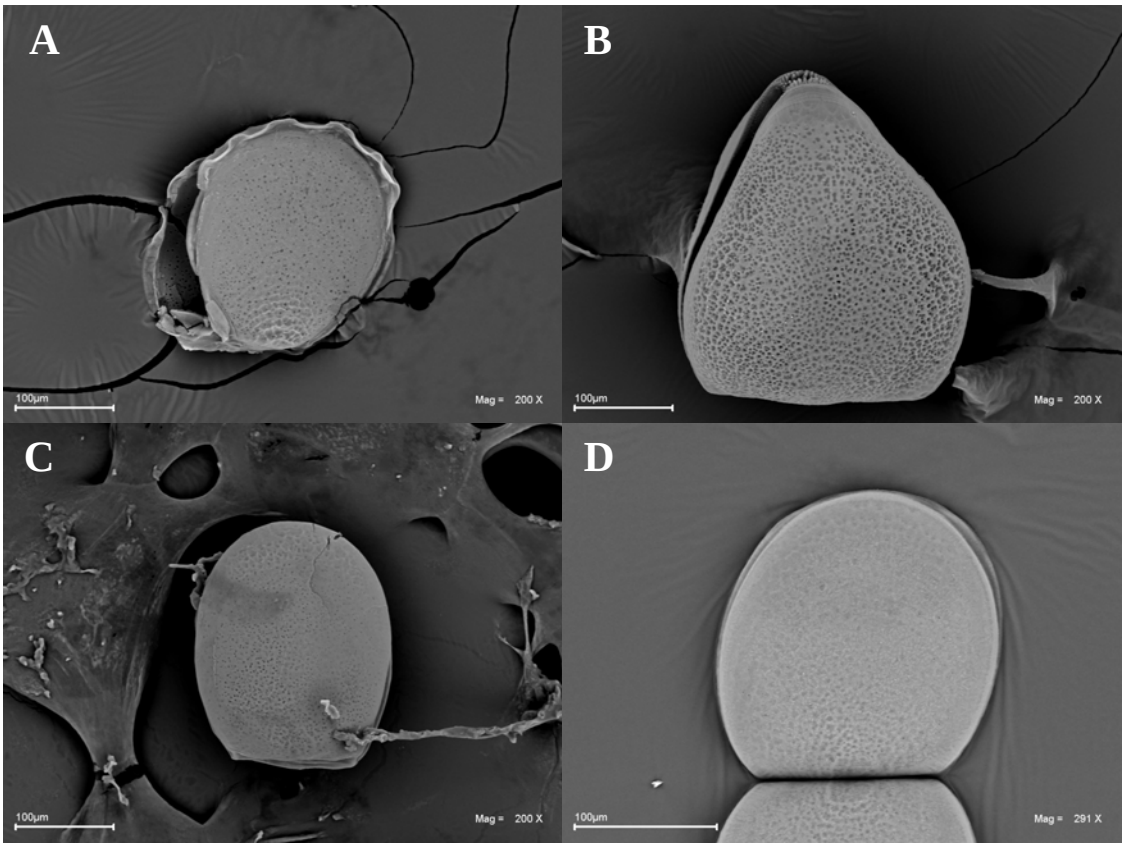

Figure 1.3. Scanning electron micrographs of the exterior valves of from (A) *Lampsilis dolabraeformis*, 200X, (B) *Alasmidonta arcula*, 200X, (C) *Elliptio shepardiana*, 200X, and (D) *Elliptio spinosa*, 291X. All scale bars=100µm.

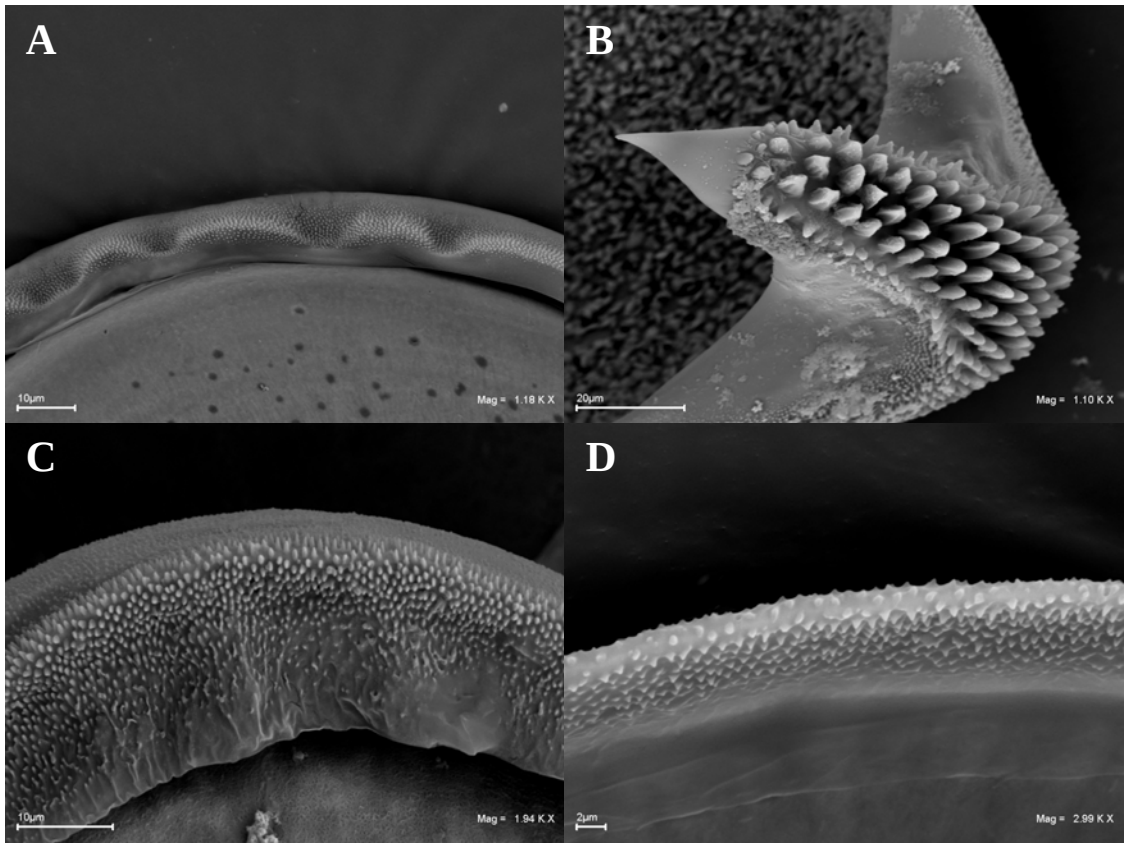

Figure 1.4. Scanning electron micrographs of interior flanges from (A) *Lampsilis dolabraeformis*, 1180X, (B) *Alasmidonta arcula*, 1100X, (C) *Elliptio shepardiana*, 1940X, and (D) *Elliptio spinosa*, 2990X.

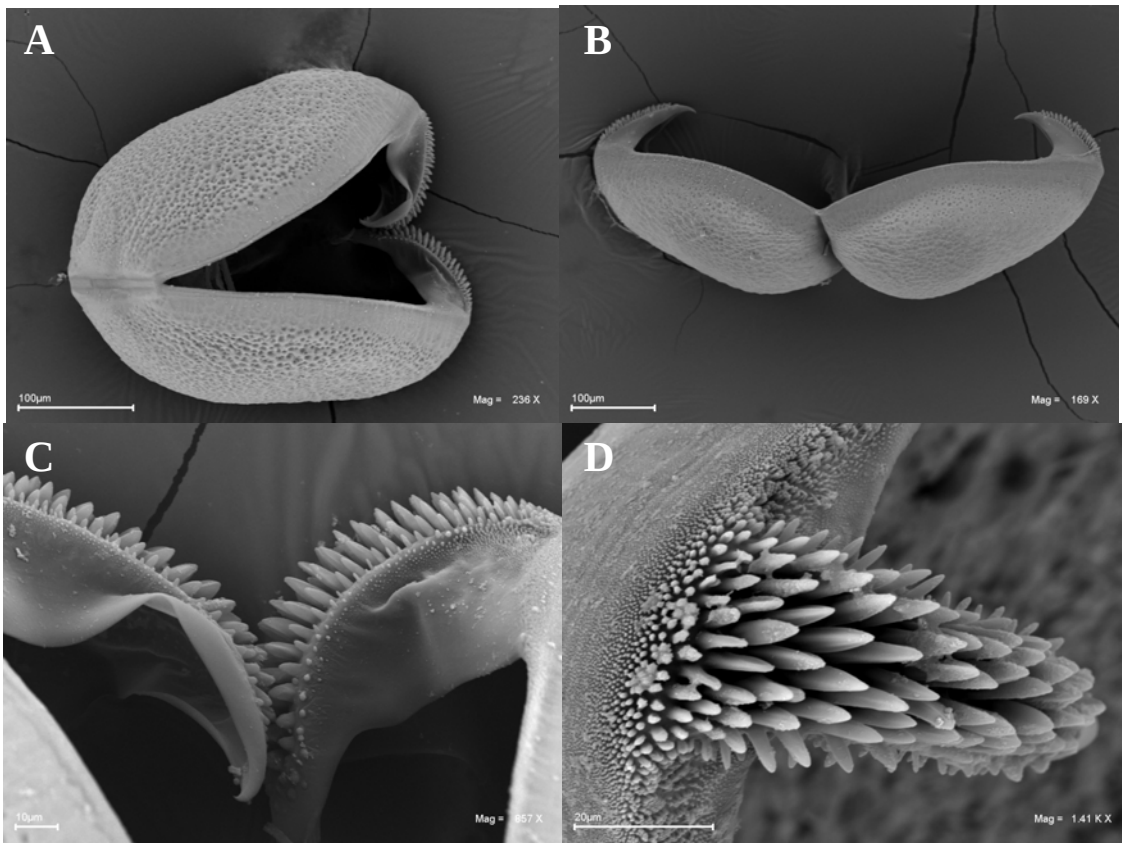

Figure 1.5. Scanning electron micrographs of *Alasmidonta arcula*. (A,B) Side profile of exterior valves, 236X, 169X respectively and (C,D) Styliform hooks, 857X, 1410X respectively.

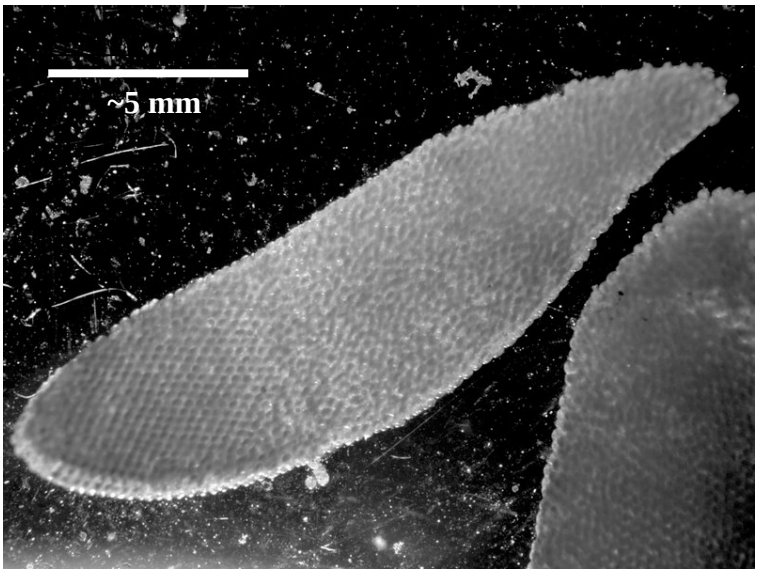

Figure 1.6. *Elliptio spinosa* conglutinate packet containing immature glochidia. Image taken at 6.3X.

CHAPTER 3

DEXAMETHASONE TREATMENT OF NON-HOST FISH TO INDUCE METAMORPHOSIS OF JUVENILE FRESHWATER MUSSELS[1]

[1] Johnson, Jennifer, Jason Wisniewski , and Robert Bringolf. To be submitted to Journal of Molluscan Studies.

Introduction

North America is home to approximately 36% (~300 species) of the world's freshwater mussel (Bivalvia: Unionoida) diversity (Graf and Cummings 2007). Unfortunately >70% of that unique mussel diversity is at risk of becoming endangered or extinct (Neves et al. 1997; Williams et al. 1993). Reasons for the declines are not well understood but urbanization, invasive species, dams, and pollution, siltation and scouring from poor land management practices have all likely contributed. Mussel populations may also be limited by their complex life cycle; for a brief period, mussel larvae (glochidia) are obligate parasites that depend on certain species of fish to metamorphose from glochidia to independent juveniles (Kat 1984). Host fish species have not been identified for many mussel species, including some of the most critically imperiled (Haag and Warren Jr 2003; Watters and O'Dee 1998). Host fish identification is critical for development of mussel management plans, which may be dependent on captive propagation programs.

In recent years, university, state, and federal agencies have made great strides in mussel conservation by advancing propagation techniques. Tank designs that include air-driven water-recirculation, automatic feeding, controlled water chemistry and flow have been used successfully for rearing juvenile and adult mussels in culture settings (Henley et al. 2001). Advancements such as the "Mucket Bucket" system (Barnhart 2006), have been valuable for improving survival and growth of juvenile mussels in laboratory settings. The "Mucket Bucket" system is beneficial in the fact that it's relatively inexpensive, compact, and allows for replication and for keeping groups of juveniles isolated from one another for a variety of purposes. The advancement of tank designs has led to successful propagation of a variety of imperiled species across the U.S. Since 2000, the Genoa National Fish Hatchery (Genoa, WI)

has released over 2 million juveniles of seven mussel species (http://www.fws.gov/midwest/genoa/mussel_recovery.html). In 2009 alone, the Virginia Department of Game and Inland Fisheries at the Aquatic Wildlife Conservation Center (Marion, Virginia), successfully propagated seven imperiled species of freshwater mussels, totaling ~860,500 juveniles (http://www.dgif.virginia.gov/awcc/freshwater-mussel-restoration/).

Development of mussel propagation programs generally depends on identification and availability of suitable host fish for the mussel species of interest. There are currently few options for propagation of mussel species with unknown host fish. In vitro culture techniques, a method in which the glochidia are placed in culture media in a petri dish, has been successful for metamorphosis of generalist mussel species, i.e. those whose glochidia can metamorphose on a variety of fish species (McGregor and Alexander 2008). Unfortunately, there are currently no methods for propagating host-specialist species (those that metamorphose on one or two species of fish). Therefore it is prudent and imperative to explore methods for inducing metamorphosis of juveniles when host fish are not known or are unavailable.

Little is known about the mechanism(s) that constrain which fish species are suitable hosts for a mussel glochidia but investigators have long speculated that the host fish immune response dictates whether or not glochidia can successfully attach and metamorphose. If the immune response theory is true, suppression of the host fish immune system may allow for glochidia attachment and metamorphosis on non-host fish. When the immune system of fish is suppressed there is often a higher instance of disease or parasitism. For example, parasite loads increased significantly on Atlantic salmon (*Salmo salar*) that were treated for 48 hours with 240 µg/L of dexamethasone, a known immunosuppressant (Nielsen and Buchmann 2003).

To date, only one published study has tested the use of immunosuppressant drugs to induce juvenile mussel metamorphosis on non-host fish. Kirk and Layzer (1997) reported promising results with two mussel species when they used intraperitoneal (IP) cortisol implants in non-host fish to facilitate juvenile mussel metamorphosis. Currently little is known about optimal immunosuppressant treatment methods and doses/concentrations for mussel propagation, or how corticosteroid immunosuppressants in the water may affect glochidia viability. Further investigation into immunosuppression of non-host fish could prove to be pivotal in the recovery of imperiled mussels, particularly species for which a host fish species has not been identified. Fish immunosuppression may also provide researchers with insight about the mechanisms that determine host-fish suitability.

The goal of this study was to determine if a synthetic corticosteroid, dexamethasone, could be used to immunosuppress non-host fish to facilitate glochidia attachment and metamorphosis into juvenile mussels. Specific objectives included: 1) evaluate dexamethasone treatment methods (immersion versus injection), 2) determine optimal dexamethasone dose and duration, and 3) perform an acute toxicity test to assess effects of dexamethasone on glochidia viability.

Methods

Test Species

I worked with *Lampsilis dolabraeformis* (Lea, 1838) to study the effects of fish immunosuppression on glochidia metamorphosis. Though *L. dolabraeformis* is endemic to the Altamaha River, its anatomy and life history are representative of other, more widespread and common members of the tribe Lampsilini. Additionally, *L. dolabraeformis* is considered

abundant and stable in the basin where it occurs (Wisniewski 2005). O'brien (2003) reported that largemouth bass (*Micropterus salmoides*) were a suitable host and bluegill (*Lepomis macrochirus*) were not a suitable host for *L. dolabraeformis*. Bluegill, largemouth bass, and *L. dolabraeformis*, are common, readily available, and can be maintained in the laboratory which makes them well-suited for experimentation.

Mussel and Fish Collection

I collected brooding female *L. dolabraeformis* from the mainstem Altamaha River near Jesup, GA. The Altamaha Basin encompasses 36,976 km² entirely within Georgia, USA and major tributaries include the Ohoopee, Ocmulgee, and Oconee rivers. I used visual and tactile search methods involving SCUBA and snorkeling. Brooding mussels were identified by gently opening mussel shells to look for inflated marsupial gills, confirmation that the female is brooding glochidia. Brooding *L. dolabraeformis* were transported in coolers (Cope 2003) to the Whitehall Fish Lab at the University of Georgia. Mussels were maintained between 5-10ºC in a Living Stream (Frigid Units Inc., Toledo, Ohio) to limit release of glochidia by the female mussels.

Fish develop humoral immunity to glochidia (Dodd et al. 2006; Meyers et al. 1980); therefore, it was essential that fish used in the experiments did not have any previous exposure to mussels. Largemouth bass (76-127mm) and bluegill (76-102 mm) were obtained from private, state, and federal fish hatcheries in Georgia and South Carolina, USA and from ponds at the Whitehall Forest on the campus of the University of Georgia (Athens, GA). Collected fish had no known prior exposure to mussels.

Dexamethasone Treatments

Dexamethasone (Dex), a synthetic corticosteroid, was obtained from Sigma-Aldrich® (St. Louis, Missouri, USA) and stored at 4^{o}C in the dark. Injection and immersion methods used for fish immunosuppression were similar to those used in previous studies (Kirk and Layzer 1997; Nielsen and Buchmann 2003). For immersion treatments, Dex was first dissolved in acetone to make a stock solution (16.7 mg/mL), and an appropriate volume of stock solution was added to glass aquaria (exposure tanks) containing 15 L of dechlorinated tap water to achieve the target treatment concentrations of 250µg/L, 500 µg/L, and 1000 µg/L. The control group was exposed to only dechlorinated tap water. For injections, Dex was dissolved in olive oil and fish were injected intraperitoneally (IP) with 200 µg/g. Sham injected fish received 100 µl of pure olive oil, which was equivalent to the most injected in any of the Dex-injected fish. For each trial, metamorphosis success (% M) was determined for *L. dolabraeformis* glochidia on Dex-treated and untreated non-host fish.

Experimental Design

Dexamethasone Trial 1 (January 2009). I compared the effects of immunosuppression on %M by two different Dex dosing regimens: immersion or IP injection. I randomly assigned 48 bluegill to one of six treatment groups: control (no injection or immersion), sham injection, 200 µg/g injection, and immersions of 250 µg/L, 500 µg/L, or 1000 µg/L for 48 hours. Following treatment all fish were placed in clean water for recovery and maintained for 48 hours before being inoculated with *L. dolabraeformis* glochidia for the host trial (details below). Untreated largemouth bass, the primary host for *L. dolabraeformis*, were inoculated as well for comparison of metamorphosis results and to ensure that glochidia were healthy and viable. A two-way

nested analysis of variance (ANOVA) was performed to detect any differences between treated and untreated groups, and differences among the treatment types ($\alpha = 0.05$). Largemouth bass treatments were not included in the two-way nested ANOVA.

Dexamethasone Trial 2 (March 2009). I tested the effect of immunosuppression on %M with five species of fish: bluegill, channel catfish (*Ictalurus punctatus,* 127-178mm) goldfish (*Carassius auratus,* 25-51 mm), golden shiner (*Notemigonus crysoleucas,* 76-102 mm), and eastern mosquitofish (*Gambusia holbrooki*, 25-35 mm). Based on the results of Trial 1, I incorporated only the 1000 µg/L Dex immersion treatment concentration for Trial 2. Unexposed groups of each fish species served as controls and largemouth bass were included to ensure that glochidia were viable and for comparison of metamorphosis results. Similar to Trial 1, fish in this trial were exposed to Dex for 48 hours but unlike Trial 1, the fish were immediately inoculated with *L. dolabraeformis* glochidia following the Dex immersion. The host trials are described below.

Dexamethasone Trial 3 (October 2009). Trial 3 was similar to Trial 2 except duration of Dex immersion was 7 days rather than 48 hours and different fish species were included: Bluegill, channel catfish, fathead minnow (*Pimephales promelas*, 50-76 mm*)*, and lake sturgeon (*Acipenser fulvescens,* 152-178 mm). Water in each exposure tank was renewed and Dex (1000 µg/L) was reapplied on days two, four, and six. On day seven, each treatment group was moved to clean water and then inoculated with *L. dolabraeformis* glochidia. Unexposed groups of each fish species served as controls and largemouth bass were included to ensure that glochidia were viable and for comparison of metamorphosis results.

Host Trials

Glochidia were extracted from 3-5 brooding female *L. dolabraeformis* by puncturing the inflated marsupium with a 5 ml syringe and gently flushing it with culture water (O'Brien and Brim-Box 1999; O'Dee and Watters 1998; Rogers 2001; Zale and Neves 1982). Extracted glochidia were collected in a petri dish and a subsample was tested for viability by exposing the glochidia to a saturated sodium chloride solution; viable glochidia responded by closing their valves in response to an irritant (O'Dee and Watters 1998; Rogers 2001). Glochidia were pooled from all females with >80% viability. Fish were separated by species into 19-L buckets containing 10 L of water and inoculated with approximately 4,000 glochidia per liter for one hour (O'Dee and Watters 1998). The buckets were aerated to keep glochidia suspended in the water column where they could contact the fish and attach to gill or fin tissue. Fish were then transferred to monitoring tanks within a recirculating system (AHAB® Aquatic Habitats Inc., Apopka, FL.) The AHAB unit is a closed system composed of racks of self-cleaning tanks through which water flows and then re-circulates back into a main sump for treatment including removal of solids, biofiltration, clarification with activated carbon and UV sterilization. Temperatures ranged from 21-24º C among the trials. Filter cups (2" pvc pipe with a 153 μm mesh screen on one end) were fitted to the outlet on each monitoring tank to catch the dead glochidia and metamorphosed juveniles as they sloughed off the fish.

Beginning the day after inoculation of glochidia (day 1), I checked daily for sloughed glochidia and metamorphosed juveniles by rinsing the filter cups into a Bogrov tray and looking at the contents under a stereomicroscope. Glochidia and juveniles were counted, photographed and recorded. Juvenile mussels were separated from dead glochidia and placed in a rearing system (Barnhart 2006) within their respective treatment groups to grow. Filter cups were

monitored until there were no glochidia or juveniles observed for five consecutive days. Juvenile metamorphosis success (% M) was calculated by the following formula: [(# of juveniles/ (juveniles + glochidia)) x 100]. Juvenile metamorphosis rates were compared between Dex-treated and untreated groups for each fish species

Lymphocyte Counts

Absolute lymphocyte counts were evaluated in bluegill (76-102 mm) to validate that Dex treatment resulted in immunosuppression and to provide additional information about the optimal Dex concentration and duration for immersion treatments. Methods for creating stock solutions for immersion were similar to those used for Trial 1.

Immersion treatment groups included baseline (fish sampled from the culture tank), solvent control (acetone), Dex 250µg/L, Dex 500µg/L, and Dex 1000µg/L. Each immersion treatment consisted of nine fish maintained in a 39-L aquarium with 35 L of dechlorinated tap water. Exposure water was renewed (50%) on days two, four, and six. Four fish were sampled for the baseline group on day 0. On days three, five, and seven following immersion, three fish were randomly sub-sampled from each treatment for blood samples (non-repeated measures). Fish were euthanized with an overdose of MS-222 and blood was collected from the caudal vein in hematocrit tubes coated with ethylenediamine tetraacetic acid (EDTA). Blood smears were prepared for each fish on glass microscope slides and the smears were stained for enumeration of leukocytes (lymphocytes, monocytes, granulocytes, and thrombocytes) on each slide. I was interested in the inflammatory response, so all cells were classified either as lymphocytes or "other leukocytes". Absolute lymphocyte numbers were calculated by multiplying the percentage of lymphocytes by the total white blood cell count (per µl). Means (N=3) of the

percentage of lymphocytes were calculated for each day fish were sampled. Means were compared with a repeated-measures ANOVA followed by a Tukey's post hoc analysis (α = 0.05).

Dexamethasone Glochidia Acute Toxicity Test

Acute toxicity of Dex to glochidia was evaluated according to standard guidelines (ASTM 2006). Brooding *L. dolabraeformis* (species used in other Dex trials) were not available at the time the acute toxicity tests were performed (August 2010). Instead, acute toxicity tests were performed with glochidia from another member of the same genus, *L. fasciola* (Rafinesque, 1820). In July, 2010 brooding *L. fasciola* were obtained from the North Carolina Wildlife Resources Division Hatchery in Marion, NC. Glochidia for the toxicity test were pooled from two female mussels (viability = 93%). Briefly, there were four Dex treatments and a control, with three replicates of each treatment. To prepare the stock solution, Dex was dissolved in acetone to 20µg/L. Test solutions were prepared in moderately hard reconstituted water (ASTM 2006) and were placed in 150-mL beakers. Each beaker contained 100 mL of test solution and approximately 300 glochidia. Target Dex concentrations included: 0, 500, 1000, 2000 and 4000 µg/L. Test solutions were not renewed during the experiment. At 24 and 48 hrs after glochidia were initially placed in the test solutions, subsamples of approximately 100 glochidia from each replicate were tested for viability by determining the number of glochidia that closed their valves in response to addition of a saturated solution of sodium chloride (ASTM 2006; O'Dee and Watters 1998; Rogers 2001). Water chemistry (temperature, specific conductivity, dissolved oxygen, and pH) was measured with a multi-probe (Hydrolab Quanta) in one replicate of each treatment group at time 0 hr and 48 hr. Dexamethasone test concentrations were not verified

analytically. Glochidia viability for treatments was compared with a repeated-measures ANOVA followed by a Tukey's post hoc analysis ($\alpha = 0.05$). All statistical analyses were evaluated at $\alpha=0.05$ level of significance.

Results

Injection and Immersions

Lampsilis dolabraeformis metamorphosis success on largemouth bass, the confirmed host for this mussel species, was high for all three trials (85.5%, 74.9%, 42.6%), indicating that the glochidia were healthy and viable. In Trial 1, there was a significant difference between treated and untreated groups ($p= 0.027$), but no difference among the types of treatments ($p= 0.806$, Table 2.1, Figure 2.1). The highest metamorphosis rates on bluegill were from the fish immersed in Dex at 500µg/L (4.7%; 12 juveniles) and 1000 µg/L (2.5%; 8 juveniles). Unfortunately, 6 of the 8 injected bluegill died before metamorphosis was complete but metamorphosis rate on the 2 bluegill that survived Dex injection (200 ug/g) was 3.0% (5 juveniles). The control and sham injected fish produced only one juvenile between the two treatments. Though the total number of juveniles produced by Dex-treated fish was relatively low, the high variability in %M within Dex treatments suggests that at least some fish exposed to Dex were affected by the treatment. Additionally, all bluegill treatment groups that produced metamorphosed juvenile *L. dolabraeformis* released the juveniles approximately 2 days sooner than those from the largemouth bass (Figure 2.2). Timing of sloughing of glochidia was similar among all bluegill treatments (Figure 2.3).

For Trials 2 and 3, I chose to work with only a Dex immersion concentration of 1000 µg/L because this treatment produced some juveniles in Trial 1 (8 total) and the variability of

%M for 1000 µg/L was lower than the other immersion treatments (Figure 2.1). Additionally, IP injections were labor intensive and fish receiving injections experienced high mortality rates. In Trial 2, none of the fish species treated with Dex other than bluegill produced juvenile mussels (Table 2.1). Similar to Trial 1, there was limited metamorphosis success on Dex-treated bluegill (%M = 3.4; 7 juveniles, Table 2.1), though untreated bluegill also produced juveniles (%M = 1.1; 7 juveniles, Table 2.1). Similar to Trial 1, glochidia from all bluegill metamorphosed before the largemouth bass (Figure 2.4) and the rate at which glochidia were sloughed was similar among fish species (Figure 2.5).

Trial 3 results were generally consistent with Trials 1 and 2. There was some metamorphosis on Dex-treated bluegill (%M = 1.2; 12 juveniles) and untreated bluegill (%M = 2.1; 15 juveniles, Table 2.1). There was no metamorphosis on the other fish species (treated or untreated; Table 1). Consistent with the other trials, juveniles from bluegill groups released earlier than from the largemouth bass (Figure 2.6) and patterns of glochidia sloughing over time were similar among all species (Figure 2.7).

Absolute lymphocyte counts

Absolute lymphocyte counts were not significantly different among Dex immersion treatments for days 5 and 7 ($p= 0.62$, 0.66 respectively). However, on day 7 post–immersion the absolute lymphocyte counts dropped substantially, though not statistically significant ($p= 0.127$), in the 1000 ug/L treatment (Figure 2.8), suggesting that the fish were immunocompromised by day 7.

Dexamethasone Acute Toxicity Test

Water chemistry was similar among treatments; temperature ranged from 20 - 22ºC, dissolved was 7 - 9 mg/L, pH was 6.3 – 8.2 and specific conductance was 0.334 – 0.341 µS/cm. There was no apparent effect of direct exposure to Dex on *L. fasciola* glochidia at 24 or 48 hrs of exposure (Figure 2.9). Glochidia viability for all treatment groups remained above 85% for the entire sampling period.

Discussion

Statistical Methods

I acknowledge that the true experimental replicate for Dex treatment is the tank to which the Dex was applied; however, due to logistical constraints I was unable to replicate Dex treatments and therefore, for statistical purposes, I calculated the mean number of juvenile mussels produced per monitoring tank. Individual monitoring tanks contained 2-3 fish, and were replicated four times in Trial 1 and three times in Trials 2 and 3. Statistical analyses were not performed on results from Trial 2 or 3 because of the lack of metamorphosis on Dex-treated fish.

Immunosuppression

The goal of this study was to determine if Dex, a synthetic corticosteroid, could be used to induce metamorphosis of juvenile mussels on immuno-compromised non-host fish. I attempted to determine the optimal exposure route and dose for Dex treatment. In addition, I tested if Dex had any toxic effects on glochidia. Injection methods were generally more laborious, time consuming, harmful to the fish (high mortality rate) and produced similar numbers of juveniles from treated fish as did the Dex immersion methods. The high fish

mortality using IP injections led me to focus on immersion methods which resulted in production of some metamorphosed juveniles on bluegill. However, relatively few juveniles were produced despite the Dex-treated groups being statistically higher than the controls.

Although Dex was not particularly useful for inducing metamorphosis on non-hosts for the mussel species I worked with, my results indicate that the fish immune system was suppressed by exposure to Dexamethasone at a concentration of 1000 µg/L for 7 days. Lower concentrations and shorter durations did not cause a reduction of total lymphocyte counts which suggests that the fish may not have been sufficiently immunocompromised. Despite the lower lymphocyte counts the treatment regimen, 1000 µg/L Dex for 7 days, did not result in increased %M of juvenile mussels, which suggests more research is necessary to determine the optimal dose and duration of Dex for inducing juvenile metamorphosis on non-host fish. Additionally, I treated several fish species with Dex but only attempted metamorphosis with one mussel species (*L. dolabraeformis)*. It is possible that immunosuppression may be more successful with other mussel species. Dexamethasone was not harmful to glochidia at concentrations as high as 4000 µg/L so higher concentrations could be tested with fish and glochidia exposed simultaneously. Furthermore the results of my study, though certainly not definitive, suggest that host fish immunity may not be the primary factor for determining appropriate species of host fish, as has been commonly speculated previously. These results could inform future studies of mechanisms that determine host and non-host fish species for mussels.

Some interesting trends were evident from my study. For example, all juveniles produced from non-host fish (bluegill), treated with Dex or not, metamorphosed 1-3 days earlier than juveniles that metamorphosed on the host fish (largemouth bass, Figures 2.2, 2.4, 2.6). One possible explanation could be that antibodies in host fish increase significantly 10 days after

initial exposure to glochidia (Rogers-Lowery et al. 2007) and conceivably the effects of the immunosuppressant wore off and the non-host fish was finally able to slough off the juveniles. Unfortunately I was unable to culture any of the juveniles for more than a few weeks to determine if there were differences in fitness between the juveniles produced from the primary host and juveniles produced from the non-host. Evaluating juvenile fitness is an important area of future research if immunosuppression of non-host fish is going to become a viable option for propagation of juvenile mussels.

Another possibility for the difference in time to metamorphosis is that different species of fish may have different time periods for juvenile metamorphosis. Although I did not find any previous studies that specifically investigated the difference in time to metamorphosis between fish species, results from published host trials illustrates this could be a possible explanation (Haag and Warren Jr 2003; O'Dee and Watters 1998; Rogers 2001). For example, O'Dee and Watters (1998) reported that smallmouth bass were primary hosts for *L. radiata* (Lamark 1819) and juveniles metamorphosed between days 32-40; whereas, longear sunfish (*Lepomis megalotis*) were marginal hosts and juveniles metamorphosed between days 28-30.

Treatment of fish with Dex has resulted in higher incidences of parasites and bacteria on immunosuppressed fish (Lindenstrom 1998; Nielsen and Buchmann 2003). Nielsen and Buchman (2003) demonstrated that Atlantic salmon (*Salmo salar*) treated with Dex had significantly higher loads of *Gyrodactylus derjavini* (Monogenea) than unexposed treatments. Likewise, Antonio and Hedrick (1994) reported a higher incidence of bacterial disease in channel catfish exposed to Kenalog, a synthetic corticosteroid. These studies have illustrated that corticosteroids can be used to immunosuppress fish and that this condition results in an increased parasite and bacterial load. However, only one previous study investigated the use of

immunosuppressant treatments to enhance parasitism of glochidia on fish and the resulting metamorphosis of juvenile freshwater mussels (Kirk and Layzer 1997). Kirk and Layzer (1997) treated non-host fish with cortisol and documented enhanced metamorphosis on treated fish compared to non-treated fish. However, relatively few juveniles (maximum of 17 per fish) were produced in each experiment and the results varied between each set of experiments. Despite the relatively low numbers of juveniles produced by Kirk and Layzer (1997), proof of concept was established and additional efforts to optimize the dosing regimen may result in enhanced production of juvenile mussels in the absence of primary host fish.

Dexamethasone has recently been shown to only inhibit the non-specific (innate) immune response, not the humoral response (Lovy et al. 2008). Kirk and Layzer (1997) speculated the corticosteroid they used, cortisol, successfully suppressed both the humoral and innate responses. Given the marginal success of Kirk and Layzer study and the limited results in my study, it may be that the humoral response in fish plays a larger role in determining host suitability than previously thought. Additional research is needed to understand mechanisms controlling host suitability.

All treatment groups, including the largemouth bass, had similar patterns of glochidia sloughing (1-3 days post inoculating). The first 24 hours is the time period when a difference in rates of epithelial tissue encapsulation of the glochidia is expected between host and non-host fish. If the fish is a host, encapsulation occurs more quickly than that of glochidia on a non-host (Rogers-Lowery and Dimock 2006). Encapsulation on a non-host is slower and has an irregular formation of tissue surrounding the glochidia (Rogers-Lowery and Dimock 2006). This suggests that the glochidia depend, at least in part, on the innate immune response (responsible for encapsulation) for metamorphosis to occur. If the innate immune response, an integral part of

the process, was suppressed by Dex it would explain why immunosuppressiondid not work in my study. If this theory is true, suppressing the known primary host with Dex should result in reduced juvenile metamorphosis. Unfortunately, in these trials I did not explore this possibility. For future research into mechanisms controlling host fish suitability, immunosuppression of host-fish is highly recommended.

This study has generated several questions regarding the mechanisms that control host fish suitability. A number of scenarios may explain why Dex was not more effective at inducing metamorphosis on non-host fish. Further study of the fish immune system has the potential to provide more insight into the mechanisms of host suitability; if researchers determine the specific factors involving host fish suitability and mussel metamorphosis it could provide managers with valuable alternatives for producing juvenile mussels in the absence of suitable host fish. Immunosuppression of non-host fish still requires much research but is promising and it may bide critical time to identify natural host fish and develop best management practices that are greatly needed for the conservation of freshwater mussels.

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Table

Table 2.1. Summary of fish immunosuppression trials with Dexamethasone and resulting metamorphosis of *Lampsilis dolabraeformis*.

Trial	Fish sp.	Treatment	Juveniles	% Metamorphosis (±Stdev)
1	*Micropterus salmoides*	Control	1417	85.5 ± 9.6
	Lepomis macrochirus	Injection 200 µg/g	5	2.4 ± 2.4
		Sham	0	0
		Control	1	0.7 ± 1.4
		Immersion (250 µg/L)	2	1.3 ± 1.5
		Immersion (500 µg/L)	12	4.7 ± 5.0
		Immersion (1000 µg/L)	8	2.5 ± 1.3
2	*M. salmoides*	Control	1021	74.9 ± 5.7
	L. macrochirus	Immersion (1000 µg/L)	7	3.4 ± 4.3
		Control	7	1.1 ± 1.9
	Carassius auratus	Immersion (1000 µg/L)	0	0
		Control	0	0
	Gambusia holbrooki	Immersion (1000 µg/L)	0	0
		Control	0	0
	Ictalurus punctatus	Immersion (1000 µg/L)	0	0
		Control	0	0
	Notemigonus crysoleucas	Immersion (1000 µg/L)	0	0
		Control	0	0
3	*M. salmoides*	Control	117	42.6 ± 8.8
	L. macrochirus	Immersion (1000 µg/L)	12	1.2 ± 1.2
		Control	15	2.08 ± 1.9
	Acipenser fulvenscens	Immersion (1000 µg/L)	0	0
		Control	0	0
	I. punctatus	Immersion (1000 µg/L)	0	0
		Control	0	0
	Pimephales promelas	Immersion (1000 µg/L)	0	0
		Control	0	0

Figures

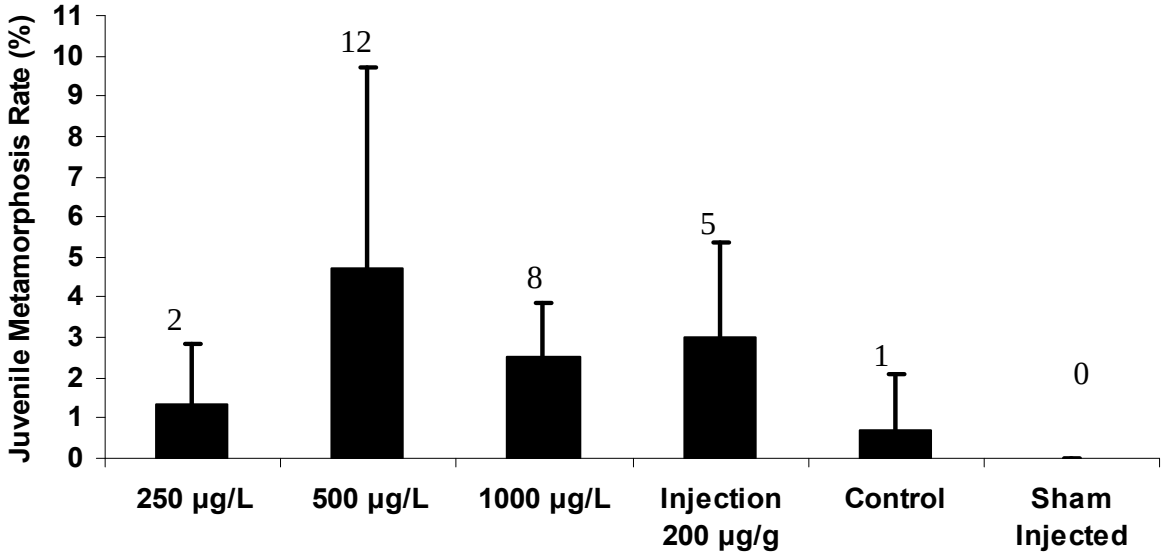

Figure 2.1. Metamorphosis success of *Lampsilis dolabraeformis* on bluegill (n=4) exposed to dexamethasone by 48-hour immersion or intraperitoneal injection. Numbers above each bar represent the number of juveniles produced in that treatment group. Error bars represent one standard deviation. There was a statistically significant difference between treated and untreated (Control and Sham Injected) groups ($p = 0.027$). There were no statistically significant differences among treatments ($p = 0.806$).

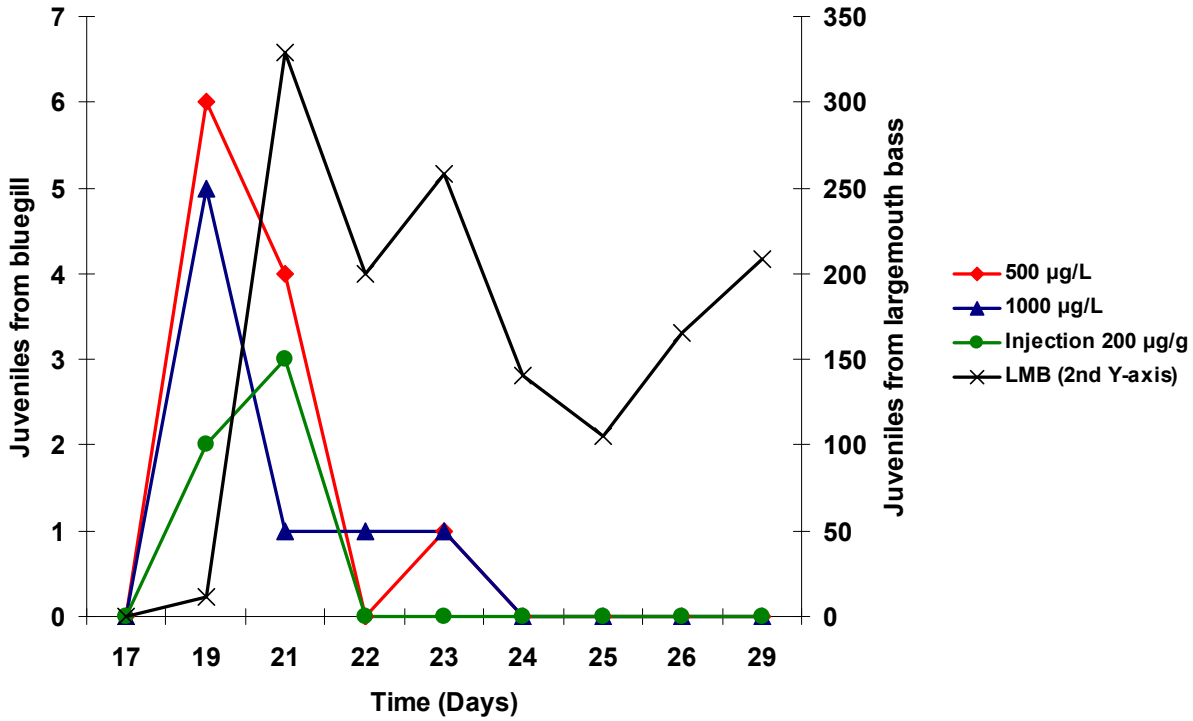

Figure 2.2. Timing of release of *Lampsilis dolabraeformis* juveniles from bluegill treated with Dexamethasone (48-hour immersion: 500 µg/L and 1000 µg/L, injection 200 µg/g), and from the primary host, largemouth bass (LMB). X-axis is days from initial exposure to glochidia.

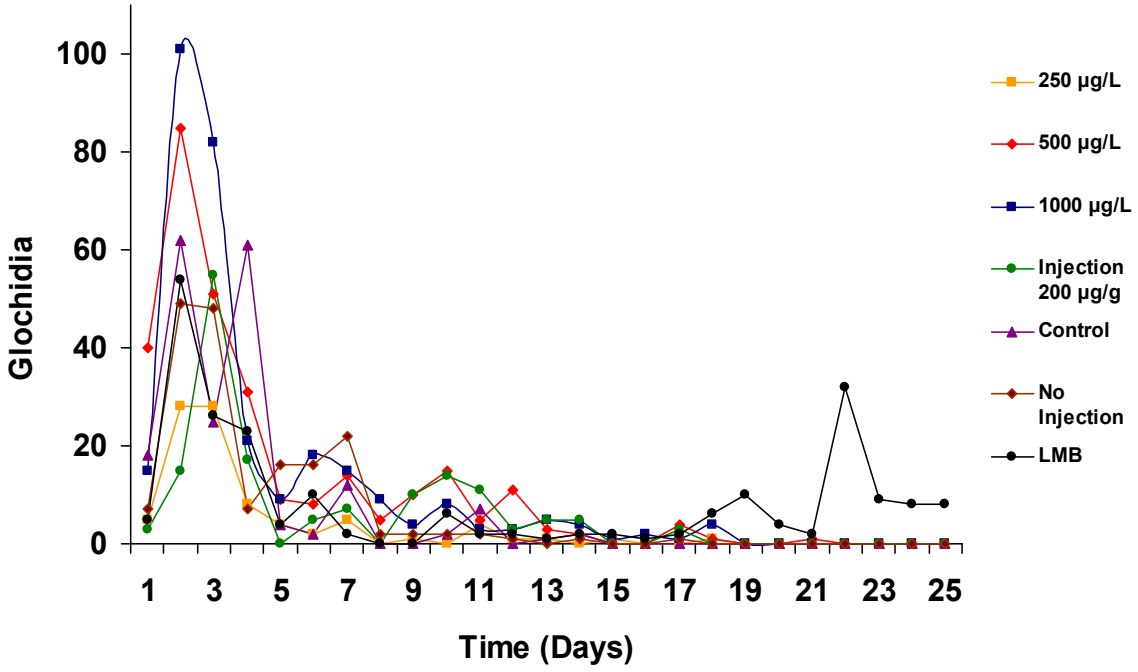

Figure 2.3. Timing of release of sloughed *Lampsilis dolabraeformis* glochidia from bluegill treated with Dexamethasone (48-hour immersion: 250, 500, 1000 µg/L, injection 200 µg/g) and from the primary host, largemouth bass (LMB).

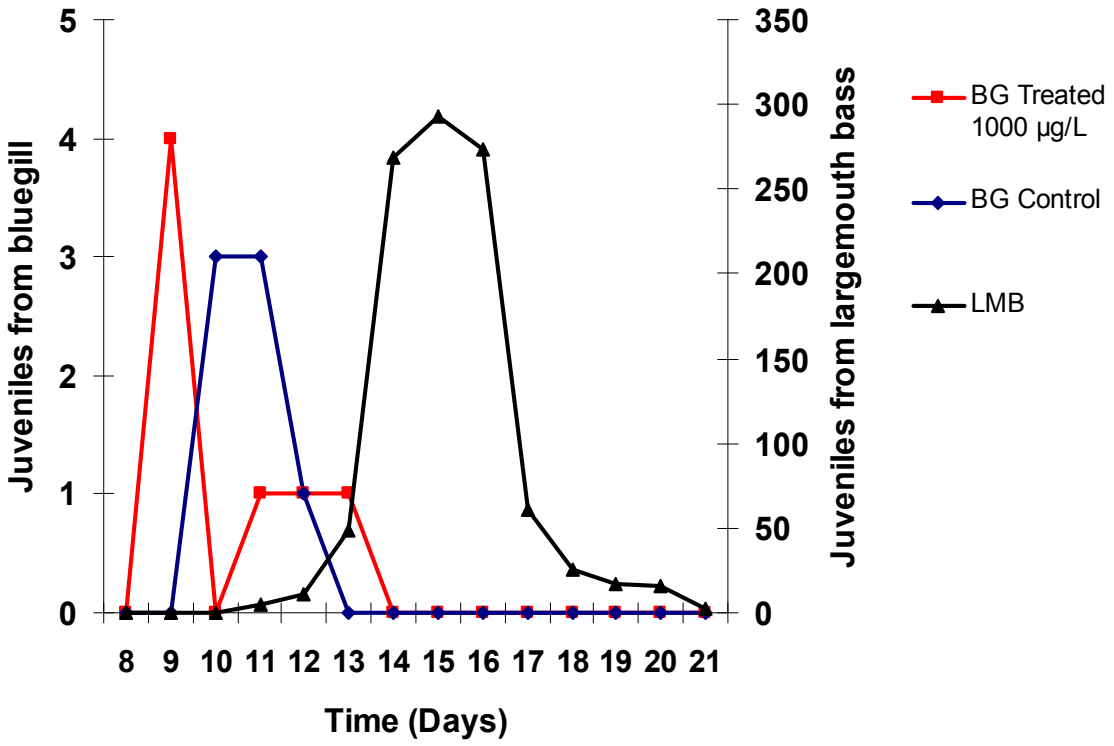

Figure 2.4. Timing of juvenile metamorphosis for *Lampsilis dolabraeformis* on bluegill (BG) treated with Dexamethasone (1000 µg/L) for 48 hours, untreated (Control) bluegill, and the primary host largemouth bass (LMB). X-axis is days after initial exposure to glochidia.

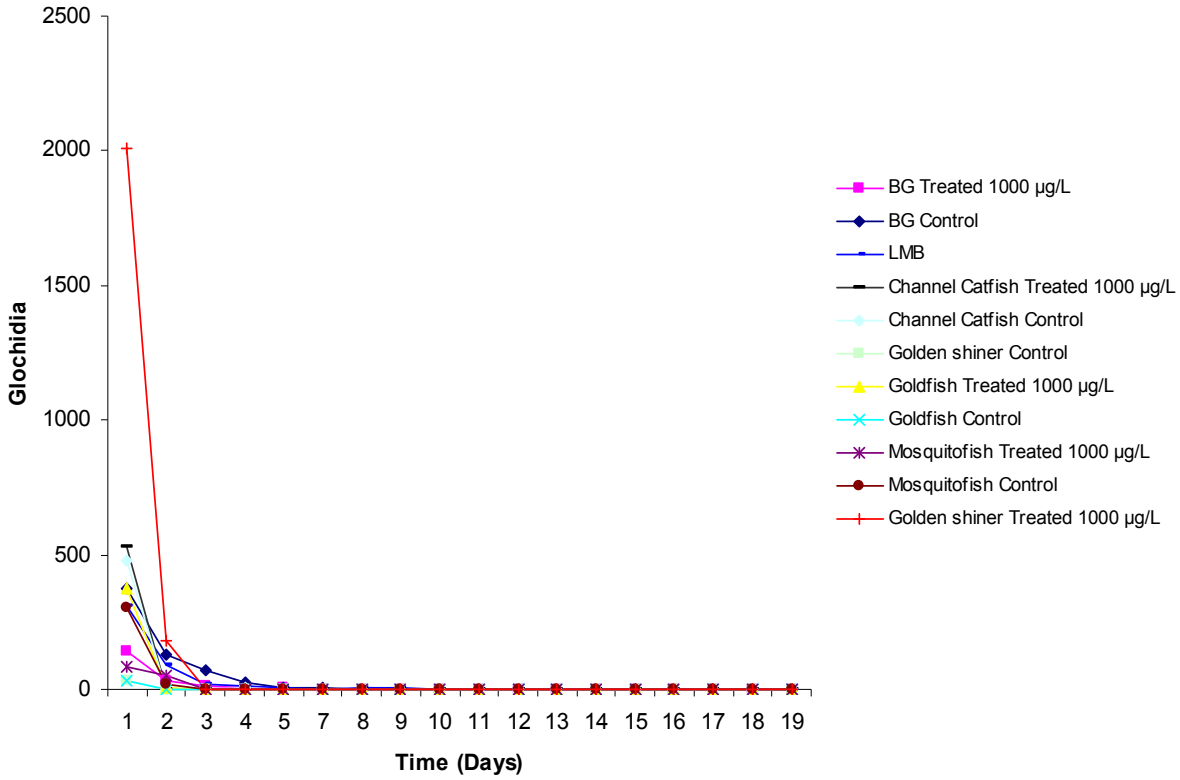

Figure 2.5. Timing of release of sloughed *Lampsilis dolabraeformis* glochidia for untreated (Control) and 1000 µg/L Dexamethasone 48-hour treated bluegill (BG), channel catfish, golden shiners, goldfish, and mosquitofish and the primary host largemouth bass (LMB). X-axis is the days following initial glochidia exposure to fish.

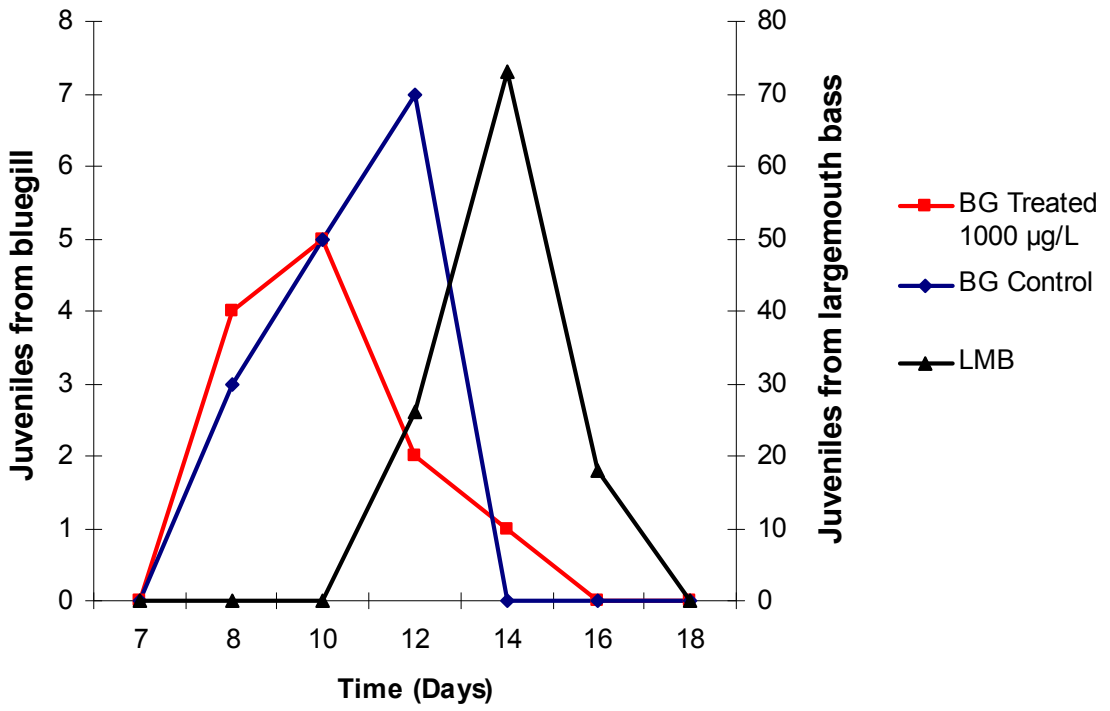

Figure 2.6. Timing of juvenile metamorphosis success for *Lampsilis dolabraeformis* on bluegill (BG) treated with 1000 µg/L of dexamethasone for 7 days, untreated bluegill (Control), and largemouth bass (LMB). X-axis is days after initial exposure to glochidia.

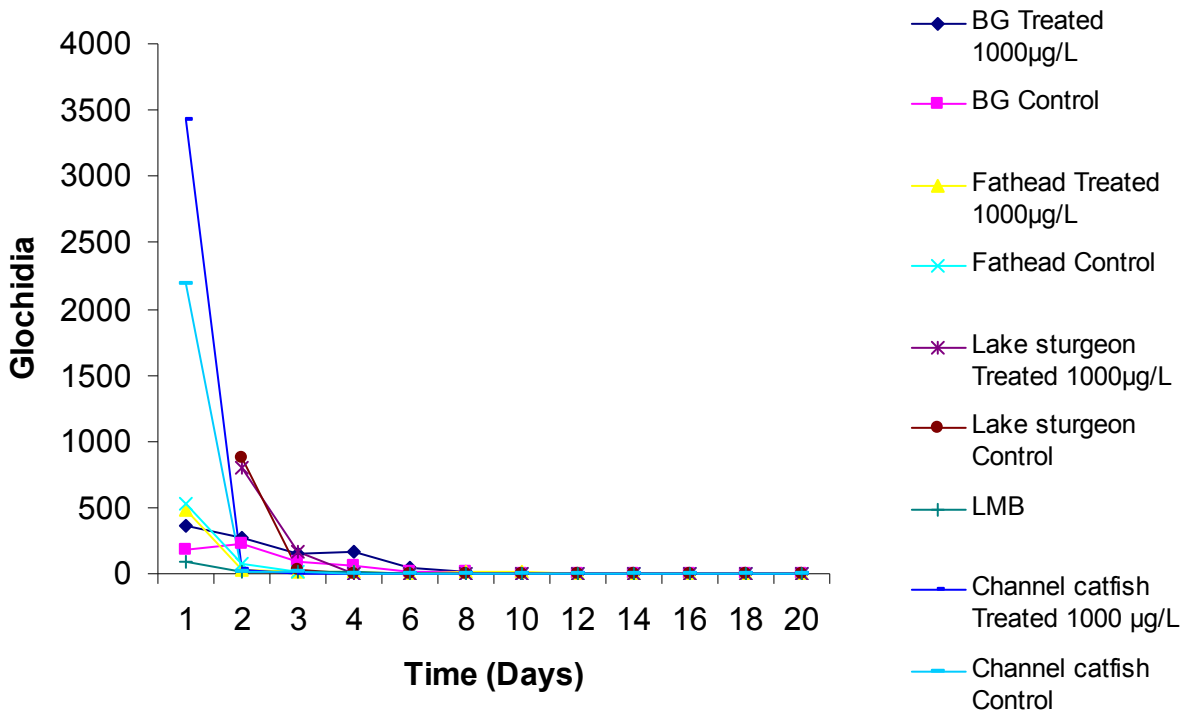

Figure 2.7. Timing of release of sloughed *Lampsilis dolabraeformis* glochidia for untreated (Control) and 1000 µg/L Dexamethasone 48-hour treated bluegill (BG), fathead minnows, channel catfish, lake sturgeon and the primary host largemouth bass (LMB). X-axis is the days following initial glochidia exposure to fish.

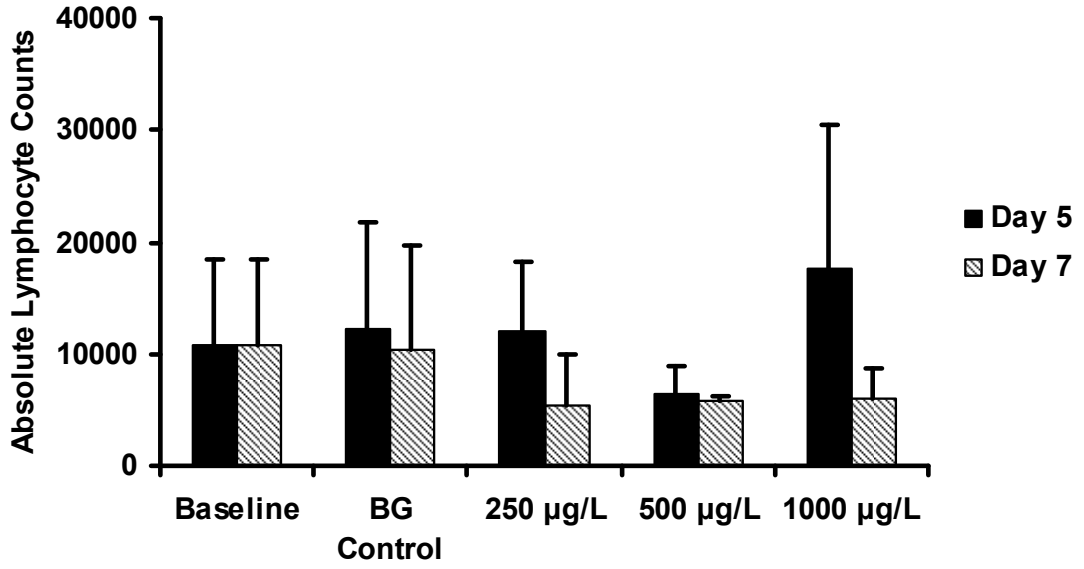

Figure 2.8. Absolute lymphocyte counts for bluegill at five and seven days after immunosuppression with dexamethasone (250µg/L, 500µg/L, and 1000µg/L). Error bars illustrate one standard deviation. Absolute lymphocyte counts were not significantly different between days 5 and 7 ($p = 0.08$). Absolute lymphocyte counts were not significantly different among Dex immersion treatments for days 5 and 7 ($p = 0.62$, 0.66 respectively).

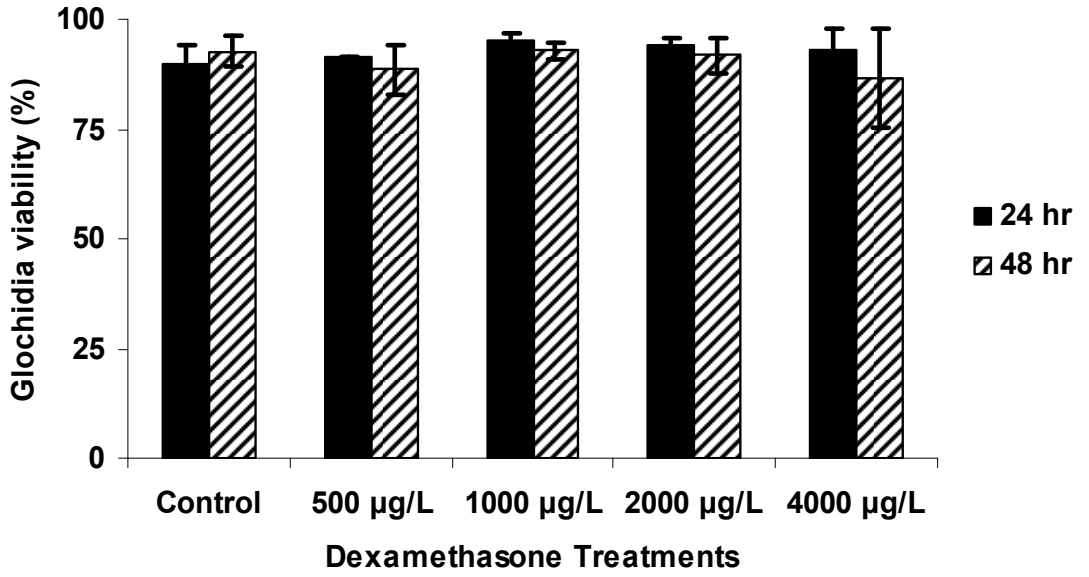

Figure 2.9. Viability of *Lampsilis fasciola* glochidia following exposure to waterborne Dexamethasone (500µg/L, 1000µg/L, 2000µg/L, and 4000µg/L). Viability was determined by subsampling glochidia (~1000/replicate, N=3) and observing their response to a saturated sodium chloride solution. Error bars illustrate one standard deviation. Viability was not significantly different ($p = 0.9$) among treatments at either time point (24 or 48 hr).

CHAPTER FOUR

DISCUSSION

Early life history information from my study will provide managers with some of the essential ecological information necessary when formulating management and recovery plans for mussels of the Altamaha River basin. For instance, host identification for the *A. arcula* was listed as a top priority in the 2005 Georgia Wildlife Action Plan. Mussel recovery plans must consider host fish information to determine if suitable host fish are present in the basin. Furthermore, without host fish mussel population augmentation will likely be unsuccessful in the long-term. I identified host fish for seven species of freshwater mussels of the Altamaha Basin, three of which had no previous known host: *E. shepardiana, L. splendida*, and *A. arcula*.

My study also provided some of the first detailed descriptions for glochidia of endemic mussels of the Altamaha River basin. Glochidia morphology may be useful for identifying glochidia attached to fish collected in the wild, thereby providing some host information. Glochidia description may be more applicable in regions with fewer species of mussels of a given genus because morphological variation with a genus is often limited. Detailed descriptions of glochidia morphology may provide identification of key taxonomic characteristics that help scientists and resource managers unequivocally identify the adults from which the glochidia were obtained and may provide additional information about phylogenetic relationships among mussel species.

Future studies should focus resources on investigating reproductive biology and early life history of imperiled mussel species. Efforts to protect and enhance imperiled mussel species will

be greatly improved by knowledge of brooding periods, optimal temperatures and flows, host fish, and descriptions of glochidia morphology. Knowledge of mussel early life history can help answer questions about why mussel populations are declining (ie. loss of fish host or preferred habitat), which can ultimately lead to the preservation of freshwater mussel biodiversity.

Furthermore, metamorphosis rates of mussels in the natural environment are not well understood and should be further evaluated. Lab inoculations of glochidia simulate the most optimal conditions for attachment. High metamorphosis rates in the lab may not necessarily correlate to high success of attachment and metamorphosis in the natural environment. There may be unknown limiting factors (ie. intra- and interspecific competition) preventing the mussel species from completing their life cycle. Furthermore, to categorize a host fish as "primary" or "marginal" may be an oversimplified description. For example, Martel and Lauzon-Guay (2005) were able to investigate density of glochidia of the generalist species *Anodonta kennerlyi* (Lea, 1860) in three lakes on Vancouver Island, British Columbia and determined that glochidia density on fish was always higher on the species of fish that cohabitated with *A. kennerlyi*. However, there were always glochidia found on the two salmonid species in the lakes, although in much smaller densities. The authors speculated the use of salmonids as hosts may be advantageous because the salmon swim over a wide range of the lakes, thereby dispersing the juveniles over a greater area than other fish species. Interestingly, all three lakes differed in densities of glochidia on the four species of fish tested, illustrating different dominant host species for the three separate populations of *A. kennerlyi*. Martel and Lauzon-Guay (2005) highlights our lack of understanding of the mussel host relationships in the wild. Speculating further, perhaps the salmonids are simply a "back up" for when their primary host is unavailable. In my study, results from the *A. arcula* host trial indicate the robust redhorse is a marginal host

for this species. If "Liem's paradox" (i.e. Specialization retained, although not necessarily utilized until competition is present; Liem 1980) holds true, the robust redhorse could be *A. arcula*'s specialized host when there is high interspecific competition; thereby, making the robust redhorse biologically more important than *A. arcula*'s primary host. Studies like mine and Martel and Lauzon-Guay (2005) further highlight the need for more natural history investigations of mussels, both in the laboratory and natural environment settings.

Another goal of this study was to investigate the utility of immunosuppression of non-host fish to enhance glochidia metamorphosis. Results of the trials were initially promising but ultimately treatment of fish with dexamethasone did not result in production of large numbers of juvenile mussels. The lack of successwith Dex may be explained in a number of ways. and raises questions regarding the mechanisms controlling host suitability. One potential explanation that cannot be ignored is that host suitability is, in fact, not determined by the fish's immune system. Alternatively, the immune system may play a key role in this process but both the innate and humoral immune response of host fish may be involved in determining host suitability. Dexamethasone suppresses only the innate immune system; therefore, my study is not definitive for this approach. Further study of the fish immune system is needed to provide additional insight into the mechanisms of host suitability. Despite the limited success with juvenile production in my study, the use of immunosuppression to induce juvenile metamorphosis on non-host fish remains promising and could eventually prove to be a valuable tool for restoration of freshwater mussels, particularly for threatened and endangered populations for which host fish have not be identified. If researchers determine the specific factors involving mussel metamorphosis on host-fish, it could provide managers with valuable time to develop best management practices that are greatly needed for the conservation of freshwater mussels.

The chapters of this thesis focus on mussel conservation, particularly through mussel propagation. However, future efforts that involve propagation and possible stocking of freshwater mussels should proceed with caution and extreme care. Before any restoration efforts begin, researchers and managers must carefully plan their approach. Suitable habitat, genetic variation within and among the mussel populations, and appropriate age and sex ratios need to be clearly understood. Furthermore, researchers should investigate possible interactions with other aquatic species (ie. competition or hybridization). Ultimately, stocking of mussels should not be the only approach to mussel conservation. Educating the general public on best land use practices may indeed be the most pivotal conservation step to minimize habitat degradation.

In conclusion, North America's unique freshwater mussel diversity has a critical role in aquatic ecosystem structure and function. Unfortunately this fauna has experienced and is experiencing an imminent threat of being vastly reduced. Research and management efforts are making great strides but more research and restoration efforts are needed. My study will hopefully be a part of the foundation upon which future conservation decisions are made.

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