QUANTIFYING THE FRESHWATER MUSSEL - FISH HOST RELATIONSHIP TO INFORM CONSERVATION: GULF STURGEON AS POTENTIAL HOSTS AND EFFECTS OF EXOGENOUS CORTISOL

by

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(Under the Direction of Robert B. Bringolf)

ABSTRACT

Among the many global cases of drastic declines in faunal biodiversity, the North American freshwater mussel fauna represents an extreme case. This project first examined the potential host relationships between mussels native to the Apalachicola-Chattahoochee-Flint river basin (ACF) and a single species, the Gulf sturgeon. *Amblema neislerii* (fat threeridge) alone was found to successfully metamorphose (3.5%) on Atlantic sturgeon, the sister subspecies to Gulf sturgeon. This project second examined the physiological aspect of the mussel-fish host relationship via investigation of the effects of exogenous cortisol on larval metamorphosis. This investigation found that administration of cortisol to potential host fish can affect mussel metamorphosis in many ways: increased initial glochidial attachment, increased proportion of successful metamorphosis, higher number of juveniles produced, and a protracted time period of juvenile production. These findings contribute to basic research into the physiological mechanisms underlying the mussel-fish host relationship and applied research in captive propagation techniques.

INDEX WORDS: Freshwater mussel, host fish, Gulf sturgeon, Apalachicola-Chattahoochee-Flint (ACF), cortisol, immunosuppression, metamorphosis

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

FRESHWATER MUSSELS

Freshwater ecosystems around the world are experiencing declines in faunal biodiversity (Richter et al. 1997); among the most well documented examples of this phenomenon are the freshwater mussels in the Southeast region of North America (Bogan 1993; Neves et al. 1997). With approximately 300 described mussel species and subspecies, North America is the most phylogenetically rich region in the world (Turgeon 1998; Williams et al. 1993). Of these species, 10% are already extinct and 65% of the remaining species are classified as threatened, endangered, or vulnerable to extinction (Haag and Williams 2014). The projected rates of extinction within this taxon are comparable to rates described for tropical rainforests, the most threatened terrestrial biome (Ricciardi and Rasmussen 1999).

As generally long-lived benthic filter-feeders, mussels are vulnerable to many anthropogenic threats (Bogan 1993; Cope et al. 2008). Mussel habitat destruction can result from agricultural sediment pollution, removal of gravel beds by dredging for maintenance of transportation routes, and inundation of habitat upstream of hydroelectric dams (Williams et al. 1993; Wood and Armitage 1997). Habitat downstream of dams is often degraded because of drastically altered hydrologic regimes (Vaughn and Taylor 1999; Watters 1999). Agricultural nutrient pollution and non-point municipal effluents also threaten the survival and reproductive functions of mussels (Watters 1999).

Mussels provide a wide array of ecosystem services in freshwater systems: water filtration, sediment bioturbation, nutrient cycling through and among trophic levels, and habitat improvement. Mussels act as a bridge to upper trophic levels by feeding at multiple trophic levels on phytoplankton, zooplankton, bacteria, algae, and detritus via actively-pumped filtration (Vaughn et al. 2008). Bioturbation resulting from mussel burrowing activities increases the oxygen, water, and nutrient content of sediments (Vaughn et al. 2008). Physical construction of habitat by living and dead shells, along with biodeposition of nutrients, improve habitat for congeners, a variety of macroinvertebrates, and fish (Gutiérrez et al. 2003; Vaughn and Spooner 2006). Recently, live mussels have also been found to harbor developing eggs of co-occurring fish, which suggests a possible co-dependence of mussels and fish (Wisniewski et al. 2013). Thus, local extirpation of mussel assemblages may have significant, negative cascading effects through multiple trophic levels (Spooner et al. 2012; Vaughn et al. 2008).

As part of their life history, unionid mussels complete a metamorphosis (usually two to four weeks) in which larvae (glochidia) must attach to the gills or fins of fish before entering the juvenile stage. To successfully begin metamorphosis, the attached glochidia must trigger a limited immunological reaction on the part of the host fish where the adjacent epithelial tissue of the host fish grows to encapsulate the attached glochidium. During encapsulation (encystment), the glochidium either undergoes metamorphosis to the juvenile stage or is destroyed by the host's immune system and sloughed off (Rogers-Lowery and Dimock 2006). This obligatory parasitic life stage, thought to serve as a dispersal mechanism, introduces another potential threat to mussel populations (Haag 2012; Strayer 2008). Elimination of host fish by overfishing, dam construction, and other threats may result in local extirpation of specialist mussels reliant upon that fish (Kelner and Sietman 2000; Vaughn and Taylor 1999; Watters 1996).

Mussels exhibit a wide range of host specialization and reproductive strategies. Unionids capable of successfully undergoing metamorphosis on only a small number of species or genera are referred to as specialists. Alternatively, unionid species capable of metamorphosis on many species in multiple fish families are referred to as generalists (Barnhart et al. 2008; Haag 2012). A fish species that successfully produces juveniles of a given mussel species with a high level of success is considered to be a 'primary' host for that unionid. A 'secondary' or 'marginal' host is one that produces juveniles at a greatly reduced level (Fritts et al. 2012; Haag 2012). The variety of host infection mechanisms include simple broadcasting of glochidia, production of glochidia-laden mucus webs, use of prey-mimicking mantle lures, and prey-mimicking conglutinates (discrete packets of glochidia) (Barnhart et al. 2008). With steadily increasing knowledge of basic mussel biology, the discussion of ecological categories is growing. Some species use the broad life history strategy of long life span, slow growth, and late maturation, while other species are characterized by short life span, fast growth, and early maturation (Haag 2012).

Dedicated research efforts focusing on the mussel-fish host relationship and other aspects of mussel natural history emerged in the early 20th century in response to major mussel population declines. Two primary areas of this early research were the identification of fish host species and the description of factors involved in larval metamorphosis and host specificity (Haag 2012). While the volume and variety of mussel research projects have expanded greatly in the last several decades, an incomplete understanding of these two aspects of the host relationship persists (Haag and Williams 2014). In spite of the many host identification trials conducted over the last century, fish host data are reported for only about one third of the approximately 300 species of freshwater mussels in North America (Haag and Williams 2014). Of this relatively small proportion of documented host relationships, much of the research was

conducted prior to the adoption of quantitative techniques for conducting host trials, rendering the conclusions incomplete or questionable (Haag and Warren 2003). Through daily monitoring and individual identification of all sloughed glochidia/juveniles, a variety of metrics now allow a much more refined description of the host relationship than was available previously.

The continued incomplete understanding of the mussel-fish host relationship represents a major obstacle to effective management of mussel species in danger of extinction. Natural resource managers tasked with the conservation of imperiled mussels require knowledge of not only the mussels' critical habitat but also the host fish critical to their reproductive cycle and population viability. Toward this end, host identification trials allow managers to design and implement conservation actions focused on both an imperiled mussel species as well as the fish species upon which the mussel is reliant. In addition to habitat restoration, captive propagation of mussels in hatcheries for the restoration and augmentation of degraded populations has become a valuable tool in conservation programs (USFWS 2003). Investigation of the mechanisms underlying the interaction between glochidium and fish host immune response may improve the efficacy of propagation projects.

STURGEON AS MUSSEL HOSTS

The structure of most fish host identification trials focuses on a single mussel species and evaluates its use of multiple fish species as potential hosts. This is done by exposing a variety of co-occurring fish species to the glochidia of a single mussel species and monitoring the sloughed glochidia or juveniles produced from each fish. An alternative to this approach is a design that focuses on a particular fish species to identify the mussels that may use it as a host. The present

study employed such a design to evaluate the potential role of extirpated native sturgeon as hosts to mussels in the Apalachicola-Chattahoochee-Flint (ACF) River Basin.

Gulf sturgeon (Gulf of Mexico sturgeon), *Acipenser oxyrinchus desotoi*, is a sister sub-species of the Atlantic sturgeon, *Acipenser oxyrinchus oxyrinchus*. While the Atlantic sturgeon's range extends from Florida to Newfoundland, with multiple distinct population segments, the Gulf sturgeon is confined to its namesake gulf and is considered threatened throughout its range (Atlantic Sturgeon Status Revew Team 2007). It inhabits the coastal rivers of the eastern Gulf of Mexico from early spring to late fall and overwinters in the marine waters of the Gulf (Hightower et al. 2002). Gulf sturgeon saw marked declines in the first half of the 20th century resulting from overfishing, habitat destruction, and impoundment construction (Limburg and Waldman 2009). Gulf sturgeon, listed as threatened under the Endangered Species Act in 1991, once migrated throughout the ACF Basin (Zehfuss et al. 1999). However, construction of the Jim Woodruff Lock and Dam (JWLD) in 1952 prevented such migrations, which resulted in their extirpation from 78% of their historic upstream range in the basin (USFWS 1995). The U.S. Fish and Wildlife Service currently estimates the Gulf sturgeon population below the JWLD to be only 1292 individuals (95% CI: 616-1,968) (A. Kaeser, US Fish and Wildlife Service, personal communication). Fish passage construction and operational modifications of the JWLD have been considered as means to provide for upstream spawning movement of Gulf sturgeon (USFWS 2009).

The ACF Basin drains large portions of southeast Alabama, southwest Georgia, and a portion of northwest Florida before flowing into the Gulf of Mexico. The drainage encompasses a large area that has historically seen high levels of both agricultural land use and urban development, specifically Atlanta, GA, one of the largest urban areas in the country. Alterations

in natural flow regimes and pollution (e.g., sediment, nutrient, pharmaceutical, heavy metals) associated with urban/suburban and agricultural land uses are likely significant factors in the population declines seen in ACF mussel and anadromous fish populations alike (Richter et al. 1997).

The ACF basin is also home to 32 species of mussels, including eight endemic species and six species federally listed as threatened or endangered (Brim Box and Williams 2000; Neves et al. 1997; Williams et al. 1993). Recent research found the Gulf sturgeon to be a primary host (88% successful metamorphosis) for the federally threatened *Elliptoideus sloatianus* (Purple Bankclimber), which occurs primarily in the Flint River upstream of JWLD (Fritts et al. 2012). *E. sloatianus* also uses secondary hosts (darters) at lower rates of metamorphosis (<30%), and these secondary hosts may have allowed *E. sloatianus* to sustain populations above JWLD in the absence of a primary host, the Gulf sturgeon (Brim Box and Williams 2000; Fritts et al. 2012). These data suggest that the return of Gulf sturgeon upstream of JWLD could provide significant benefits to at least some endemic and imperiled mussels of the ACF, but to date sturgeon have been evaluated as a potential host for only two of the native mussel species: *E. sloatianus and Hamiota subangulata* (Fritts and Bringolf 2014; Fritts et al. 2012). These benefits may come in the form of not only a means of glochidial metamorphosis, but also as a means to connect otherwise isolated sub-populations of mussels along sturgeon migration routes. In this way, the reintroduced Gulf sturgeon could act as biological conservation corridors for mussels and thereby improve genetic exchange in the larger meta-populations (Berg et al. 1998; Schwalb et al. 2011). Through reintroduction and expansion of the range of Gulf sturgeon, not only their own population, but co-occurring mussel populations,

may achieve increased levels of long-term population sustainability (Flowers et al. 2009; Schwalb et al. 2013; Zehfuss et al. 1999).

EFFECTS OF CORTISOL ON LARVAL METAMORPHOSIS

The specific biochemical factors mediating the success or failure of successful glochidial metamorphosis remain largely unknown; however, research suggests the immunological characteristics of the potential host act as a primary determinant (Dodd et al. 2005; Meyers et al. 1980). The mussel-fish host relationship is an essential component of the mussel life cycle and thus the persistence of a mussel population, so a better understanding of the underlying physiological factors may yield more informed management actions of both wild populations and captive propagation programs aimed at conservation of critically endangered species.

Glochidial metamorphosis is initiated by attachment of a glochidium to fish gills or fins, initiating a minor, but necessary, immune response as epithelial cells migrate to surround the glochidium (encapsulation) in a matter of hours (Rogers-Lowery and Dimock 2006; Waller and Mitchell 1989). Encapsulation also triggers a broader immune response in the tissue surrounding the glochidium: inflammation, hyperplasia, and increased number of leukocytes (Arey 1932; Karna and Millemann 1978; Meyers et al. 1980; Waller and Mitchell 1989). The degree of this response varies between hosts: a lesser response is seen in host fish and naïve fish (not previously exposed to glochidial infestation); a greater response is seen in non-host fish and fish previously exposed to glochidia (‘resistant’) (Dodd et al. 2005; Rogers-Lowery and Dimock 2006; Waller and Mitchell 1989). In both non-host and resistant fish, the encapsulation process is significantly altered: there is a slower rate of epithelial migration, an irregular formation of the cyst, and an abbreviated period of encapsulation before sloughing of glochidia (Rogers-Lowery

and Dimock 2006; Waller and Mitchell 1989). This altered process of cyst formation indicates and contributes to decreased rates of metamorphosis (Dodd et al. 2006; Rogers-Lowery and Dimock 2006; Waller and Mitchell 1989).

Cortisol, the primary corticosteroid, is released as part of the stress response in teleosts and has system-wide immunosuppressant effects (Bonga 1997; Harris and Bird 2000; Pickering and Pottinger 1989). Susceptibility to bacterial, fungal, and ectoparasitic infections are seen in fish with elevated levels of cortisol (Davis et al. 2003; Pickering and Pottinger 1989). Specific systemic responses include reduced hyperplasia, inflammation, and leukocytes (Pickering 1984). Two separate investigations have examined the immunosuppressive effects of exogenous cortisol on glochidial metamorphosis. Kirk and Layzer (1997) injected four fish species with cortisol-laden cocoa butter implants and immediately exposed them to glochidia of two mussel species (*Venustaconcha sima* and *Villosa taeniata*). Cortisol treatment resulted in limited production of *V. sima* juveniles on one of two tested non-host fish species and limited production of *V. taeniata* juveniles on one of three tested non-hosts. By inducing production of juveniles on non-host fish species, Kirk and Layzer first demonstrated that exogenous cortisol administered to the host could be used to alter the outcome of glochidial metamorphosis. Dubansky et al. (2011) continued such investigations in a single mussel-fish pair: *Utterbackia imbecillis* and *Lepomis macrochirus* (bluegill sunfish). Treatment of *L. macrochirus* with exogenous cortisol (via intraperitoneal injection of cortisol-laden coconut oil) resulted in increased glochidial attachment (+34.9%) and increased metamorphosis success (+30%).

OBJECTIVES

The overall goal of this project was to improve understanding of mussel-fish host relationships as a part of the basic reproductive biology of freshwater mussels. This was accomplished by examining the potential host suitability of Gulf sturgeon for ACF mussel species and by evaluating the role of cortisol in larval metamorphosis. The specific objectives of this study were to: 1) determine potential for Gulf sturgeon to serve as hosts for ACF mussels, and 2) estimate the effects of exogenous cortisol treatment administered to hosts on the level of initial glochidial attachment, the rate of glochidial metamorphosis, the number of juveniles produced, and the time period over which juveniles were released.

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CHAPTER 2

EXAMINATION OF GULF STURGEON AS HOST FOR MUSSELS OF THE APALACHICOLA – CHATTACHOOCHEE – FLINT RIVER BASIN[1]

[1]Nelson, J. M., and R. B. Bringolf. To be submitted to Freshwater Mollusk Biology and Conservation.

INTRODUCTION

Freshwater ecosystems around the world are experiencing declines in faunal biodiversity (Richter et al. 1997); among the most well documented examples of this phenomenon are freshwater mussels in the Southeast region of North America (Bogan 1993; Neves et al. 1997). With almost 300 described species and subspecies, North America is the most phylogenetically-rich region in the world for freshwater mussels (Turgeon 1998; Williams et al. 1993). Of these species, 48.5% are now federally listed as either threatened or endangered (Bogan 1996; Turgeon 1998). The projected rates of extinction within this taxon are comparable to rates described for tropical rainforests, the most threatened terrestrial biome (Ricciardi and Rasmussen 1999). The potential widespread loss of native mussel species represents a major impairment of natural freshwater systems. Mussels provide a wide array of ecosystem services: water filtration, sediment bioturbation, nutrient cycling through and between trophic levels, and habitat improvement (Vaughn et al. 2008).

In addition to the suite of threats (e.g., habitat degradation/fragmentation, pollution, invasive species) common to most aquatic organisms, mussels are made vulnerable by their reliance on host fish species to complete their reproductive cycle. Unionid mussels must pass through a metamorphosis (usually two to four weeks) in which larvae (glochidia) must attach to the gills or fins of fish before entering the juvenile stage. To successfully begin metamorphosis, the attached glochidia must trigger a limited immunological reaction on the part of the host fish where the adjacent epithelial tissue of the host fish grows to encapsulate the attached glochidium. During encapsulation, the glochidium either undergoes metamorphosis to the juvenile stage or is destroyed by the host's immune system and sloughed off (Arey 1921; Rogers and Dimock 2003). The host relationship for a given mussel-fish host pair is often characterized by the percentage of

attached glochidia that successfully metamorphose to the juvenile stage ('%M'). A fish species demonstrating high compatibility (e.g., >60% metamorphosis) with a mussel is termed a 'primary' host, while a fish species showing low compatibility (e.g., <30% metamorphosis) is termed a 'secondary' or 'marginal' host (Barnhart et al. 2008). When explicitly defined, these terms qualifying the host relationship often vary by source because of the relatively recent adoption of %M as the standard metric for description of the host relationship and the inherently arbitrary nature of designating categories along a continuum such as %M.

Mussels reliant upon highly migratory fish for reproduction are especially vulnerable to the effects of dams, which often prevent spawning migrations of native fish species (Flowers et al. 2009; Kelner and Sietman 2000; Schwalb et al. 2011; Smith and Hightower 2012). One example of a highly impounded, large river system is the Apalachicola-Chattahoochee-Flint (ACF) River Basin. The ACF drains large portions of southeast Alabama, southwest Georgia, and a portion of northwest Florida; an area that has historically experienced high levels of both agricultural land use and urban development. The ACF hosts 16 large dams that fragment habitat and prevent large scale spawning runs of native migratory species, including Gulf sturgeon (Gulf of Mexico sturgeon; *Acipenser oxyrinchus desotoi;* Flowers et al. 2009).

Gulf sturgeon is a sister sub-species to the Atlantic sturgeon, *Acipenser oxyrinchus oxyrinchus,* and is the only sturgeon species native to the ACF Basin. While the Atlantic sturgeon's range extends from Florida to Newfoundland, with multiple distinct population segments, the Gulf sturgeon is confined to its namesake gulf and is considered threatened throughout its range (Atlantic Sturgeon Status Revew Team 2007). It inhabits the coastal rivers of the eastern Gulf of Mexico from early spring to late fall and overwinters in the marine waters of the Gulf (Hightower et al. 2002). During the first half of the 20th century marked declines in

Gulf sturgeon populations occurred because of overharvesting, habitat destruction, and impoundment construction (Limburg and Waldman 2009); the declines resulted in a federal listing under the Endangered Species Act in 1991. Gulf sturgeon migrated throughout the ACF Basin (Zehfuss et al. 1999) until construction of the Jim Woodruff Lock and Dam (JWLD) in 1952 prevented farther spawning migrations and resulted in their extirpation from 78% of their historic upstream range (Flowers et al. 2009; USFWS 1995). Fish passage construction and operational modifications of the JWLD have been considered as means to provide for upstream spawning movement of Gulf sturgeon (USFWS 2009).

The ACF basin is home to 32 species of mussels, including eight endemic species and six species federally listed as threatened or endangered (Brim Box and Williams 2000; Williams et al. 1993). Recent research found the native Gulf sturgeon to be a primary host (88% successful metamorphosis) for the federally threatened *Elliptoideus sloatianus* (Purple Bankclimber), which occurs primarily in the Flint River upstream of JWLD (Fritts et al. 2012). *E. sloatianus* also uses secondary hosts (darters) at lower rates of metamorphosis (<30%), and these secondary hosts may have allowed *E. sloatianus* to maintain populations above JWLD in the absence of Gulf sturgeon (Fritts et al. 2012). These data suggest that the return of the native sturgeon species upstream of JWLD could provide significant benefits to the endemic and imperiled mussels of the ACF, but to date sturgeon have been evaluated as a potential host for only two native ACF mussel species: *E. sloatianus*, *Hamiota subangulata* (Fritts and Bringolf 2014; Fritts et al. 2012). These benefits may come in the form of not only a means of glochidial metamorphosis, but also as a means to connect otherwise isolated sub-populations of mussels along sturgeon migration routes. In this way, the reintroduced sturgeon could act as biological conservation corridors for mussels, thereby improving genetic exchange in the larger meta-populations

(Schwalb et al. 2011; Smith 1985). Through reintroduction and expansion of the range of Gulf sturgeon, not only their own population, but co-occurring mussel populations, may achieve increased levels of long-term population sustainability (Flowers et al. 2009; Schwalb et al. 2011).

The goal of this study was to determine the capacity of the Atlantic sturgeon, as a surrogate for Gulf sturgeon, to serve as host for a subset of ACF mussel species. The specific objective of this project was to use specimen collection and systematic host trials to evaluate the levels of successful larval metamorphosis of ACF mussels on Gulf sturgeon hosts. An improved understanding of mussel-fish host relationships will contribute to management of imperiled species and a greater understanding of potential ecological benefits of reintroducing native fishes. The practical applications of such an improved knowledge may prove valuable to effective conservation management of mussel populations (Bogan 1993; Haag and Williams 2014).

METHODS

Test Organisms

Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*) were used to complete the host trials because of the difficulties associated with work involving the federally threatened Gulf sturgeon (*Acipenser oxyrinchus desotoi*): permit limits for capture of wild individuals, limited or no availability from hatcheries. As sister sub-species, a mussel-fish host relationship with Atlantic sturgeon most likely represents the same relationship with Gulf sturgeon. This was demonstrated by Fritts (2012) who did not find statistical differences between levels of larval metamorphosis of *E. sloatianus* on the same two sturgeon subspecies (%metamorphosis $\pm$ 95% CI, Gulf Sturgeon: 88% $\pm$ 3, Atlantic sturgeon: 89% $\pm$ 7).

Juvenile Atlantic sturgeon (44.7 ± 28.6 g) used in these host trials were acquired from the Bears Bluff National Fish Hatchery under US FWS Permit #17367. Sturgeon were held in 850-L circular tanks within a recirculating system, then moved to individual 3-L tanks within a modified recirculating aquaculture system, "AHAB" (Fig. 2.1, AHAB, Pentair Aquatic Eco-systems, Apopka, FL), for an acclimation period of at least one week. Fish were fed commercial fish feed 2mm pellets (Melick Aquafeed, Catawissa, PA) once daily at a rate of 3% body weight prior to trials; feed rate was reduced to 0.5% body weight during trials. A Hach HQ40d Multimeter (Hach, Loveland, CO) was used throughout the research period to monitor daily temperature (23.1±3.1^{o}C), dissolved oxygen (8.9±0.8mg/L), and pH (8.5±0.6); a Lamotte Colorimeter (LaMotte Co., Chestertown, MD) was used weekly to monitor ammonia (0.17±0.13mg/L).

Mussel species used in this study included: *Amblema neislerii, Elliptio crassidens, E. pullata, Glebula rotundata, Lampsilis floridensis, Lampsilis straminea, Villosa lienosa, and Villosa vibex*. Adult female mussels were collected from the Apalachicola and Flint rivers from June 2014 to June 2015. Mussels were collected by hand from the river substrate by wading, snorkeling, or scuba diving (depending on depth). All specimens were inspected in the field to identify species, sex, and presence of glochidia (evidenced by swollen marsupial gills). Brooding females were transported in aerated vessels to the University of Georgia Aquatic Science Lab. Mussels were kept in 1-L individual beakers submerged within 50-L aquaria (to separate aborted glochidia) with constant aeration and regular water changes to maintain water quality.

A federal endangered species permit (USFWS #TE10239A-0) allowed for host work with listed mussels in the ACF Basin, and a Georgia Scientific Collecting Permit (29-WBH-11-22)

covered collection of non-listed mussels. A federal endangered species permit (NOAA NMFS #17367) allowed for use of juvenile Atlantic sturgeon for host trials at the University of Georgia.

Host Trials

Host trials were conducted between June 2014 and July 2015 according to methods previously reported by Fritts et al. (2012). Briefly, glochidia were acquired by carefully prying open the mussel valves, then inserting a syringe and needle filled with fresh water into the water tube membrane of a gravid gill. Water was gently aspirated in the water tube to rupture the membrane and release glochidia, which were captured in a petri dish. Sub-samples of 50-100 glochidia per female were exposed to a NaCl solution to ascertain viability of individual females (Zale and Neves 1982). Preferred viability for all glochidia used in trials was ideally >80%, but this was not possible in one case (*E. crassidens*, 79%). Viable glochidia were pooled by species, the quantity of glochidia was estimated via sub-sample enumeration in a known volume, and inoculation baths were prepared at 4000 glochidia/L in a 50-L glass aquarium with vigorous aeration. For each mussel species trial, 3-4 sturgeon were used to allow estimation of variance. Sturgeon were submerged in the inoculation bath for 15 minutes, then rinsed with fresh water to remove any unattached glochidia.

Following inoculation, sturgeon were housed in individual 3-L tanks in a re-circulating aquaculture system (Fig. 2.1, AHAB; Aquatic Habitats, Inc.). Water quality was monitored for temperature, dissolved oxygen, pH (daily), and ammonia (weekly). Each 'self-cleaning' AHAB tank was constantly flushed with water, and each outflow was equipped with a 153 µm Nitex mesh ('filter cup') to capture any sloughed glochidia and metamorphosed juveniles released from the sturgeon. Filter cups were checked every two to three days for glochidia/juveniles, which

were then individually counted under a Leica WILD MZ8 dissecting stereomicroscope (Leica Microsystems Inc., Buffalo Grove, IL) by gently rinsing the filters into a beaker and observing the contents in a Bogorov tray. Metamorphosed juveniles are readily identifiable from glochidia by observing movement and the presence of structures such as the heart, foot, and gills. Individual fish were removed from trials when glochidia or juveniles were not seen for at least three monitoring periods (or six days). In the event of a fish mortality during a trial, the individual was examined for attached glochidia prior to proper disposal.

Metamorphosis success (%M) was estimated for each fish replicate as a percentage by dividing recovered juveniles by the sum of recovered glochidia and juveniles. Mean %M and confidence intervals (95%) were then calculated for each species' metamorphosis rate. Following host trials, length and weight of fish were measured to allow for calculation of number of juveniles per gram of fish. The number of juveniles produced for each replicate per monitoring event was calculated and plotted to determine time period from inoculation to peak juvenile production.

RESULTS

Amblema neislerii metamorphosis success on Atlantic sturgeon was 3.5% $\pm$ 0.94 (95% CI, n=3), but %M was less than 1% for all of the other seven mussel species tested (Table 2.1). A single juvenile *E. pullata* was produced from 1387 attached glochidia (0.07%) and a single juvenile *V. vibex* was produced from 2996 attached glochidia (0.03%). The mean number of juvenile *A. neislerii* produced per gram of fish was 1.23/g ($\pm$0.27 SD) and peak juvenile production occurred 14 days post-inoculation (Fig. 2.2).

DISCUSSION

Mussel-fish host trials have proven essential to our growing understanding of the basic biology, ecology, physiology, and conservation of imperiled mussels. The National Strategy for the Conservation of Native Mussels lists identification of fish hosts as the first objective within the overall goal of increasing fundamental knowledge of basic biology and habitat requirements of mussels (NNMCC 1997). Recognition of the mussel extinction crisis has led to a large increase in both the number of researchers and the breadth of scientific disciplines applied to mussel research (Haag 2012). In spite of these growing efforts, basic host data are known for only about one third of native mussels in North America (Haag 2012). However, many of the host trials were conducted prior to the adoption of laboratory techniques for the quantitative evaluation of host relationships; these early studies resulted in incomplete data (Haag 2014).

The only tested mussel species showing successful metamorphosis on Atlantic sturgeon in this study was *A. neislerii*, which was federally listed as endangered in 1998. The low %M of *A. neislerii* on sturgeon observed in this study aligns with previous host research for the species. Fritts and Bringolf (2014) characterized *A. neislerii* as a host generalist that demonstrated variable, but generally low, levels of metamorphosis on 23 fish species across 7 families. Should sturgeon be restored to their historic range in the ACF, the actual benefits to *A. neislerii* may be much greater than is suggested by the low %M found in this study because as large-bodied highly migratory fish, sturgeon annual spawning migrations may restore and reconnect widely separated mussel populations through improved genetic exchange (Schwalb et al. 2011).

In contrast to the detailed and extensive host data available for *A. neislerii*, such data are generally lacking for other mussels in the ACF (Table 2.2). Mussels in the genus *Elliptio* and other pleurobemines (tribe Pleurobemini) generally use darters, sculpins, or minnows as hosts.

The remaining five mussel species examined in this study (tribe Lampsilini) are most often reported to use sunfishes as hosts (Haag 2012). The lack of reported hosts and host trials conducted with sturgeon or other diadromous fishes may reflect the difficulties associated with their acquisition for research or possibly a bias related to their historic absence from lotic systems following widespread construction of dams in North America.

Two of the mussel species in this study, *E. crassidens and E. pullata*, had an initial glochidia viability of about 80% from a single brooding female per species. Ideally, glochidia would be combined from multiple females for a host trial, but sampling constraints precluded collection of additional brooding females. Glochidia with less than 90% initial viability may result in a disproportionate decrease in infectivity on tested fish species in a host trial (Fritts et al. 2014). The lack of metamorphosis success of these two species on sturgeon should be interpreted with caution and may represent false negative results. Replication of the trials with >90% viable glochidia pooled from multiple females is recommended to better ascertain the putative non-host relationships and confer greater confidence in those results.

Taken together, the eight mussels examined in this study and the two species (*E. sloatianus, Hamiota subangulata*) examined in related studies (Fritts and Bringolf 2014; Fritts et al. 2012), will serve as a strong foundation for continued research into the ecological relationships between Gulf sturgeon (or their surrogates) and the 32 native mussels of the ACF. ACF mussel host trials should also be extended beyond sturgeon to include other diadromous species, such as the Alabama shad (*Alosa alabamae*) and striped bass (*Morone saxatilis*), whose ACF populations will likely benefit from possible structural or operational modifications of the JWLD (Smith and Hightower 2012; Smith et al. 2011; USFWS 2009).

Because of the federally threatened status of Gulf sturgeon, trials examining their host suitability for Gulf slope mussels are limited in number (Fritts and Bringolf 2014; Fritts et al. 2012; Keller and Ruessler 1997). Although this study did not demonstrate that the native sturgeon is a primary host for any of the examined mussel species, identification of both host and non-host fish species is required for effective conservation management of imperiled mussel species. In this way, the study has added to our general understanding of mussel-fish host relationships and the specific ecological role played by sturgeon in the context of the mussel fauna native to the ACF basin.

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Figure 2.1. Recirculating aquaculture system (Aquatic Habitat, AHAB, Pentair Aquatic Eco-Systems) used to hold Atlantic sturgeon during mussel host trials. Note 153 µm mesh filter cups at tank outflows.

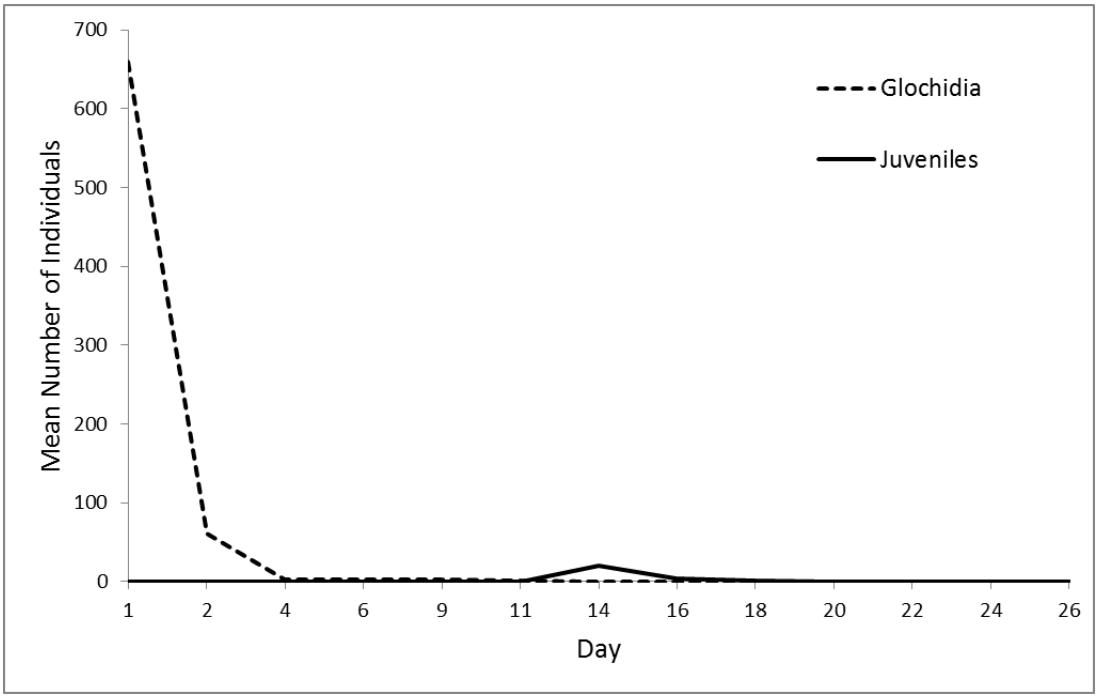

Figure 2.2. Mean daily juvenile production of *A. neislerii* (n=3). Three juvenile Atlantic sturgeon, *Acipenser oxyrinchus oxyrinchus*, were exposed to glochidia of *A. neislerii* for 15 minutes in an aquarium at a glochidial density of 4000 glochidia per Liter. Effluent from individual tanks were monitored for sloughed glochidia and juveniles. Peak juvenile production occurred 14 days post-inoculation.

Table 2.1. Results of ACF mussel host trials on Atlantic sturgeon.

Mussel spp.	Adult Females	Glochidia Viability %	%M	n	Glochidial Attachment	Juvenile Production
Amblema neislerii	3	92	3.5	3	35.3	24.7
Elliptio crassidens	1	79	0	3	7.3	0.0
Elliptio pullata	1	81	0*	3	30.0	0.3
Glebula rotundata	3	96	0	3	58.9	0.0
Lampsilis floridensis	3	93	0	3	20.7	0.0
Lampsilis straminea	1	98	0	4	91.1	0.0
Villosa lienosa	2	97	0	3	68.4	0.0
Villosa vibex	2	98	0*	3	74.6	0.3

%M is mean percentage metamorphosis success; asterisk (*) denotes a %M value rounded to zero. Glochidia Viability is the percentage of glochidia responsive to NaCl challenge following collection from one to three adult females. Number of fish replicates per species host trial is shown by 'n.' Glochidial Attachment is mean number of initially attached glochidia, standardized by weight (g) of fish. Juvenile Production is mean number of juveniles produced per fish replicate.

Table 2.2. Reported host data on examined ACF mussel species.

Mussel spp.	Fish Host spp.	Fish Family	Test	Reference
Amblema neislerii	*Dorosoma petenense*	Clupeidae	L	Fritts and Bringolf 2014
	Nocomis leptocephalus	Cyprinidae	L	Fritts and Bringolf 2014
	Notropis amplamala	Cyprinidae	L	Fritts and Bringolf 2014
	Notropis lutipinnis	Cyprinidae	L	Fritts and Bringolf 2014
	Pimephales promelas	Cyprinidae	L	Fritts and Bringolf 2014
	Pteronotropis grandipinnis	Cyprinidae	L	Fritts and Bringolf 2014
	Ameiurus brunneus	Ictaluridae	L	Fritts and Bringolf 2014
	Ameiurus melas	Ictaluridae	L	Fritts and Bringolf 2014
	Ameiurus natalis	Ictaluridae	L	Fritts and Bringolf 2014
	Ictalurus punctatus	Ictaluridae	L	Fritts and Bringolf 2014
	Gambusia holbrooki	Poeciliidae	L	Fritts and Bringolf 2014
	Morone saxatilis	Moronidae	L	Fritts and Bringolf 2014
	Lepomis auritus	Centrarchidae	L	Fritts and Bringolf 2014
	Lepomis cyanellus	Centrarchidae	L	Fritts and Bringolf 2014
	Lepomis gulosas	Centrarchidae	L	Fritts and Bringolf 2014
	Lepomis macrochirus	Centrarchidae	L	Fritts and Bringolf 2014
	Lepomis marginatus	Centrarchidae	L	Fritts and Bringolf 2014
	Lepomis megalotis	Centrarchidae	L	Fritts and Bringolf 2014
	Lepomis punctatus	Centrarchidae	L	Fritts and Bringolf 2014
	Micropterus salmoides	Centrarchidae	L	Fritts and Bringolf 2014
	Etheostoma fusiforme	Percidae	L	Fritts and Bringolf 2014
	Etheostoma inscriptum	Percidae	L	Fritts and Bringolf 2014
	Etheostoma olmstedi	Percidae	L	Fritts and Bringolf 2014
Elliptio crassidens	*Alosa chrysochloris*	Clupeidae	N	Howard 1914
Elliptio pullata	*Lepomis macrochirus*	Centrarchidae	L	Keller and Ruessler 1997
	Micropterus salmoides	Centrarchidae	L	Keller and Ruessler 1997
Glebula rotundata	*Lepomis cyanellus*	Centrarchidae	L	Parker 1984
	Lepomis macrochirus	Centrarchidae	L	Parker 1984
	Trinectes maculatus	Achiridae	N	Parker 1984
	Anchoa mithilli	Engraulidae	N	Parker 1984
	Lepisosteus oculatus	Lepisosteidae	N	Parker 1984
	Morone chrysops	Moronidae	N	Parker 1984
	Cyprinus carpio	Cyprinidae	N	Parker 1984
Lampsilis floridensis	*Micropterus salmoides*	Centrarchidae	L	Keller and Ruessler 1997
	Lepisosteus osseus	Lepisosteidae	L	Keller and Ruessler 1997
	Lepisosteus platyrhincus	Lepisosteidae	L	Keller and Ruessler 1997
Lampsilis straminea	*Lepomis macrochirus*	Centrarchidae	L	Keller and Ruessler 1997
	Micropterus salmoides	Centrarchidae	L	Keller and Ruessler 1997
	Notemigonus crysoleucas	Cyprinidae	L	Keller and Ruessler 1997
	Notropis texanus	Cyprinidae	L	Keller and Ruessler 1997
	Ictalurus punctatus	Ictaluridae	L	Keller and Ruessler 1997
	Gambusia affinis	Poeciliidae	L	Keller and Ruessler 1997
Villosa lienosa	*Lepomis macrochirus*	Centrarchidae	L	Keller and Ruessler 1997
	Micropterus salmoides	Centrarchidae	L	Keller and Ruessler 1997
	Ameiurus nebulosus	Ictaluridae	L	Keller and Ruessler 1997
	Ictalurus punctatus	Ictaluridae	L	Keller and Ruessler 1997
Villosa vibex	*Lepomis cyanellus*	Centrarchidae	L	Haag 1999
	Micropterus salmoides	Centrarchidae	L	Haag 1999
	Micropterus coosae	Centrarchidae	L	Haag 1999
	Micropterus punctulatus	Centrarchidae	L	Haag 1999

'L' denotes host data from trials conducted in a laboratory setting; 'N' denotes host data from natural infestations.

CHAPTER 3

EFFECTS ON MUSSEL METAMORPHOSIS OF EXOGENOUS CORTISOL TREATMENT TO HOST FISH[2]

[2]Nelson, J. M., and R. B. Bringolf. To be submitted to the North American Journal of Aquaculture.

INTRODUCTION

Freshwater communities around the world are now experiencing significant declines in faunal biodiversity (Richter et al. 1997). The projected extinction rates of North American freshwater fauna are comparable to those of tropical rainforest communities, considered one of the most stressed terrestrial biomes (Ricciardi and Rasmussen 1999). Among the most well documented examples of these phenomena are the freshwater mussels (Order Unionoida) of North America (Bogan 1993; Neves et al. 1997). With approximately 300 of the roughly 800 described mussel species and subspecies, North America is the most phylogenetically-rich region in the world (Turgeon 1998; Williams et al. 1993).

Of the approximately 300 North American mussel species, 10% are already extinct while 65% of the remaining species are now classified as threatened, endangered, or vulnerable to extinction (Haag and Williams 2014). As benthic filter-feeders, mussels are vulnerable to a number of anthropogenic threats (Bogan 1993; Cope et al. 2008). Habitat destruction and degradation commonly result from channel dredging and agricultural pollutants in the form of sediment and nutrients. Hydroelectric dams fragment local populations, inundate upstream habitat, alter natural flow and temperature regimes, and block movement of host fish species (Williams et al. 1993; Wood and Armitage 1997). Through burrowing and filter-feeding activities, mussels provide an array of ecosystem services in freshwater systems. Mussels act as a bridge to upper trophic levels (such as muskrats and birds) by feeding on phytoplankton, zooplankton, bacteria, algae, and detritus via actively-pumped filtration (Vaughn et al. 2008). Bioturbation resulting from burrowing activities increases the oxygen, water, and nutrient content of sediments (Vaughn et al. 2008). Mussel shells serve as substrate and, along with biodeposition of nutrients, mussels improve habitat for a variety of aquatic organisms (Gutiérrez

et al. 2003; Vaughn and Spooner 2006). Thus, local extirpation of mussel assemblages may have significant, negative cascading effects across multiple trophic levels (Vaughn et al. 2008).

A unique feature of freshwater mussel life history is a metamorphosis stage (usually for two to four weeks) in which parasitic larvae (glochidia) must attach to the gills or fins of fish before entering the juvenile stage. While attached, the glochidia either successfully undergo metamorphosis to the juvenile stage or are rejected by the host and sloughed off (Haag 2012). Approximately 80% of North American mussel species are classified as "specialists" because they successfully metamorphose on only a small number of host species or genera. Alternatively, other species termed "generalists" are capable of metamorphosis on many fish species in multiple families (Barnhart et al. 2008; Haag 2012).

In addition to direct anthropogenic threats, effective management for the conservation of imperiled mussels is hindered by the absence of certain basic biological and ecological knowledge, including the physiological mechanisms underlying host specificity (Haag and Williams 2014). Because the mussel/fish-host relationship is integral to the mussel life cycle and, therefore, the persistence of a population, a better understanding of factors affecting successful metamorphosis of larvae will inform management of wild populations and improve captive propagation programs. Such improvements may prove essential to ongoing efforts aimed at conservation of critically endangered species.

Although the specific biochemical factors mediating the success or failure of glochidial metamorphosis remain largely unknown, research suggests the immunological characteristics of the potential host act as a primary determinant (Barnhart et al. 2008; Dodd et al. 2005; Meyers et al. 1980). Metamorphosis is initiated by attachment of a glochidium to fish gills or fins, initiating a minor, but necessary, immune response as epithelial cells migrate to surround the

glochidium (encapsulation) in a matter of hours (Rogers-Lowery and Dimock 2006; Waller and Mitchell 1989). Encapsulation also triggers a broader immune response in the tissue surrounding the glochidium: inflammation, hyperplasia, and increased number of leukocytes (Arey 1932; Karna and Millemann 1978; Meyers et al. 1980; Waller and Mitchell 1989). The degree of this response varies between hosts: a lesser response is seen in host fish and naïve fish (not previously exposed to glochidial infestation); a greater response is seen in non-host fish and fish previously exposed to glochidia (resistant) (Dodd et al. 2005; Rogers-Lowery and Dimock 2006; Waller and Mitchell 1989). In both non-host and resistant fish, the encapsulation process is significantly altered in a number of ways: a slower rate of epithelial migration, an irregular formation of the cyst, and an abbreviated period of encapsulation before sloughing of glochidia (Rogers-Lowery and Dimock 2006; Waller and Mitchell 1989). This altered process of cyst formation indicates and contributes to decreased levels of metamorphosis (Dodd et al. 2006; Rogers-Lowery and Dimock 2006; Waller and Mitchell 1989).

Cortisol, the primary corticosteroid, is released as part of the stress response in teleosts and has system-wide immunosuppressant effects (Bonga 1997; Harris and Bird 2000; Pickering and Pottinger 1989). Specific systemic responses include reduced hyperplasia, inflammation, and leukocytes (Pickering 1984), which all contribute to reduced immunocompetence. Indeed, fish with elevated levels of cortisol have shown increased susceptibility to bacterial, fungal, and ectoparasitic infections (Davis et al. 2003; Pickering and Pottinger 1989). Furthermore, Kirk and Layzer (1997) previously demonstrated that administration of exogenous cortisol to a known non-host fish species resulted in limited successful glochidial metamorphosis. Dubansky et al. (2011) similarly found that exogenous cortisol administration to host fish resulted in a 35% increase in glochidial attachment success and a 30% increase in metamorphosis success. The

stress response, and cortisol in particular, seems to play a role in host specificity; the use of exogenous cortisol to induce or increase metamorphosis in otherwise marginal or non-host fish may provide a valuable tool for culture of species for which the primary host(s) are unknown. The objective of the present study was to further investigate the potential for exogenous cortisol to increase glochidial attachment and metamorphosis on marginal or non-host fish.

METHODS

Five trials were conducted from February 2015 to August 2015 in which various fish were treated with exogenous cortisol and exposed to glochidia of a single mussel species (*Ligumia subrostrata*) to examine the effects of cortisol on attachment and metamorphosis success. The level of immunocompatibility between a mussel species and host fish varies greatly across fish taxa. To account for this range of potential host relationships, five fish species were chosen from four families: largemouth bass, *Micropterus salmoides* (14.0 $\pm$ 1.8 cm, 25.9 $\pm$ 9.8 g); bluegill sunfish, *Lepomis macrochirus* (9.7 $\pm$ 1.4 cm, 13.1 $\pm$ 5.3 g); channel catfish, *Ictalurus punctatus* (12.8 $\pm$ 1.6 cm, 14.4 $\pm$ 5.2 g); goldfish, *Carassius auratus* (12.6 $\pm$ 1.9 cm, 31.4 $\pm$ 12.8 g); and a hybrid of blue tilapa, *Oreochromis aureus*, and Nile tilapia, *Oreochromis niloticus* (15.8 $\pm$ 2.5 cm, 64.9 $\pm$ 29.2 g). The tilapia were acquired from a lab population at the University of Georgia Aquatic Biology and Ecotoxicology Lab; all other fish were acquired from local state and private hatcheries. Glochidia from the common pondmussel, *Ligumia subrostrata*, were used in all five trials; 20 gravid female pondmussels were acquired from a pond at Auburn University.

Prior to trials, fish were held in 575-L rectangular tanks (tilapia were held in 65-L aquaria) in a recirculating system. Fish were fed commercial feed pellets at a rate of 3% body weight per day prior to trials; feeding rate was reduced to 0.5% body weight per day during

trials. A Hach HQ40d Multimeter (Hach, Loveland, CO) was used daily throughout the trials to monitor temperature (23.2 $\pm$ 2.9 ^{o}C), dissolved oxygen (8.77 $\pm$ 0.44 mg/L), and pH (7.4 $\pm$ 0.5). A LaMotte colorimeter (LaMotte Co., Chestertown, MD) was used weekly to monitor ammonia (0.22 $\pm$ 0.17 mg/L).

In each trial, four treatments were used: a control (no injection, otherwise handled identically to all other treatments), a procedural control (injection of coconut oil only, 'Sham'), low cortisol (0.05 mg/g fish wet weight), and high cortisol (0.20 mg/g fish wet weight). Cortisol concentrations were determined based on previous work (Dubansky et al. 2011; Vijayan et al. 1991; Woo et al. 1987). Individual fish were randomly assigned to one of the four treatments and trials were initiated with seven replicates per treatment for a total of 28 experimental units. Consistent with previous work (Dubansky et al. 2011; Gamperl et al. 1994), fish were anaesthetized with 200-500 mg/L MS-222, depending on species, and cortisol (hydrocortizone 98%, Thermo Fisher Scientific, Fair Lawn, NJ) was administered via intra-peritoneal injection (23 gauge, 1" needle) in a suspension of melted coconut oil (34 ^{o}C). The resulting implant was expected to be slowly metabolized, releasing cortisol and mimicking a state of chronic stress. After injection of cortisol/coconut oil, fish were held for three days to allow endogenous cortisol to return to baseline levels (following handling) in individual covered 2.5-L infestation tanks (custom-made) with a conical bottom to later allow for suspension (via aeration) of glochidia during the infestation procedure.

Three days after initiation of fish cortisol treatments, *L. subrostrata* glochidia were prepared following standard procedures (Zale and Neves 1982) to estimate numbers and assess viability via a salt challenge. Viable glochidia from multiple females (two to four per trial) were mixed prior to infestation; viability ranged from 85.3 to 95.7%. Fish were individually exposed

to suspended glochidia for 15 minutes at a concentration of 2000 viable glochidia per liter (5000 viable glochidia per 2.5-L infestation tank). To evacuate the infestation tanks for unattached glochidia, aeration was ceased to allow unattached glochidia to settle, and tanks were twice flushed with ~30% tank volume over the course of half an hour. Fish were held in the infestation tanks for six hours to allow for glochidial encapsulation (Rogers-Lowery and Dimock 2006) and were then transferred to individual 3-L tanks in a recirculating Aquatic Habitat culture system (AHAB, Pentair, Aquatic Eco-systems, Apopka, FL). Water was constantly circulated through the AHAB tanks, for which each outflow was equipped with a custom screen filter with 153 µm Nitex mesh ('filter cup'). Every other day, filter cup contents were rinsed into Bogorov enumerating trays and examined under a Leica WILD MZ8 dissecting stereomicroscope (Leica Microsystems Inc., Buffalo Grove, IL) for sloughed glochidia and juveniles. A fish was removed from trials when neither glochidia nor juveniles were seen for at least three monitoring periods (six days). In the event of a fish mortality, the gills were closely examined under a stereomicroscope for attached glochidia prior to proper disposal. Metamorphosis success was estimated for each individual fish as a percentage by dividing recovered juveniles by the sum of recovered glochidia and juveniles as described by Fritts et al. (2012). The above methods differed slightly for the two fish species initially tested: *M. salmoides* and *L. macrochirus*. In both cases, the viable glochidial density during infestation was 1000 glochidia/L rather than 2000 glochidia/L, and the subsequent holding period in infestation tanks was two days rather than three. In some trials, the number of replicates per treatment group were reduced by mortalities from the initial number (n = 7): catfish (n = 7), goldfish (n = 7), tilapia (n = 4-5), largemouth bass (n = 6-7), bluegill (n = 6-7).

To determine if treatment methods altered circulating cortisol levels, a separate series of trials were conducted to measure circulating plasma cortisol concentrations in fish at the time of exposure to glochidia. Proxy fish from the same populations for each of the five species previously tested were handled and treated identically to those used to estimate the effects of cortisol on glochidial metamorphosis. As previously described, exogenous cortisol was administered to randomly assigned fish in four treatment groups (Control, Sham, 0.05 mg/g cortisol, 0.20 mg/g cortisol) and held for three days in infestation tanks, consistent with the glochidia metamorphosis trials. Rather than infesting with glochidia, proxy fish were euthanized with an overdose of MS-222 (500 mg/L), and blood was collected in heparinized 1-ml centrifuge tubes from the caudal blood vessel by severing the caudal peduncle with a scalpel. Whole blood was spun in a centrifuge (Eppendorf 5415C) at 1000 rpm for 15 minutes; plasma was decanted with a Pasteur pipette and placed in clean centrifuge tubes, frozen, and stored in a -80 ºC freezer until analysis. Plasma cortisol was quantified using a commercial (Enzo Life Sciences Inc.) cortisol ELISA kit (ADI-900-071) and a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA). In some trials, the initial number of replicates (tilapia, largemouth bass, and bluegill n = 3; goldfish n = 4) were reduced by mortalities: tilapia n = 2-3, largemouth bass n = 2-3, bluegill n = 3, goldfish n = 3-4.

The effects of exogenous cortisol on larval metamorphosis were evaluated through the use of four metrics: initial attachment of glochidia per gram of fish wet weight (attachment/g), percentage of successful metamorphosis (%M), number of juveniles produced per gram of fish wet weight (juveniles/g), and the time period over which juveniles were released from fish. Differences among treatment means for the above metrics were analyzed using R statistical software. Because %M is represented as a percentage (bounded proportions), an arcsine-square

root transformation was used to achieve normality. ANOVA (α=0.05) was used to test the null hypotheses that administration of cortisol did not result in significant differences between treatment groups. Significant F-statistics were followed by pairwise comparisons with Tukey's HSD to identify differences among treatments. A Shapiro-Wilk test was used to test for normal distribution of residuals for initial glochidia attachment, juvenile production, and plasma cortisol; data were transformed with a square root function if needed to achieve normality.

RESULTS

Mean levels of glochidia attachment varied, some significantly, among treatment groups within fish species trials. Glochidia attachment increased significantly on goldfish treated with 0.20 mg/g cortisol (10.9 glochidia per gram of fish, $F_{3,24}$=6.107, $p<0.01$), relative to both the Sham (3.6 glochidia per gram of fish, $p<0.01$) and 0.05 mg/g cortisol (3.0 glochidia per gram of fish, $p<0.01$) groups, but not the Control group (5.3 glochidia per gram of fish, $p=0.055$). Although not statistically significant, mean initial glochidia attachment in both cortisol treatments was greater than the Control and Sham groups for catfish and tilapia (Fig. 3.1). In largemouth bass and bluegill trials, there was neither a significantly different mean level of glochidial attachment among treatment groups nor a trend suggesting an effect of cortisol.

Mean levels of %M and number of juveniles produced (per gram of fish) varied among treatments with distinct trends across fish species trials. Cortisol treatment of tilapia resulted in a dose-dependent increase in mean %M, with significantly ($F_{3,13}$=3.73, $p<0.05$) higher %M on tilapia treated with 0.20 mg/g cortisol (55%) compared with all other treatment groups (Control: 14%; Sham: 12%; 0.05 mg cortisol per gram of fish: 28%; Fig. 3.2). Similarly, tilapia treated with 0.20 mg/g cortisol produced significantly more juvenile mussels per gram of fish (8.3

juveniles; $F_{3,13}$=3.95, $p<0.05$) than all other groups (Control: 1.4; Sham: 1.1; 0.05 mg cortisol per gram of fish: 4.5; Fig. 3.3). The Control ($p=0.05$) and Sham ($p<0.05$) groups produced 1 juvenile/g compared to 8 juveniles/g in the 0.20 mg/g cortisol group (Fig. 3.3). Both catfish and goldfish failed to produce juveniles in any of the four treatment groups. In largemouth bass and bluegill trials, there were no significant differences among treatment groups for mean %M or mean juvenile production, nor were there trends suggesting an effect of cortisol.

Peak production of juveniles following inoculation on largemouth bass and bluegill occurred on the 16th and 12th days, respectively, and did not differ among treatment groups (Fig. 3.5). Peak juvenile production on tilapia occurred on the 9th or 11th day, varying by treatment group. Treatment of tilapia with 0.20mg cortisol resulted in an extension of the total time period over which juveniles were produced (14 days post-inoculation) relative to the Control and Sham groups (11 days post inoculation).

Administration of cortisol to proxy fish resulted in increased levels of circulating plasma cortisol in largemouth bass ($F_{3,6}$=46.27, $p<0.001$) and bluegill ($F_{3,8}$=324.5, $p<0.001$) (Fig. 3.4). Mean plasma cortisol in tilapia was higher in cortisol treatments, but was not statistically significant; no clear trend among groups was observed in goldfish. Plasma cortisol results for catfish were not available as the ELISA did not yield reliable data.

DISCUSSION

This study expanded on pervious work by employing four metrics (initial attachment, %M, juvenile production, and time period of juvenile production) to examine the effects of exogenous cortisol on larval metamorphosis among five fish species across a wide phylogenetic

range. The resulting data suggest the existence of a continuum of cortisol-induced effects, varying by the natural host specificity of the mussel-fish pair.

Metamorphosis was not successful in the Control or Sham groups in the catfish and goldfish trials. This finding establishes these species as non-hosts for *L. subrostrata* (Fig. 3.2). The failure of exogenous cortisol treatment to induce any level of metamorphosis on non-hosts in this study is not surprising when compared to similar findings. Kirk and Layzer (1997) saw limited successful metamorphosis in two non-host mussel-fish pairs following administration of exogenous cortisol; however they also saw no induction of metamorphosis in three other tested non-host pairs. In contrast to the catfish and goldfish trials, mean %M levels were high in Control and Sham groups in largemouth bass (Control: 66%, Sham: 68%) and bluegill trials (Control: 81%, Sham: 82%), which suggests that these species serve as primary hosts for *L. subrostrata*. This finding is consistent with early host work and natural infestations showing that several centrarchids (four members of the genus Lepomis and *Micropterus salmoides*) are hosts for *L. subrostrata* (Lefevre and Curtis 1912; Stern and Felder 1978). The ability of *L. subrostrata* to subvert the primary hosts' immune systems, as evidenced by the high %M in the Control and Sham groups, may preclude an additional measureable increase in %M following inhibition of the immune system by exogenous cortisol. A physiological interplay such as this would explain the absence of a cortisol treatment effect in both largemouth bass and bluegill trials. In the tilapia trial, %M levels in Control and Sham groups were 14% and 12% respectively, suggesting that tilapia serve as a marginal host for *L. subrostrata*. The dose-dependent treatment effect of cortisol observed in tilapia %M (28% in the 0.05 mg/g cortisol group; 55% in the 0.20 mg/g cortisol group) supports results reported by Dubansky et al. (2011) following treatment of bluegill with 0.20 mg/g exogenous cortisol. The effects of cortisol treatment on juveniles/g

among tilapia followed a very similar trend compared to the effects on %M (Fig. 3.2). The similarity is not surprising, given that the two metrics are closely related because of the narrow weight range of study fish.

Similar to the results of %M and juveniles/g, there was no treatment effect of cortisol on mean attachment/g in either of the two primary host species (Fig. 3.1). However, compared to control groups, mean attachment/g was elevated in all cortisol treatment groups in the three fish species (catfish, goldfish, tilapia) putatively categorized as a non-host or marginal host, though only in the goldfish trial was the increase statistically significant. As with %M, the increase in initial attachment with cortisol administration was observed in the preliminary work by Dubansky et al. (2011).

The plasma cortisol levels measured in a series of proxy trials showed a clear increase in cortisol treatment groups for both largemouth bass and bluegill (Fig. 3.4). Because of the consistent cortisol treatment effects observed in tilapia, a direct measure of tilapia plasma cortisol was expected to reflect such treatment effects. Although mean levels of tilapia plasma cortisol were elevated above Control and Sham groups, the differences were not significant. The absence of a significant treatment effect in the proxy trial tilapia treatment groups may be caused by one of many factors. Increased ambient temperature may increase metabolism and the clearance rate of exogenous cortisol, but the recorded water temperature difference varied by less than 1 ^{o}C between the tilapia larval metamorphosis trials and the trials measuring plasma cortisol in proxy fish. Repeated warming and cooling of cortisol/coconut oil stocks, as occurs during administration, may potentially affect potency, but hydrocortisone has been demonstrated as highly stable across a range of temperatures (Sarkar et al. 2011). The absence of an observed increase in goldfish and tilapia plasma cortisol may be the result of human error in the

administration of treatment injections or an artifact of the small sample size (n=3). Alternatively, the lack of treatment effect in tilapia plasma cortisol levels may be related to a delay in the physiological response to the clearance of plasma cortisol following complete metabolism of the hydrocortisone-laden implant. In the case of tilapia and goldfish, the three-day period that fish were held in inoculation tanks may have been sufficient to metabolize the implant and clear the exogenous cortisol, but insufficient to regain full functionality of the immune system. The appropriate length of time (48 vs 72hrs) to hold fish prior to inoculation was investigated in a preliminary trial with bluegill, and the resulting plasma cortisol levels were nearly identical from 48 to 72 hours. However, as evidenced by the contradictory results between tilapia plasma cortisol levels and the treatment effects on larval metamorphosis, the time necessary to absorb and metabolize cortisol from the implant, clear the exogenous cortisol, and regain immune function likely varies by species.

In summary, this study found that administering cortisol to potential host fish can affect the larval metamorphosis of freshwater mussels in a number of ways: increased initial glochidial attachment, a greater proportion of those glochidia that successfully undergo metamorphosis, higher number of juveniles ultimately produced, and a protracted time period over which those juveniles are released from the fish. The above effects of exogenous cortisol may be greatest within the context of a host relationship involving a marginal host species, but further research is necessary for confirmation. A comparison of results between the non-hosts, marginal host, and primary hosts suggests an interactive effect between exogenous cortisol and host specificity. These results integrate well with previous research suggesting that distinct subsets of immunological and physiological properties, varying by host specificity, govern the interaction between glochidium and fish (Bauer and Vogel 1987; Meyers et al. 1980; Rogers-Lowery and

Dimock 2006; Waller and Mitchell 1989). Likewise, the specific cortisol-mediated factors that resulted in the treatment effects seen in this study may comprise a distinct subset of the total set of immunological features involved in larval metamorphosis.

Ongoing research efforts continue to improve our, as yet, poor understanding of the specific mechanisms involved in larval metamorphosis. This study contributes to the emerging body of research supporting cortisol as a significant factor involved in the dynamics of the host relationship (Dubansky et al. 2011; Kirk and Layzer 1997; K. Douda, Czech University of Life Sciences Prague, personal communication). Future research may expand on this by using exogenous cortisol and host specificity as predictor variables in experiments working toward the isolation and identification of the roles of individual immune features within larval metamorphosis.

The findings of this study may also be applied to the improvement of lab techniques for the captive propagation of imperiled mussels as part of ongoing conservation efforts. To address the high number of mussels currently at risk of extinction, the number of mussel conservation hatcheries has grown rapidly in the last decade. In cases where the primary fish host is known for a given imperiled mussel species, hatcheries can produce large quantities of juveniles for the augmentation and restoration of naturally reproducing populations. However, in cases where the primary fish host is unknown, this study represents the development of a promising technique for improving production of juvenile freshwater mussels on marginal hosts until future research identifies a primary host species. In the case that a primary host species proves difficult to rear in hatcheries, this technique may provide an avenue for increased production of juvenile mussels on a marginal host better suited to a captive environment.

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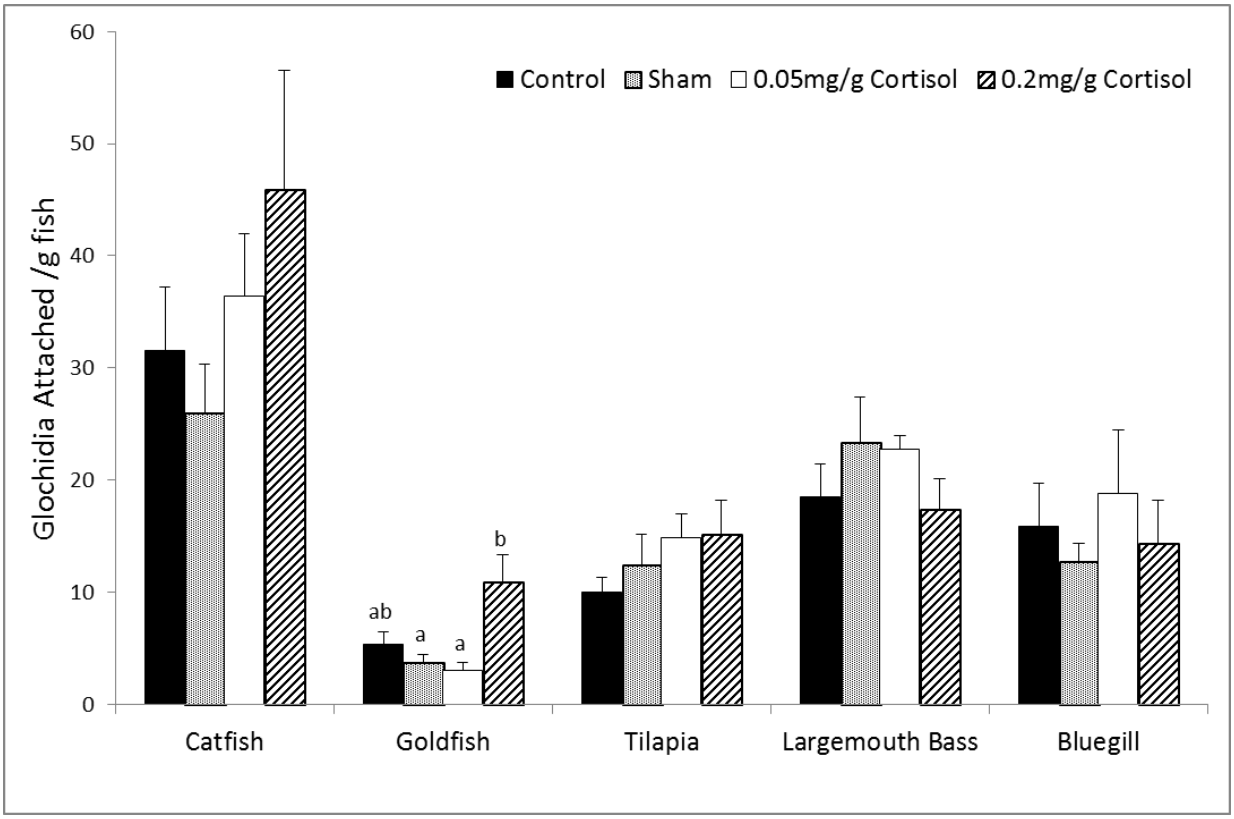

Figure 3.1. Comparison of initially attached *L. subrostrata* glochidia per gram of fish (mean ± SE) on five fish species following host-administered exogenous cortisol. Lower case letters denote significant differences between treatments within fish species as determined by Tukey's HSD following ANOVA. Fish in the control group were not injected, but otherwise handled identically; fish in the sham-injection group were injected with coconut oil only. Treatment groups in some species trials were reduced by mortalities from the initial number of replicates (n = 7): catfish (n = 7), goldfish (n = 7), tilapia (n = 4-5), largemouth bass (n = 7), bluegill (n = 6-7).

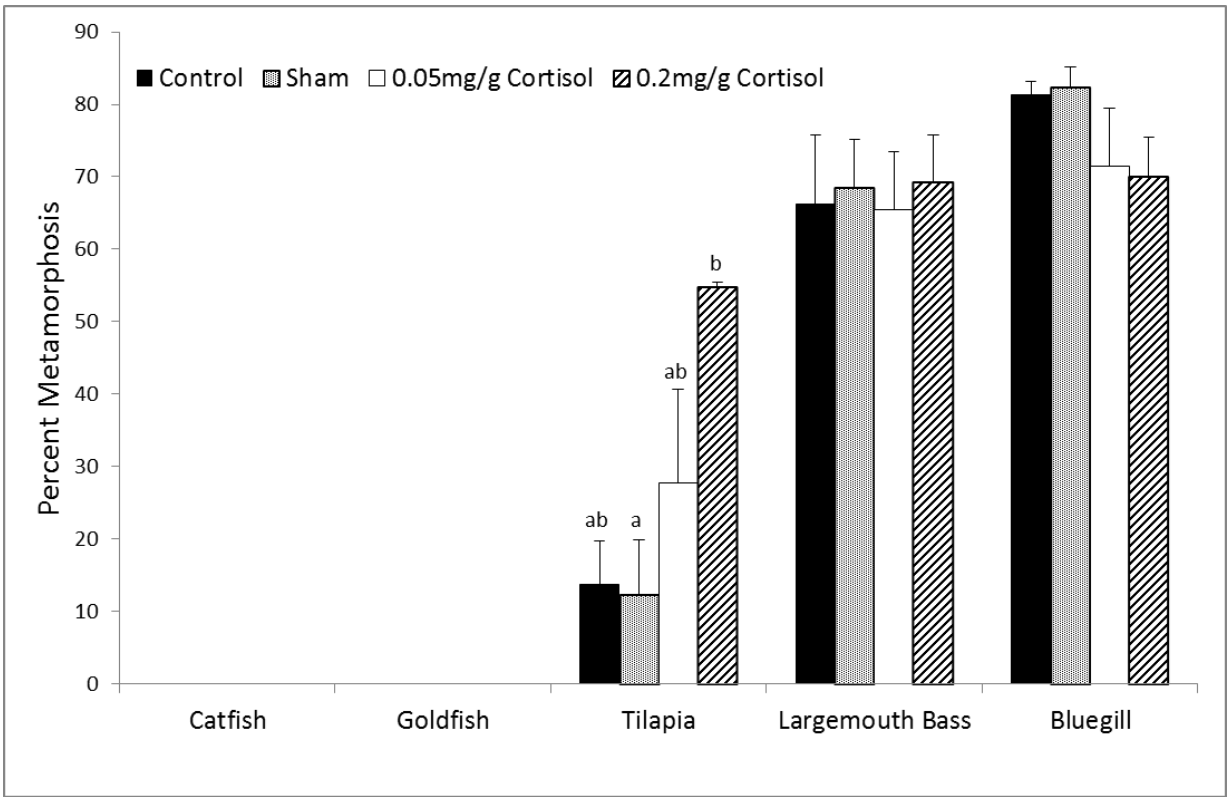

Figure 3.2. Comparison of percentage of metamorphosis success (mean ± SE) of *L. subrostrata* glochidia on five fish species following host-administered exogenous cortisol. Lower case letters denote significant differences between treatments within fish species as determined by Tukey's HSD following ANOVA. Fish in the control group were not injected, but otherwise handled identically; fish in the sham-injection group were injected with coconut oil only. Treatment groups in some species trials were reduced by mortalities from the initial number of replicates (n = 7): catfish (n = 7), goldfish (n = 7), tilapia (n = 4-5), largemouth bass (n = 6-7), bluegill (n = 6-7). No juveniles were produced on either catfish or goldfish.

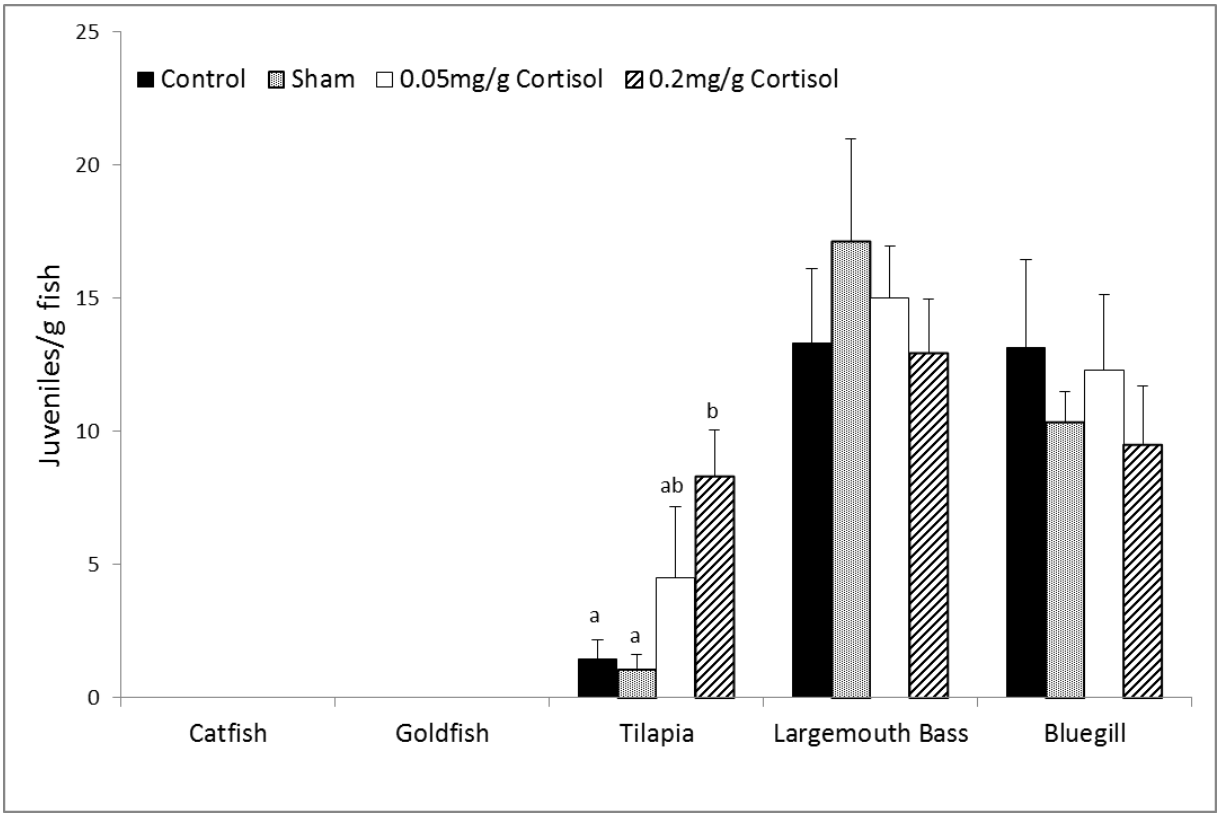

Figure 3.3. Comparison of number of *L. subrostrata* juveniles produced (mean ± SE) on five fish species following host-administered exogenous cortisol. Lower case letters denote significant differences between treatments within fish species as determined by Tukey's HSD following ANOVA. Fish in the control group were not injected, but otherwise handled identically; fish in the sham-injection group were injected with coconut oil only. Treatment groups in some species trials were reduced by mortalities from the initial number of replicates (n = 7): catfish (n = 7), goldfish (n = 7), tilapia (n = 4-5), largemouth bass (n = 6-7), bluegill (n = 6-7). No juveniles were produced on either catfish or goldfish.

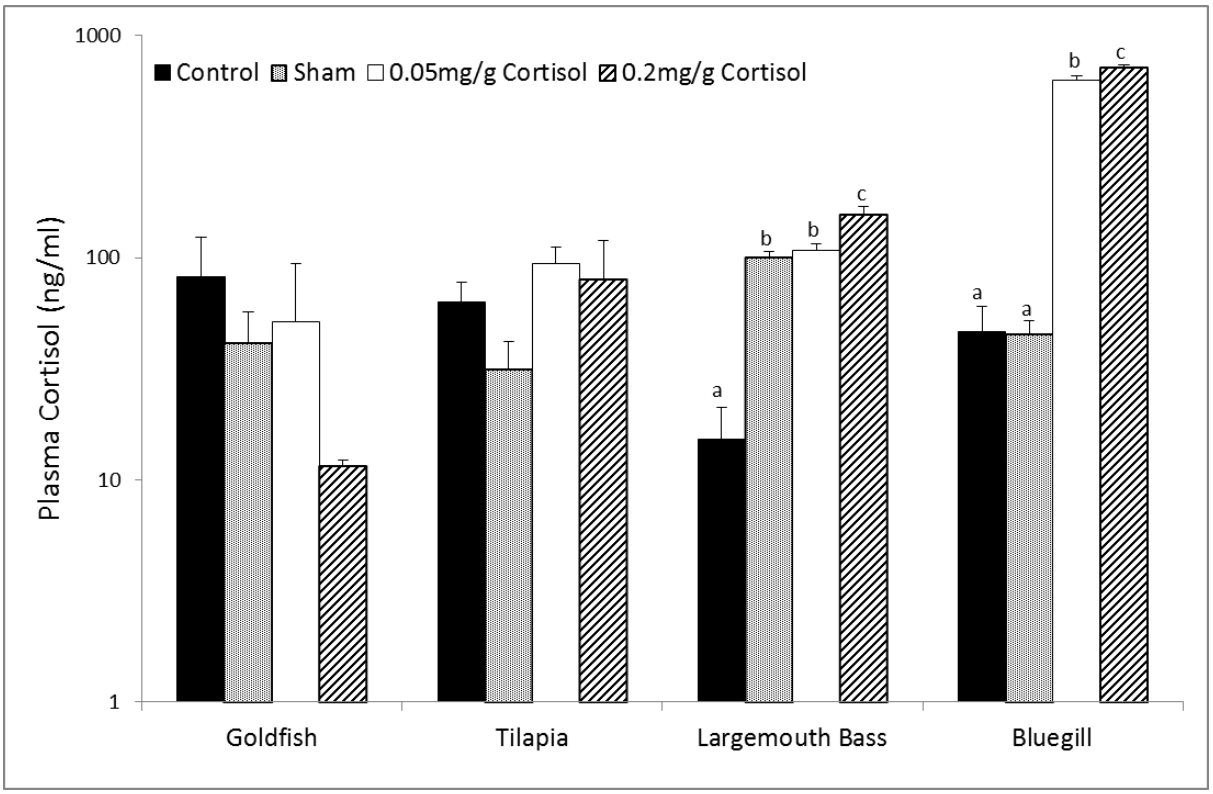

Figure 3.4. Comparison of circulating plasma cortisol levels (mean ± SE) in four fish species following administration of exogenous cortisol. Lower case letters denote significant differences between treatments within fish species as determined by Tukey's HSD following ANOVA. Fish in the control group were not injected, but otherwise handled identically; fish in the sham-injection group were injected with coconut oil only. In some species trials the initial number of replicates (tilapia, largemouth bass, and bluegill n = 3; goldfish n = 4) were reduced by mortalities: tilapia n = 2-3, largemouth bass n = 2-3, bluegill n = 3, goldfish n = 3-4.

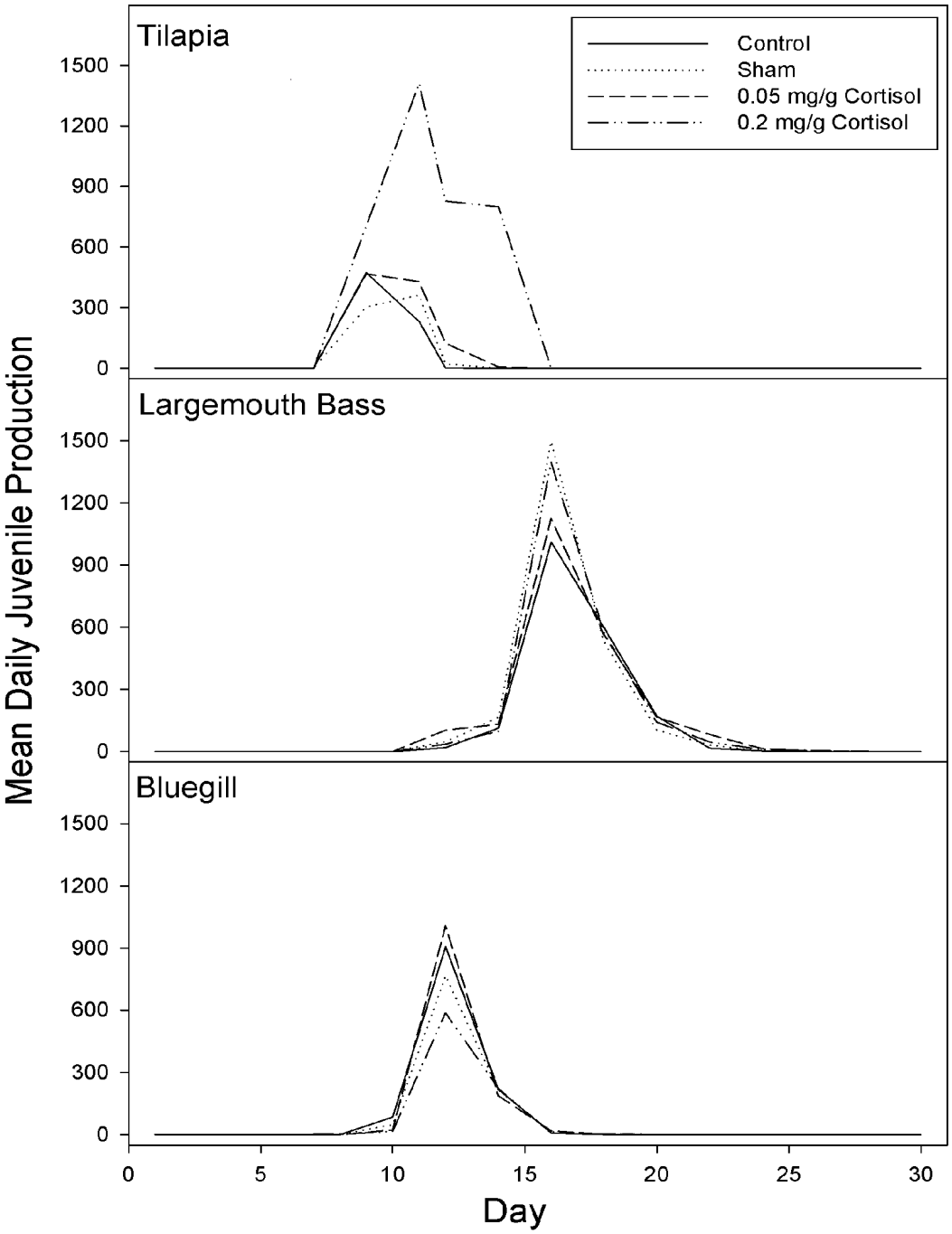

Figure 3.5. Comparison of time period over which *L. subrostrata* juveniles were produced from three fish species following administration of exogenous cortisol. Fish in the control group were not injected, but otherwise handled identically; fish in the sham-injection group were injected with coconut oil only. Treatment groups in some species trials were reduced by mortalities from the initial number of replicates (n = 7): tilapia (n = 4-5), largemouth bass (n = 6-7), bluegill (n = 6-7).

CHAPTER 4

DISCUSSION

Within the context of widespread imperilment of both terrestrial and aquatic biota, the status of the North American freshwater mussel fauna represents an extreme case. The cumulative direct and indirect effects of human activities have resulted in the extinction of 10% of the estimated 300 mussel species and imperilment of 65% of the remaining species (Haag and Williams 2014). Scientific inquiry into the natural history of mussels began in earnest just over 100 years ago and was primarily motivated by commercial applications. In recent decades, the impetus for research has shifted toward conservation in light of major population declines across the continent (Haag 2012). Mussels' dependence upon fish hosts, during their obligate parasitic life stage, renders them especially vulnerable to anthropogenic effects on aquatic systems. In spite of the volume of research examining mussels, many aspects of the mussel-fish host relationship remain poorly understood. Further investigation of this relationship is necessary for not only basic biological and ecological understanding of these fascinating animals, but also as a means to inform management actions whose objective is their continued existence.

Through a series of fish host identification trials, the first portion of this research project examined the potential host relationships between mussels native to the ACF Basin and a single species, the Gulf sturgeon. Should the Gulf sturgeon, itself federally listed as threatened, be reintroduced to its historic range above the JWLD, it may serve as host to a number of the native mussel species, six of which are threatened or endangered (Brim Box and Williams 2000; Fritts

et al. 2012; Williams et al. 2008). Of the seven mussels tested in this study, *Amblema neislerii* (fat threeridge) alone was found to successfully metamorphose (3.5%) on Atlantic sturgeon, the sister subspecies to (and surrogate of) Gulf sturgeon. These findings, coupled with related work (Fritts and Bringolf 2014; Fritts et al. 2012), are a major step toward understanding the ecology and reproductive biology of the ACF mussel fauna. Future work should continue sturgeon host trials, but also expand trials to examine other diadromous ACF species such as Alabama shad and Gulf striped bass, whose movements are also limited by dams.

The second portion of this research project examined the physiological aspect of the mussel-fish host relationship via investigation of the effects of exogenous cortisol on larval metamorphosis. In spite of a long-standing recognition of the immunological nature of the factors governing metamorphosis, a comprehensive understanding of the molecular and cellular mechanisms involved has yet to be achieved (Arey 1921; Barnhart et al. 2008; Rogers-Lowery and Dimock 2006). Cortisol suppresses the vertebrate immune system and increase susceptibility to infections, specifically ectoparasitic infections (Davis et al. 2003; Harris and Bird 2000). Previous research found host-administered exogenous cortisol to (1) induce larval metamorphosis on known non-host species (Kirk and Layzer 1997) and to (2) increase both glochidial attachment and metamorphosis success (Dubansky et al. 2011). Building on the aforementioned research, this study expanded the scope of investigation into the effects of exogenous cortisol by increasing: the number of levels of cortisol treatment, the number of fish species, and the number of metrics used to evaluate any treatment effects. This expanded investigation has found that administering cortisol to potential host fish can affect the larval metamorphosis of freshwater mussels in a number of ways: increased initial glochidial attachment, increasing the proportion of those glochidia that successfully undergo

metamorphosis, higher number of juveniles ultimately produced, and a protracted time period over which those juveniles are released from the fish. Furthermore, a comparison of results between the non-hosts, marginal host, and primary hosts suggests that the potential effects of exogenous cortisol depend on and vary by the natural host relationship of the particular mussel-fish pair under examination. These findings contribute to both basic research into the physiological mechanisms underlying the mussel-fish host relationship and applied research in captive propagation techniques. In cases where the primary fish host is unknown, administration of exogenous cortisol may increase production of juvenile freshwater mussels on marginal hosts until future research identifies a primary host species.

An improved understanding of the mussel-fish host relationship is a fundamental necessity of any management effort with the objective of conserving this highly imperiled taxon. Systematic identification of fish hosts is essential to the design and implementation of effective management actions in North America and around the world. Captive propagation programs currently and increasingly serve as a conservation bridge until such a time as fish hosts and critical habitat are identified and restored. In the absence of robust empirical research to inform conservation efforts, mussel populations in the ACF, in North America, and across the world will continue to be threatened by and give way to extinction.

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