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Aspects of the Physiological and Behavioral Defense Adaptations of the Mountain Madtom (Noturus eleutherus)

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J. Brian Alford, Major Professor

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Carolyn R. Hodges
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(Original signatures are on file with official student records.)
Aspects of the Physiological and Behavioral Defense Adaptations of the Mountain Madtom

(Noturus eleutherus)

A Thesis Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Meredith Leigh Hayes

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Abstract

Madtoms (*Noturus* spp.) are a highly endemic clade of miniature catfishes that faces widespread imperilment. Little is known about the ecology of these secretive fishes, and understanding the behavioral and physiological adaptations madtoms have evolved to resist pathogens and competitors is necessary for conservation.

Madtoms nest under cover and provide extensive paternal care. Attempts to rear eggs in captivity result in high mortality rates from infection, leading to questions about how wild nests resist disease. In many fishes, males produce antimicrobial substances that confer protection to eggs. To determine if guardian males deter disease in nests, Mountain Madtoms (*Noturus eleutherus*) were captively spawned and survivorship calculated. Four clutches were produced; one was consumed, one was incubated by the male, and two were isolated. Hatching success was 10% for the clutch cared for by the male and 60% and 18% respectively for the isolated clutches. This does not support the hypothesis that the guardian male generates higher hatching rates, but more observations are required to make a definitive conclusion. To detect antimicrobial activity in the epidermal mucus of madtoms, hydrophobic peptides extracted from mucus of male *N. eleutherus* were assayed against several pathogens. The proteins did not inhibit the growth of any strain, but further research is needed to determine whether the inactivity was a result of an absence of antimicrobial properties in madtom mucus.

Because madtoms rely upon cavities for shelter, they must compete for these spaces with other speleophilic taxa or risk exposure and increased predation. While it’s been suggested that invasive crayfish exclude madtoms from cover, no studies have been conducted to demonstrate this. I experimentally tested competitive exclusion between two species of
invasive crayfish (*Orconectes juvenilis* and *O. virilis*) and Mountain Madtoms by manipulating crayfish density, territory establishment, relative size, and length of exposure. Length of exposure and relative size were inversely correlated with madtom occupancy and health (*P*<0.005, both). Juvenile madtoms experienced 100% mortality. Thus, invasive crayfish have a size-specific competitive advantage over madtoms, and may have played a role in the decline of the Chucky Madtom (*Noturus crypticus*) through habitat exclusion and predation on juveniles.
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An Introduction to Madtoms

Madtoms (*Noturus* spp.) are a clade of diminutive, secretive, venomous catfish (Ictaluridae) endemic to North America. These mysterious fishes are nocturnal, sheltering in cavities beneath large rocks by day and emerging to cruise the benthos by night. Relatively little is known about madtoms due to their small size, cryptic coloration, and elusive nature. Less than half of the known taxa have had life history studies completed, and relatively few studies have delved into their complex ecological roles. While there are currently 29 described *Noturus* species, making it the most diverse genus in the family Ictaluridae, several complexes are likely masquerading as a single species that warrant a closer look (Egge and Simons 2006). By the time a unique species is discovered, it is often already critically endangered (Near and Hardman 2006; Warren et al. 2000). More research is desperately needed to understand the life history and ecology of these fishes before more populations become extinct.

Madtoms are characterized diagnostically from other ictalurids by possessing a connection between their fatty adipose fin and their caudal fin. Additionally, all species are known to display pectoral and dorsal spines associated with venom glands that protect the small-bodied fish from predators (Birkhead 1967). Based on early descriptions of some species (Mayden 1983), they feed mainly at dusk and dawn, avoiding both direct competitors for prey as well as sight-feeding predators (e.g., the black bass *Micropterus* spp.). They primarily use their olfactory and gustatory senses to skim the substrate with their barbels to locate and consume aquatic insects like larval chironomids, ephemeropterans, and trichopterans as well as some aquatic crustaceans (Burr and Stoeckel 1999; Mayden and Burr 1981). They range in size from 36 to 265 mm (standard length, SL), with only the Stonecat (*Noturus flavus*) regularly
attaining body sizes above 150 mm SL (Burr and Stoeckel 1999). Morphologically, most species are dark in color and fairly drab, enabling them to blend in with the stream bottom in the low light that they prefer (Taylor 1969). Species in subgenera *Noturus* and *Schilbeodes* tend to be uniformly pigmented, whereas those in subgenus *Rabida* may be boldly marked with bands, saddles or mottling (Mayden 1983). They do not exhibit sexual dimorphism in coloration like many striking fishes of the Southeastern U.S.

Excepting the more widespread stonecat, madtoms are found only east of the Missouri River and predominantly south of and including the Ohio River basin due to their requirement for waters sufficiently warm enough for spawning (Taylor 1969). Most madtoms inhabit riffles or pools in clear flowing streams where cover may be found in the form of slab rocks, woody debris, cobble, vegetation, and even castoff manmade items like cans and bottles. They rely upon these sheltered cavities for protection from sight-feeding predators who hunt during the day, and for spawning and egg-rearing.

Spawning occurs primarily in June and July of the second or third summer of life, with madtoms becoming reproductively mature at one or two years of age (Burr and Stoeckel 1999; Mayden 1983; Taylor 1969). Beginning in the spring, male madtoms develop swelling in the lips and genital papillae, as well as enlarged cephalic muscles that are thought to assist in nest building and defense. Females lay relatively few, large eggs in a cavity excavated by the male (Burr and Stoeckel 1999; Taylor 1969). There appears to be a tradeoff in fecundity for the extensive parental care provided by the male throughout the incubation period. The paternal guardian defends the nest from predators, fans the eggs with his tail for aeration, and cleans eggs with his mouth (Bulger et al. 2002; Burr and Stoeckel 1999; Mayden and Burr 1981;
Mayden 1983). He may remain with the nest for several days post hatch before the larvae disperse (Mayden 1983; Mayden and Burr 1981; Starnes and Starnes 1985). This behavior represents one of the most intensive forms of parental care in North American fishes. This nesting phase is a critical stage in the lifecycle of madtoms, and demersal, adhesive eggs like theirs are particularly vulnerable to predation and disease as they are laid in dense clumps that attract high numbers of predators and facilitate the spread of pathogens (Bunn et al. 2000; Cabrita et al. 2009; Scott and O’Bier 1962). Juvenile madtoms are known to be preyed upon by some birds, snakes, piscivorous fish, and crayfish (Burr and Stoeckel 1999; Mayden 1983; Mayden and Burr 1981). Mortality during this critical phase has an exaggerated impact on recruitment and population growth more so than at any other time in a fish’s life, making the egg and juvenile stages particularly important to consider when determining management protocols (Bunn et al. 2000; Sifa and Mathias 1987).

The North American fish fauna is currently in the midst of a conservation crisis, with species of *Noturus* facing an inordinate rate of imperilment (Jelks et al. 2011). Several aspects of their life history serve to make them especially susceptible to population declines, extirpation, and even extinction. More than half of the nearly 30 described species exhibit extreme endemism, with some having a range restricted to a single stream system and short, fragmented patches of suitable habitat (Burr and Stoeckel 1999). As a result, natural stochastic events (*i.e.* drought, disease, flood, etc.) and anthropogenic activities pose a threat to the persistence of such small populations (Burr et al. 2005; Near and Hardman 2006). Stream alteration in the form of channelization, impoundment, introduced species, and lack of riparian vegetation buffers combined with land-use changes that cause sedimentation, contamination
from pesticide runoff, and elevated nutrient concentrations have been well-documented as causes for the declines of several species of madtom over the past few decades (Burr et al. 2005; Kuhajda et al. 2016a; Shute et al. 2005; Utz 2014). Benthic species often suffer disproportionately from sedimentation as the cavities and interstitial spaces they and their prey items inhabit become clogged and uninhabitable. Unlike many freshwater fish species whose primary sensory mechanism is sight, madtoms rely upon olfactory and gustatory senses which may be disrupted by complex organic chemicals that contaminate streams as agricultural runoff (Etnier and Jenkins 1980). With half of all Noturus species currently federally listed for protection and at least one considered extinct (The IUCN Red List 2017), it is now more important than ever to add to the relatively small body of scientific research on these disappearing creatures. More insight into their physiological and behavioral adaptations to their environment is necessary for the development of management recommendations and recovery protocols. For this reason, I have chosen to study two specific fields of madtom defense mechanisms: (1) antimicrobial proteins in madtom epidermal mucus and its role in parental care, and (2) the interspecific habitat competition between madtoms and invasive crayfish species.
PART I: Antimicrobial Properties of Paternal Care in the Mountain Madtom (*Noturus eleutherus*)
Abstract

Madtom catfish exhibit some of the most elaborate parental care of freshwater fishes in North America. While their egg cleaning and guarding behaviors have been thoroughly studied, one potential aspect of care-giving has received little to no attention: disease prevention. Guardian males in many fish species secrete specialized antimicrobial substances that inhibit microbial infection in their eggs, and many more species have been found to possess antimicrobial compounds in their epidermal mucus that help them resist pathogens. Madtom eggs are notoriously susceptible to apparent fungal infection when incubated in captivity without the guardian male present. In contrast, clutches cared for by the male in the wild appear resistant to pathogens and have very high hatching success. In light of the current decline of madtom populations throughout their range, the need for successful propagation protocols and ecological studies is crucial. In this study, I hypothesized that the presence of the guardian male increases the survivorship of clutches by conferring disease resistance through physical contact with his epidermal mucus. To test this hypothesis, I first determined the survivorship of captively-spawned Mountain Madtom (*Noturus eleutherus*) eggs with and without the guardian male present. Four clutches were produced during the summers of 2016 and 2017; one was consumed by the parents, one was incubated by the guardian male, and two were isolated from the guardian male. The survivorship was 10% for the eggs remaining with the male, and for the two isolated clutches, 60% and 18% respectively. The hypothesis that clutches cared for by the male will have a higher hatching success was not supported, but the sample size was too small to draw a firm conclusion. Second, I screened for antimicrobial activity of Mountain Madtoms’ epidermal mucus through assays against several strains of
common fungal and bacterial pathogens. No discernible antimicrobial activity was detected against any strain. While there was no evidence of antimicrobial benefits of paternal care in Mountain Madtoms in this study, further research is necessary to thoroughly explore this topic.
Chapter 1: Parental Care & Antimicrobial Activity in Fishes

Male parental care in fish is not an unusual phenomenon. There are at least 43 families of fish in which the male is the sole care-giver, ranging from lungfish (Lepidosirenidae) to blennies (Blenniidae) to darters (Percidae) (Blumer 1979). Among these, the madtom catfishes (Ictaluridae) provide some of the most extensive paternal care found in North America, both pre- and post-fertilization (Taylor 1969). Guardian male madtoms build nests to guarding eggs from predators, aerate eggs by agitating the water with his tail, remove dead or diseased eggs from the nest, and clean eggs by vigorously rubbing them with his body and rolling them in his mouth (Burr and Stoeckel 1999). Thus, there is no doubt that the madtom guardian male plays a pivotal role in the survival of his eggs to the larval stage. While these care-giving behaviors have been widely studied, one potential aspect of male parental care in madtoms has been given little or no attention: possible antimicrobial benefits transferred to the eggs by the guardian males.

The water column in a freshwater ecosystem is teeming with a vast array of microbes, and nearly every surface is covered in biofilms of potentially harmful bacteria, fungi, viruses, and protozoa (Locke et al. 1984). However, the prevalence of disease outbreaks in fish is relatively low, even after cuts and wounds expose sterile tissues (Ellis 2001). Only relatively recently have scientists come to discover the means by which fishes fend off pathogenic invasion: an antimicrobial coat of slime covering the epidermis. Goblet cells embedded in the skin are constantly secreting this mucus which is composed of a variety of structural and specialized cells including glycoproteins, immunoglobins, enzymes, and antimicrobial peptides (Esteban 2012). Antimicrobial compounds have been detected in the epidermal mucus of nearly
every teleostian species tested, indicating this is an ancient and widely conserved form of innate immunity. Indeed, this slime coat is the first line of defense for fishes living in aquatic environments that carry heavy pathogenic microbial loads (Ebran et al. 1999).

Demersal, oviparous animals like madtoms are especially susceptible to bacterial and fungal colonization because they are in constant contact with the benthos and the biofilms present there (Giacomello et al. 2006). It is one of the leading causes of mortality of eggs in hatcheries, and extensive, costly research has been conducted to develop methods that prevent such infections and improve hatching success rates of commercial aquaculture operations (Komar et al. 2004; Mitchell et al. 2009). It has recently been determined that care-giving males in several different species are able to combat microbial infection of eggs by applying antimicrobial substances to nests. Knouft et al. (2003) demonstrated that the presence of Fringed Darter (Etheostoma crossopterum) guarding males significantly reduced egg infection compared to those where the male was absent. When exposed to the males’ mucus during bacterial and fungal assays, microbial growth was indeed inhibited. In two species of blenny (Ophioblennius atlanticus atlanticus and Salaria pavo), nesting males secrete an antimicrobial substance onto eggs from enlarged anal glands (Giacomello et al. 2006; Pizzolon et al. 2010). Male Three-spined Sticklebacks (Gasterosteus aculeatus) produce a “glue” that provides antimicrobial benefits to nests (Little et al. 2008). Likewise, madtoms provide paternal care to nests and lay demersal eggs, and due to the widely conserved presence of antimicrobial compounds in fish epidermal mucus, can also be expected to express antimicrobial properties in their slime coat.
Due to their current conservation status as one of the most imperiled genera of freshwater fishes in North America, some madtom species have been or are currently being propagated in attempts to augment or restore wild populations. Many more species have been petitioned as federally endangered or threatened under the U.S. Endangered Species Act (Center for Biological Diversity 2010), and recovery strategies will likely include propagation. Unfortunately, captive spawning of madtom species is notoriously challenging. Cases of spawning in aquaria are relatively rare, and guardian males are extremely prone to abandoning or consuming their own eggs (Bowen 1980; Bulger et al. 2002; Clark 1978; Fitzpatrick 1981; Stoeckel 1993). When isolated from the guardian male, eggs are highly susceptible to disease, and fungal infections account for the vast majority of mortalities in hatcheries (pers. comm., J.R. Shute, Conservation Fisheries, Inc.; Petty et al. 2013; Stoeckel 1993). However, clutches collected from the wild at more advanced stages (i.e., nests that have spent more time with the guardian male) have less disease-related mortalities and significantly higher hatching success than less developed eggs (Burr and Mayden 1982; Dinkins 1984; Mayden et al. 1980; Starnes and Starnes 1985). Thus, it is likely that the guardian male provides some level of care that prevents disease outbreaks (Blumer 1985; Stoeckel 1993). In light of the new evidence for the role antimicrobial compounds produced by adult fish plays in some forms of parental care, I hypothesize that a similar phenomenon is occurring in madtom catfishes. A better understanding of how nests are able to resist pathogenic colonization in the wild is necessary for improving rearing techniques in captivity. Identifying the presence of antimicrobial compounds and the effects they have on overall nest viability will greatly benefit rare Noturus propagation efforts. The research objectives of my study were as follows:
1. Quantify hatching success of egg clutches with guardian male present versus nests with guardian male absent;

2. Demonstrate inhibitory effects of madtom epidermal mucus on pathogens through microbial assays.
Chapter 2: Captive Spawning and Egg Survivorship

Methods

Mountain Madtoms (*Noturus eleutherus*), subgenus *Rabida*, were chosen as the study species. They are considered a species of “least concern” (The IUCN Red List 2017), and they have a relatively widespread distribution, occurring in streams of the Mississippi River system, the Ouachita, Black, and St. Francis systems, in much of the Ohio River Basin, and in the Cumberland and Tennessee drainages (Starnes and Starnes 1985). Nests were collected from the Powell River during the summers of 2014 and 2015 and reared to adulthood at the culture facilities at Conservation Fisheries, Inc. (CFI) in Knoxville, TN. They were kept in 75 L tanks on a recirculating system equipped with sponge filters, fans to create current, bubblers to oxygenate the water, a layer of sand-gravel substrate, and cover objects to provide refuge (Appendix A).

To condition the madtoms for captive spawning, CFI’s broodstock conditioning protocols were implemented following Petty et al. (2013). Day length was manipulated with an astronomic timer and programmed for CFI’s latitude and longitude to imitate natural photoperiod. Water temperature was controlled by ventilation with outdoor air during the winter and maintained at or around 23.5°C during the summer, using air conditioning when necessary to keep temperatures under 25°C. Broodstock were fed *ad libitum* on live black worms, live chopped red worms, live *Gammarus pulex* (an amphipod), frozen brine shrimp, and frozen bloodworms.

In late spring of 2016 and 2017, the adult madtoms were grouped into breeding groups: one male and one female, or one male and two females per tank. Spawning habitat was added in the form of terra cotta plant pot dishes turned upside down to create a cavity, square slate tiles of various diameters, and halved PVC pipes (Appendix B). Behavior was assessed daily for
signs of courtship and spawning: male and female inhabiting the cavity together, sealing up access to the cavity with substrate, and cessation of feeding activity. Guardian male madtoms are notoriously sensitive to nest disturbance and will often abandon or devour eggs if exposed or bothered (Bowen 1980; Clark 1978; Dinkins and Shute 1996; Fitzpatrick 1981; Stoeckel 1993). Therefore, minimally invasive techniques were used to observe nests. Male madtoms excavate a shallow depression in the substrate inside the cavity, and the eggs are always found in the deepest portion of that cavity (Mayden and Burr 1981). In the aquaria, this depression often resulted in an area cleared of substrate, making courtship and nests observable from underneath the glass tank bottom. When observation of this nature was not possible, I used a lighted endoscope (Android Smartphone Endoscope USB Borescope 7mm 2M Waterproof Inspection Snake Camera) to look for nests with minimal disturbance.

Of the 4 clutches produced throughout the course of this study, each was randomly selected to either remain with the guardian male or be removed from the nest. The latter were transferred to a separate recirculating aquaculture system empty of all fish to exclude contact with possible ambient epidermal mucus present in the water. Eggs were incubated in covered opaque trays to simulate the lightless conditions of naturally-occurring nests and equipped with an air-stone to mimic tail-fanning by the male to eliminate oxygenation (or lack thereof) as a potentially confounding variable. Egg mortalities were recorded twice daily. All dead or diseased eggs were removed to prevent another potentially confounding variable as the guardian male typically performs this behavior himself to prevent the uninhibited spread of infection. Once all eggs hatched, egg survivorship was calculated using the following formula:

\[
\frac{\text{# live larvae}}{\text{# eggs laid}} \times 100.
\]
Results & Discussion

The Mountain Madtoms in this study were reproductively mature by the third summer of life, at age 2. This is consistent with reports of other large madtom species in subgenus *Rabida* (Burr et al. 1989; Burr and Mayden 1982; Burr and Mayden 1984; Burr and Stoeckel 1999; Dinkins and Shute 1996; Mayden and Burr 1981). Males began to develop typical nuptial characteristics (enlarged cephalic epaxial muscles, swollen lips, and swollen genital papillae) and females became gravid by late April when water temperatures stayed consistently above 18°C. Age 3 fish came into spawning condition approximately 3 weeks earlier than did age 2 individuals. Females retained distended abdomens until August, and males exhibited breeding morphology until September.

Of the three types of structures available for shelter in each tank, male madtoms appeared to prefer the plant pot dishes, presumably because the single small entry hole was easily defendable (Appendix B). The males created nests by excavating a depression in the center of the cavity. They swept the substrate towards the edges, and piled substrate in the entry hole to completely seal the cavity (Appendix B). Some studies indicate that females participate in nest construction or cavity enhancement (Bulger et al. 2002; Chan 1995; Dinkins 1984; Fitzpatrick 1981), but in this study, only the male prepared nests for spawning. Both sexes excavated depressions under shelter structures throughout the year, but this activity seemed unrelated to spawning. Only those cavities manipulated by males and sealed off with substrate served as spawning sites. At some point, the female would enter the nest, and the breeding pair would remain there together for three or four days courting and spawning. During this time, neither fish would emerge or feed. This observation supports the assumptions
that male madtoms do not feed while nesting based on collections of nesting males with empty stomachs (Burr and Dimmick 1981; Burr and Mayden 1982; Clark 1978; Mayden et al. 1980). On several occasions, the female would reemerge from the nest after several days remaining extremely gravid, indicating no spawning had occurred. Occasionally, the female would emerge looking much less gravid, but no eggs would be found. I concluded that these represented instances of egg expulsion followed by consumption, which is a serious complication of spawning madtoms in captivity (Bowen 1980; Bulger et al. 2002; Clark 1978; Fitzpatrick 1981; Stoeckel 1993).

Four clutches were produced over the course of this study (Table 1), all produced by age 2 fish. Clutch size ranged from 25 to 48 eggs. These clutches appear to be quite a bit smaller than wild clutches, with 70 eggs being reported in Starnes and Starnes (1985), and other collections ranging from 49 to 115 eggs in a single nest. The difference in nest size could be a result of high natural variability, hatchery conditions and diet, or unobserved egg consumption.

The first nest was discovered on 3 May 2016 under a pot dish occupied by a single male. The water temperature was 21°C. The clutch was composed of a single mass of 48 yellow, spherical eggs adhered to one another with a few small pieces of substrate attached. The age of the eggs could not be determined, but presumably were less than days old. The clutch was removed from the male guardian and transferred to a small mesh basket in an opaque tray in a recirculating system devoid of any fish to avoid any contact with fish epidermal mucus. An airstone was added to the tray, and a piece of opaque black plastic laid over the top to simulate the lightless conditions inside the cavity (Appendix C). The embryos appeared to be developing normally. Five days after removal from the nest, signs of _Saprolegnia_ infection were visible as
opaque, white, furry masses growing inside the egg and on its surface. Diseased eggs were removed twice daily to prevent contamination of healthy ones. The first eggs hatched on the seventh day after discovery, and all had hatched by the ninth. Of the original 48 collected, 29 survived to hatching (egg survivorship = 60.4%). After hatching, many larvae failed to thrive, and another 17 mortalities occurred before larvae had absorbed their yolk. Therefore, survival to dispersal age was only 25% overall.

Table 1: Description of captively-spawned Mountain Madtom clutches.

<table>
<thead>
<tr>
<th>Date</th>
<th>Water Temp.</th>
<th>Clutch Size</th>
<th>Clutch Removed from Male?</th>
<th># Days to Hatch</th>
<th>Survivorship</th>
</tr>
</thead>
<tbody>
<tr>
<td>5/3/16</td>
<td>21°C</td>
<td>48</td>
<td>Yes</td>
<td>9</td>
<td>60%</td>
</tr>
<tr>
<td>7/12/16</td>
<td>23°C</td>
<td>38</td>
<td>Yes</td>
<td>7</td>
<td>18%</td>
</tr>
<tr>
<td>7/6/17</td>
<td>23°C</td>
<td>25</td>
<td>No</td>
<td>NA</td>
<td>0%</td>
</tr>
<tr>
<td>7/12/17</td>
<td>23°C</td>
<td>48</td>
<td>No</td>
<td>9</td>
<td>10%</td>
</tr>
</tbody>
</table>

The second clutch was discovered under a pot dish occupied by both the male and female madtom on 12 July 2016 when the water temperature was 23°C. The eggs appeared to be in the very early phases of development, and this combined with the presence of the female in the nest indicated that they were a few hours old. The clutch consisted of 38 yellow, spherical eggs in a single aggregation, and this one was also removed from the male guardian. The same incubation protocols were followed as in the first case. Mortalities correlated with
Saprolegnia infection began less than 24 h after discovery. Hatching began on the sixth day after they were spawned, and was complete by the seventh. Only 7 larvae were produced (egg survivorship = 18%).

On 6 July 2017, at approximately 1400, a pair of fish laid a nest of 25 eggs. Water temperature was 23°C. Madtoms have been assumed to spawn exclusively at night or during crepuscular hours (Burr and Mayden 1982; Mayden and Burr, 1981; Taylor 1969), but this constitutes one of the first documented cases of daytime spawning. The clutch consisted of one aggregation of 16 eggs and 9 single eggs scattered nearby. This is unusual, especially since Burr and Stoeckel (1999) reported that in 98% of studies, clutches were clumped together as a single mass. Bulger et al. (2002) reported a single spawning event of the Neosho Madtom (N. placidus) that resulted in 2 small masses. In addition, all eggs appeared to have adhered to the bottom of the tank. The only other record of a madtom nest adhered to the substrate was in the Brown Madtom (N. phaeus) (Chan 1995). However, there is anecdotal evidence that N. eleutherus are more prone to laying scattered clutches rather than single masses as in the majority of madtom species, and that their clutches will adhere to the substrate in the bottom of the nest as well as to one another (pers. comm., C. Ruble, CFI).

The eggs and the breeding pair were visible through the bare glass tank bottom, and their post-spawning behavior was observed for several hours (Appendix D). Both the male and the female fanned their tail rapidly over the eggs or in close to proximity to one another, but whether this was an effort to sweep the eggs together, aerate the eggs, or continued courtship is unknown. In addition to tail fanning, the male and female would nudge and nip one another, rest peacefully side-by-side or head-to-tail for long moments, and touch lips periodically. All of
these behaviors have been well-documented in the courtship of other madtom species (Bulger et al., 2002; Burr and Stoeckel 1999; Fitzpatrick 1981; Pfingsten and Edds 1994). Approximately 2 h post-spawning, the pair’s activity increased. Both appeared to nip at the eggs once or twice, but did not completely take them into their mouths as has been reported in other species (Bulger et al. 2002; Burr and Stoeckel 1999). The frequency of the male nipping and nudging the female increased, presumably in an attempt to chase her out of the cavity. By nightfall, their behavior had not changed significantly. At 0700 the following morning, all but 9 eggs had been consumed. The male’s abdomen was distended, indicating egg consumption, and the female ate one of the remaining eggs while being observed. All eggs had been consumed gone by 1400.

The fourth clutch was discovered on 12 July 2017. The exact date of spawning could have been as early as 9 July 2017, but because the female remained in the nest and the eggs appeared to be very early in development, they were likely produced close to the time of discovery. The clutch consisted of 48 eggs in 3 separate aggregations (Appendix E). The mating pair demonstrated similar behavior as the one described above. In an attempt to prevent egg consumption, the tank they were in was turned offline, meaning water from the recirculating system housing the other breeding groups no longer flowed into that tank. Feeding pheromones travel very quickly throughout a recirculating system and stimulate a feeding response from madtoms in tanks that have not been fed, potentially instigating the nesting fish to feed. In addition, stress from overcrowding has been implicated in egg cannibalism in captivity, so turning the tank offline should prevent the guardian male from sensing the presence of the other madtoms in the system which could lead him to consume his eggs. The
female was found outside of the nest at 0900 the following morning and was removed from the tank. Seven eggs had been eaten overnight. The male’s abdomen appeared to be swollen, thus he was likely the consumer. Over the course of next nine days, all but 13 eggs were eaten, and on 21 July, five larvae had hatched, resulting in a hatching success of 10%. In one instance, two eggs that appeared to have stopped developing were consumed by the male. Therefore, it’s possible that the eggs consumed were unfertilized or infected, and the male was consuming them to prevent the spread of disease. Such a high rate of egg mortality would be unusual based on prior accounts of hatching success for species of *Noturus*. Conditions in the hatchery such as water quality, broodstock diet, and broodstock condition may have resulted in a relatively low fertilization rate and egg viability. However, it is not confirmed that all the eggs the male consumed were inviable or diseased.

Egg consumption by the male, female, or both is a significant challenge in successful captive spawning of madtoms and has been reported in Brindled Madtoms, Margined Madtoms, Neosho Madtoms, Slender Madtoms and Freckled Madtoms, Speckled Madtoms, and even other ictalurids such as bullhead (*Ameiurus* spp.) (Blumer 1985; Bowen 1980; Bulger et al. 2002; Clark 1978; Fitzpatrick 1981; Stoeckel 1993). This phenomenon occurred regardless of food availability, but because guardian males do not feed during nesting, egg consumption is likely not driven by hunger. Nesting males in the wild have been observed to eat their clutches upon exposure or disturbance (Mayden and Burr 1981), which is presumed to be a reaction to stress. However, what factors may be causing captive fish undue stress is not readily apparent in this or other studies (Bowen 1980; Fitzpatrick 1981; Stoeckel 1999).
Overall, these results are insufficient to demonstrate higher survivorship in clutches cared for by males than those incubated in isolation. However, all indications present significant benefits of male incubation of madtom clutches if egg consumption can be prevented. Egg mortality of isolated clutches is predominantly correlated with white fungal infection likely to be *Saprolegnia* spp. (Bulger et al. 2002; Petty et al. 2013; Pfingsten and Edd 1994; Stoeckel 1993), as observed in this study. In contrast, literature shows that eggs cared for by guardian males retain a clean and healthy appearance (Bulger et al. 2002). Hatching success rates are significantly higher for all madtom species when clutches collected from the wild were in advanced stages of development (Burr and Mayden 1982; Dinkins 1984; Mayden et al. 1980; Starnes and Starnes 1985), indicating that guardian males are performing some service to nests to prevent pathogenic infection (Breder and Rosen 1966). Despite water flow, aeration, darkness, and diseased egg removal, *Saprolegnia* infection still resulted in a high rate of mortality for the two isolated clutches in this study. While comparisons could not be made with clutches incubated by the male madtom in this case as the eggs were consumed by the parents, a hatching success rates of 100% is documented for *N. eleutherus* clutches removed at well-developed stages from wild fish (Starnes and Starnes 1985), providing some evidence that the physical presence of the guardian male controls fungal-associated disease outbreaks. Before this can be experimentally demonstrated, protocols for captive spawning that (1) increase clutch production and (2) reduce occurrences of egg consumption are crucial. Maximum, efficient propagation efforts for madtom species will not be reached until male incubation is an option.
Chapter 3: Epidermal Mucus Assays

Methods

*Epidermal mucus collection:*

Epidermal mucus was harvested from the Mountain Madtoms used for captive spawning in Chapter 2. Mucus collection followed the protocols of Ross et al. (2000) with modifications. All fish were fasted for 24 h before mucus was collected. Male Mountain Madtoms in breeding condition were anaesthetized with a sub-lethal dose of tricaine methanesulfonate (Argent Finquel MS-222; approximately 0.165 g/L), rinsed with 2 mL sterile water, and removed from the water for 2 min. to stimulate slime production. Each fish was then placed singly in a polyethylene bag (Ziploc®) containing 2 mL of 50mM NaCl and gently massaged for 2 min. to slough off mucus. The NaCl solution-mucus mixture was immediately transferred to a 50 mL sterile plastic Eppendorf, and the fish returned to recovery tanks.

Differing concentrations of mucus were made by pooling NaCl solution-mucus samples from varying numbers of fish. Extract A was formed from the pooled mucus of 6 individuals, Extract B from 12 individuals, and Extract C from 30 individuals (Table 2). All extracts were buffered 1:1 with 10% (v/v) acetic acid in order to dissolve and preserve the hydrophobic antimicrobial peptides that consistently exhibit the most potent antimicrobial activity (Wei et al. 2010). Negative controls composed of sterile water were run in parallel. Extract B was boiled in a water bath for 5 min., and all extracts were cooled on ice.
Table 2: Description of epidermal mucus extract preparation; AA = 10% (v/v) acetic acid.

<table>
<thead>
<tr>
<th>Extract</th>
<th># of Samples Pooled</th>
<th>Buffer</th>
<th>Heated</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6</td>
<td>AA</td>
<td>Yes</td>
</tr>
<tr>
<td>B</td>
<td>12</td>
<td>AA</td>
<td>No</td>
</tr>
<tr>
<td>C</td>
<td>30</td>
<td>AA</td>
<td>No</td>
</tr>
</tbody>
</table>

Protein purification:

The acidified mucus extracts were prepared as described by Subramanian et al. (2008) with modifications. The extracts were centrifuged at 4,000 rpm for 5 min. at 4°C and the supernatants transferred to clean Eppendorf tubes. The hydrophobic proteins, documented as the most active antimicrobial compounds in previous studies (Ebran et al. 2000; Knouft et al. 2003; Subramanian et al. 2007), were extracted and partially purified using a reverse-phase Sep-Pak 360 mg C18 Classic cartridge (125 Å, 55-105 µm; Waters Corporation, Milford, MA, USA). A positive control sample consisting of 100 µg of the mating pheromone α-factor in 1:1 50mM NaCl and 10% acetic acid was run in parallel to determine recovery of protein and biological activity. The cartridges were activated with 8.5 mL methanol and equilibrated with 8.5 mL 10% (v/v) acetic acid. The extracts were loaded onto the cartridge and washed with 3.0 mL 0.1% (v/v) TFA. The proteins were eluted with 4.25 mL acetonitrile/water/TFA (80.0:19.9:0.1, v/v/v) mixture and eluates collected in clean Eppendorf tubes and lyophilized. The lyophilized mucus samples and negative control were resuspended in 100 µL 0.1% TFA, and the lyophilized α-factor in 50 µL sterile water. All materials were stored at -20°C until further use.
**SDS-PAGE:**

Aliquots of Extracts B and C were reserved to run on a peptide gel (Invitrogen™ Novex™ 10-20% Tricine Protein Gels) to detect presence and size of proteins extracted from the madtoms’ epidermal mucus following the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protocols developed by Schaegger and von Jagow (1987). Protein samples and both positive and negative controls (15 µL each) were mixed 1:1 with tricine SDS sample buffer (Novex™). Five microliters of molecular weight markers were diluted with 20 µL SDS buffer. Low and regular range molecular weight markers were run because size of hydrophobic proteins in Noturus spp. is unknown. Samples and markers plus buffer were loaded onto the peptide gel and run in tricine gels in a Life Technologies Bolt electrophoresis apparatus 1 h at 125 V constant voltage. The gel was fixed with 5% glutaraldehyde and stained in Coomasie Brilliant Blue (0.2% Coomassie Brilliant Blue R-250, 50% methanol, 10% acetic acid) and soaked overnight in destaining solution (10% methanol, 10% acetic acid, 2% glycerol).

**Microbial strains and culture procedures:**

The antimicrobial activity of the mucus extracts was tested against a range of pathogenic microorganisms that represent some major classifications of microbes and have been shown to be susceptible to fish epidermal mucus extracts in prior studies (Table 3): 

Saprolegnia parasitica was obtained from the ATCC, and S. ferax was isolated from diseased eggs of the Ashy Darter (Etheostoma cinereum) from CFI. Identity of the isolates was confirmed by extracting and sequencing DNA and running it through the Basic Local Alignment Search Tool (National Center for Biotechnology Information). Saprolegnia cultures were grown at room temperature on glucose peptone agar (3 g L⁻¹ sucrose, 1.25 g L⁻¹ peptone, and 15 g L⁻¹ agar) treated with three antibiotics (1 mL L⁻¹ Chloramphenicol, 1 mL L⁻¹ Ampicillin, and 10 mL L⁻¹ Streptomycin, AB-GPA) and subcultured by transferring plugs of mycelia every 7-10 days. Escherichia coli and S. epidermidis were grown at 37°C in 5.0 mL Luria-Bertani (LB) broth and brain-heart infusion (BHI) broth respectively. Candida albicans was grown at 30°C in 5.0 mL yeast extract peptone dextrose (YEPD) broth.

Table 3: Description of microbial strains used for assays.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli MG1655</td>
<td>Gram negative</td>
<td>-</td>
</tr>
<tr>
<td>Staphylococcus epidermidis ATCC 35984</td>
<td>Gram positive</td>
<td>ATCC</td>
</tr>
<tr>
<td>Candida albicans SC5314</td>
<td>Yeast</td>
<td>-</td>
</tr>
<tr>
<td>Saprolegnia ferax</td>
<td>Oomycete</td>
<td>Isolates from CFI</td>
</tr>
<tr>
<td>Saprolegnia parasitica ATCC 200013</td>
<td>Oomycete</td>
<td>ATCC</td>
</tr>
</tbody>
</table>
Assaying prepared mucus for antimicrobial activity:

The partially purified protein extracts from Extracts A, B, and C were thawed and assayed against the bacteria, yeast, and oomycete strains. LB agar Petri-plates were inoculated with 200 µL of diluted *E. coli* by spreading it over agar surface with sterile glass beads. BHI agar Petri-plates were inoculated with 200 µL of diluted *S. epidermidis* in the same manner. *Candida albicans* was diluted to a final concentration of 1 X 10⁶ cells mL⁻¹ and added to molten agar poured onto YEPD agar plates. Fresh AB-GPA Petri-plates were inoculated with plugs of mycelia from *S. parasitica* and *S. ferax* cultures. Twenty microliters of each extract were pipetted onto sterile paper discs and placed on the agar of each culture using sterile forceps. All assays were performed in duplicate. The cultures were incubated at their appropriate growing temperatures and observed periodically over the course of 24 h for antimicrobial activity.

Positive control α-factor assay:

To determine whether active proteins were recovered from the purification process the mating pheromone α-factor was run in parallel as a positive control. This protein binds to a specific cell surface receptor (Ste2p) on plasmids of *Saccharomyces cerevisiae* pBec2 and stimulates growth arrest. If the protein is recovered successfully, zones of clearances should form around the samples containing active α-factor. Cultures of *S. cerevisiae* pBec2 and *S. cerevisiae* p424 which lacks the receptor were grown overnight at 30°C in MLT broth, then washed, diluted to 1 X 10⁶ cells mL⁻¹, and plated in molten top agar poured onto selective minimal medium (MLT) agar plates. Sterile paper discs were spotted with 2 µL of 0.1% TFA (negative control) and purified α-factor and 10 µL of known active α-Factor and placed on the
inoculated plates. They were incubated at 30°C and monitored for formation of zones of clearance over the course of 48 h.

_Crude mucus assay:_

Three male Mountain Madtoms in breeding condition were removed from the water and rinsed with 3 mL sterile distilled water. After 2 min., Whatman Sterile Filter Paper (size) cut into 2 cm x 2 cm squares was pressed onto the epidermis of the fish until saturated with mucus. Four samples were taken per fish and applied to a AB-GPA plate near the growing colony edge of _S. parasitica_. Sterile filter paper was plated on a separate culture plate to serve as a negative control, and paper disks saturated with 10 µL Malachite Plus – a known fungicide effective against _S. parasitica_ – was applied to another culture plate as a positive control. Cultures were observed for fungistatic activity over the course of 24 h.

**Results**

_SDs-PAGE:_

Results of the SDS-PAGE showed that proteins approximately 10 kDa in size were recovered from the partially purified samples prepared from Extracts B and C (Figure 1). AMPs ranging from 10 to 16 kDa have been reported for epidermal mucus of other catfish species (Anbucezhian 2011; Robinette et al. 1998), which is consistent with our findings.
Figure 1: Electrophoresis of TFA solvent and partially-purified protein. Samples were applied to a tricine gel SDS-PAGE after mixing in sample buffer. LMW=low molecular weight markers; S₀=sample buffer; Neg=negative control (0.1% TFA); EC=Extract C; α=5 µL & 10 µL α-factor respectively; HMW=high molecular weight markers. Low molecular weight markers are 40, 25, 25, 10, 4.6, & 1.7 kDa. High molecular weight markers are 120, 72, 55, 43, 25, 17, & 10 kDa.

Prepared mucus assays:

The samples purified from Extract A, B, and C exhibited no antimicrobial activity. No zones of inhibition formed in any sample against any strain (Table 4). In the positive control assays, the active unpurified α-factor and the α-factor prepared alongside the mucus samples stimulated growth arrest and resulted in zones of clearance in S. cerevisiae pBec2 while the negative control (0.1% TFA) did not. No zones of clearance formed on the S. cerevisiae p424 cultures. These results are consistent with recovery of biologically active proteins. Thus, the
Table 4: Antimicrobial activity of assayed samples; - = no growth inhibition; AA = 3% (v/v) acetic acid; TFA = 0.1% (v/v) trifluoroacetic acid.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Strains Inhibited</th>
<th>Average Diameter of Zone of Inhibition (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract A in AA</td>
<td>S. ferax</td>
<td>2.2</td>
</tr>
<tr>
<td>Extract A in TFA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3% Acetic Acid</td>
<td>S. ferax</td>
<td>2.4</td>
</tr>
<tr>
<td>Prepared Extract B</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Prepared Extract C</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Prepared Extract D</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Negative Control in TFA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Crude Mucus</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Malachite Green</td>
<td>S. parasitica</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Hydrophobic peptides extracted from the fish epidermal mucus would be expected to retain any biological activity it might possess.

**Crude mucus assay:**

The crude mucus had no effect on *S. parasitica* growth. After 24 h, the colony had grown to the edges of the plates with no inhibition from the saturated filter paper. The Malachite Green control resulted in a halos of averaging 2.6 cm in diameter within 3 h. Fish epidermal mucus is constantly secreted from mucosal goblet cells in live fish, with the slime coat being
constantly replenished, and thus crude mucus removed from fish quickly loses its viability as well.

**Discussion**

The hypothesis that the epidermal mucus of *N. eleutherus* possesses antimicrobial properties was not supported. The failure to demonstrate antimicrobial activity in the proteinaceous extracts of the mucus may be attributed to a number of factors. There are many components of the innate immune system housed in the epidermal mucus of teleost fish including immunoglobins, lectins, proteases, lysoyzymes, and antimicrobial peptides (Esteban 2012). I chose to investigate the biological activity of antimicrobial peptides (AMPs) as they are an evolutionarily ancient and widely conserved class of proteins found among all branches of life that are capable of fending off infections from a wide range of bacteria, fungi, viruses, and protozoa (Zasloff 2002). Potent AMPs have been isolated and identified in teleost skin mucus from a number of species in the catfish order Siluriformes to which madtoms belong, including the Yellow Catfish (*Pelteobagrus fulvidraco*), the Engraved Catfish (*Nemapteryx caelata*), the Spotted Catfish (*Arius maculatus*), and the Channel Catfish (*Ictalurus punctatus*) (Anbuchezhian et al. 2011; Bragadeeswaran et al. 2011; Robinette et al. 1998; Su 2011). There is a possibility however that AMPs are not a particularly active component of the epidermal mucus of *Noturus* species.

While many prior studies have determined that crude mucus and mucus in aqueous solutions do not possess antimicrobial properties (Arulvasu et al. 2012; Hellio et al. 2002; Wei et al. 2010), some studies have shown that the hydrophilic components retained by these
preparations do indeed inhibit microbial growth (Ebran et al. 2000; Knouft et al. 2003; Subramanian et al. 2007). Thus, results from mucus assays appear highly contradictory, even within the same species and extraction protocols (Subramanian et al. 2008). This may be due to the external and internal parameters that influence the innate immune system and mucus production (Magnadóttir 2006). Temperature, stress, diet, genetics, infection, and handling can all suppress or enhance immune expression in epidermal mucus (Alcorn et al. 2002; Beck and Peatman 2015; Duncan and Kelsius 1996; Garbi 1996). Variables involved in the rearing and handling of the Mountain Madtoms from which mucus was harvested in this study may have suppressed the expression of any antimicrobial compounds. Incubation temperature or pH of the assays may have inactivated any AMPs present, or, while the results of the SDS-PAGE would indicate that AMPs were successfully recovered, the amount may have fallen below the minimum inhibitory concentration necessary for detectable levels of activity.

Only 5 strains of 4 species of pathogens were used to screen for antimicrobial activity in this study. While AMPs are known to be effective against a wide range of pathogenic bacteria and fungi, there appears to be a high level of selectivity of specific AMPs for certain strains (Hellio et al. 2002; Kumari et al. 2007). Perhaps, the AMPs extracted from the Mountain Madtoms may not have been effective against the particular strains tested here but would be against others. Additionally, Eban et al. (1999) demonstrated that the bacterial flora living in fish epidermal mucus was capable of disrupting the growth of certain pathogens of those fishes. The field of commensal microbiota in fish mucosal immunity is garnering much attention and expanding rapidly as researchers work to understand the significant role it plays in fish health (Gomez et al. 2013). A symbiosis between madtoms and their slime microbiome may
contribute to their immunity; this symbiosis may also provide immunity to infectious microbes in the eggs they care for.

Furthermore, in some species that practice antimicrobial parental care, the antimicrobial benefit is not a function of the epidermal mucus but rather of specialized glands that secrete compounds with disease-preventing qualities (Giacomello et al. 2006; Little et al. 2008). Investigation into the physiology of breeding male madtoms might reveal a similar phenomenon in a species that has already evolved at least one unique type of specialized gland: the venom glands at the base of their spines. The egg-mouthing behavior witnessed in most species of *Noturus* (Burr and Stoeckel 1999) might also play a role in preventing microbial infection of clutches.

In conclusion, further research is necessary to determine the presence of antimicrobial substances in *Noturus* epidermal mucus and the role it plays, if any, in the protection of eggs as well as in its own defense against pathogens. Assaying the hydrophilic components, expanding the variety of pathogens tested against, culturing and/or sequencing of the epidermal microbiome, and exploring other physiological elements of breeding male madtoms are all possible directions to go in search of how madtom guardian males are able to prevent disease in eggs.
PART II: Size-specific Advantage in Habitat Competition between Mountain Madtoms

(Noturus eleutherus) and Invasive Crayfish (Orconectes spp.)
Abstract

Nonindigenous crayfish have been implicated in the widespread decline of native fish populations where they have invaded. For example, in Little Chucky Creek, a tributary to the Nolichucky River in Tennessee, the population decline of the Chucky Madtom (Noturus crypticus) to the point of extirpation or even extinction has been partially attributed to the establishment of two nonnative crayfishes, the Kentucky River Crayfish (Orconectes juvenilis) and the Virile Crayfish (O. virilis). While it has been suggested that the crayfish exclude the cavity-dwelling fish from shelter, no studies have been conducted to demonstrate that crayfish directly outcompete madtoms for cover habitat. The objective of this study was to experimentally test the hypothesis that invasive crayfish competitively exclude the Mountain Madtom (N. eleutherus), a surrogate species for the Chucky Madtom, in a laboratory environment. Behavioral trials were conducted where shelter was the limited resource for the two potential competitors. The experiment was designed to test for the effect of crayfish density, territorial establishment, relative size difference, and length of exposure on a madtom’s ability to occupy the cover object. For acute trials (48-h duration), effects of crayfish density and territory establishment were not significant (Fisher’s Exact Test, $P=0.37$ and $P=0.24$, respectively), but average size difference significantly affected madtom occupancy of the shelter habitat (binomial logistic regression, $P=0.01$). Four chronic trials lasting 5 days were conducted on size-sorted treatments in which the madtom was larger than the crayfish or the crayfish larger than the madtom. Madtom occupancy and health (i.e. crayfish-induced disease, lesions, or death), were significantly affected by relative size ($P<0.001$ both), and mortality was 100% for juvenile madtoms. I concluded that the invasive Kentucky River and Virile crayfishes
exhibit a size-specific competitive advantage over madtoms when resources are limited, and that nonindigenous crayfish species can catalyze fish population declines at least partially through habitat exclusion and predation on juveniles.
Chapter 4: Ecological Impacts of Invasive Crayfish

Few organisms are as destructive to aquatic ecosystems as those crayfish species that become established outside of their native range. Hobbs et al. (1989) stated notably that “once an ‘exotic’ crayfish is introduced into a lake or stream, it may impose considerable environmental stress on the system and, in all too many instances, irreparable shifts in species diversity occur.” Currently, the world is undergoing a massive shift in crayfish distribution, with North American species having been translocated intentionally for commercial culture or accidentally via bait bucket introductions onto every continent on the globe except Antarctica and Australia (Holdich 1999). Crayfish are large, highly aggressive macroinvertebrates whose omnivorous, opportunistic life strategies enable them to dominate the benthos of the water bodies they invade where effective predators are lacking, allowing them to outcompete naïve native creatures who are unable to withstand their onslaught (Freeman et al. 2010; Gherardi 2007). The ecological impacts of alien crayfish are vast, spanning across multiple trophic levels and altering a stream at the population, community, and ecosystem levels (Gherardi 2007). Nicknamed “ecosystem engineers,” crayfish can destabilize riverbanks, alter detrital processing rates, increase suspended sediments, and decrease light penetration by their grazing, walking, and burrowing activities (Anastácio and Marques 1996; Creed and Reed 2004). They have been implicated in significant macrophyte reduction (Magnuson et al. 1975), the decimation of macroinvertebrate communities (Charlebois and Lamberti 1996; Stenroth and Nyström 2003), and the extirpation of native crayfishes, amphibians, and fishes (Twardochleb et al. 2013).

Benthic fishes are especially vulnerable to the detrimental impacts of invasions as they share significant life history traits with crayfish. Many feed nocturnally, prey on benthic
invertebrates, and require cavities under rocks for shelter, resulting in great potential for interspecific interactions (Guan and Wiles 1997; Holdich 1999; Momot 1995; Rahel and Stein 1988). When habitat becomes a limited resource, nonindigenous crayfish, who have been shown to be more aggressive than their native counterparts (Gherardi and Cioni 2004; Pintor et al. 2008), exclude small benthic fishes from shelter and render them more susceptible to predation (Guan and Wiles 1997; Light 2005; Rahel and Stein 1988). In addition, crayfish are known to be voracious predators of fish at all life stages (Ribbens and Graham 2004; Taylor and Soucek 2010; Twardochleb et al. 2013). Although crayfish are considered omnivores, they have been experimentally shown to prefer animal protein in the form of fish to the plant matter and detritus that also make up their diet (Ilhéu et al. 2007). As more crayfish introductions occur worldwide, understanding and quantifying how they compete with native fishes is necessary for freshwater ecosystem management and conservation.

The need for understanding the impacts of crayfish introductions is critical to the management of aquatic organisms who have a high degree of endemism, as these species are at greater risk of extirpation and/or extinction. For example, invasive crayfish have specifically been cited as a factor in the decline and possible extinction of the Chucky Madtom (Noturus crypticus), a federally endangered species with an extremely restricted range (Kuhajda et al. 2016a). The Chucky Madtom has only been collected from two locations: a single specimen from Dunn Creek in 1940 and 13 individuals from Little Chucky Creek, a tributary to the Nolichucky River in Greene County, TN (Carter et al. 2005). No Chucky Madtoms have been found since 2004. Simultaneous with this apparent population crash, two species of crayfish
were recorded as having invaded Little Chucky Creek: Kentucky River Crayfish (*Orconectes juvenilis*) and Virile Crayfish (*O. virilis*) (Kuhajda et al. 2016a).

The Kentucky River Crayfish is native to the Upper Cumberland River and Kentucky River basins in Kentucky and southern Indiana (Longshaw and Stebbing 2016); the Virile Crayfish’s native range encompasses much of the Midwest, from Maine to Colorado, from Ontario, Canada south to Texas and includes most of the Mississippi River and its tributaries (Aiken 1965). However, both have been widely introduced via bait bucket releases across the U.S., and some populations are entrenched in Mexico, England, France, and the Netherlands (Ahern et al. 2008; Chucholl and Daudey 2008; Hobbs and Walton 1966; Lodge et al. 2000; Riegel 1959; Schwartz et al. 1963). Little is known about the ecology and life history of these crayfishes beyond the knowledge that they possess those qualities that make certain species effective invaders of new systems: rapid growth, extreme aggression, high fecundity, and a tolerance for a wide range of environmental parameters (Bovbjerg 1969; Lindqvist and Huner 1999; Schwartz et al. 1963). Where they have become established, they have proved problematic for the native fauna therein, causing the decline of fish populations, displacing fish from shelter, and even preying upon fish and fish eggs (Carpenter 2000; Dorn and Mittelbach 2004; Martinez 2012). Both crayfishes may achieve very large body sizes (Ahern et al. 2008), recorded up to 135 mm total length (TL) and 79 mm carapace length (CL), which enables them to dominate native crayfishes and other competitors.

The two *Orconectes* species in my study inhabit the same type of cavities beneath slab rocks necessary to the Chucky Madtom for shelter and spawning (Bovbjerg 1969; Taylor 2000), and they have been observed occupying artificial nest structures that were intended to increase
Chucky Madtom reproduction (pers. comm., J. R. Shute, CFI). Thus, it has been hypothesized that the two nonnative crayfishes outcompeted Chucky Madtoms for cover habitat and contributed to their decline. As a result, the recovery plan for the endangered fish calls for eradication of the crayfish (Kuhajda et al. 2016b). Eradicating exotic species is almost always an expensive and laborious endeavor with often dubious success, and since few laboratory studies have rigorously examined the direct competition between crayfish and benthic fishes for shelter, it is important to determine the effect these crayfish have on the madtoms to justify such a costly effort. The purpose of this study was to test this competitive interaction hypothesis in a laboratory setting as an inclusion-exclusion experiment with shelter habitat as the limited resource. In particular, I tested whether crayfish density, prior territory establishment, relative size difference, and length of exposure affected the ability of madtoms to take and maintain shelter. Because the decline of Chucky Madtoms in Little Chucky Creek coincided with the invasion of the two nonnative crayfishes, I hypothesized that the madtom would suffer increasing exclusion as crayfish density and relative size increased, when crayfish colonized the cover habitat first, and as the length of the competition phase increased.
Chapter 5: Habitat Competition

Methods

Natural densities:

In order to reflect the current natural conditions of crayfish competition in the laboratory, the naturally occurring density of crayfish in Little Chucky Creek was measured. Three reaches at two sites were selected based on historical records of Chucky Madtom occurrence, habitat suitability, and site accessibility (Figure 2). Reach 1 stretched 100 m upstream from the Bible Covered Bridge crossing 11.2 km SW of Mosheim, TN in Greene County (N 36.12439; W 83.05320). Reaches 2 and 3 were consecutive as accessibility to appropriate sites was limited. Reach 2 ranged 100 m downstream from a footbridge crossing 9.8 km SW of Mosheim, TN in Greene County (N 36.11773; W 83.02231), and Reach 3 stretched 100 m upstream from the same point. Each 100-m reach was divided into 10-m transects. Each transect was 1 m wide and ran perpendicular to the stream, and each transect further subdivided into rows of 0.5-m squares running bank to bank. A random number generator was used to select 10 quadrats to sample in the reaches. A 0.5 m² quadrat crayfish sampler was created with PVC pipe wrapped in hardwire cloth 0.5 m high to prevent crayfish escaping and placed on each randomly selected square. All the crayfish observed within the sampler were counted. The crayfish that were captured with dipnets were measured and identified to species. Water depth was recorded, and flow and habitat qualitatively described. Natural density was calculated with the following formula:

\[
\text{Crayfish density (number / m}^2\text{)} = 0.5 \times \frac{N_i}{Q_i},
\]
Where $N_i$ is the number of crayfish observed in reach $i$, and $Q_i$ is the number of quadrats sampled in reach $i$.

![Figure 2](image)

*Figure 2: Area of Little Chucky Creek where Chucky Madtoms have historically occurred. Marker 1 indicates the first reach; marker 2 indicates the second and third reaches.*

Of the 10 quadrats sampled in Reach 1, only the 4 that contained habitat suitable for crayfish or madtoms were analyzed. The other 6 sampled contained bedrock in pools, which would not harbor either crayfish or madtoms, and thus including them in analyses would not yield an accurate result of naturally occurring densities. To account for this issue, in Reaches 2 and 3, when the randomly selected quadrat did not contain habitat in which crayfish or madtoms would reasonably occur (e.g. deep pools or no cover), it was by-passed for the next randomly assigned quadrat until 10 total measurements were reached. The natural density of crayfish in Little Chucky Creek was 2.17 crayfish m$^{-2}$ (95% C.I. ± 0.89). The average size collected was approximately 23.0 mm CL and 45.4 mm TL. All crayfish species observed were *Orconectes* spp., and all species captured were either *O. virilis* or *O. forceps*, the Surgeon Crayfish. The
other documented invasive, *Orconectes juvenilis*, was seen in the area, but none were captured within the quadrat. This indicates that there may have been a sampling bias towards certain species. In addition, many crayfish - particularly the largest individuals - were highly sensitive to the disturbance caused wading in the creek. Many crayfish were observed fleeing the area before the quadrat could be placed on the creek bottom. They were also able to escape the quadrat in cobble substrate areas, and some presumably escaped beneath rocks, unseen. Therefore, the actual density and average size are likely higher and the species composition somewhat different from what is reported here.

The area of each aquarium used in the behavior trials was approximately 0.2 m. The proportional crayfish density using the above results amounts to 0.4 crayfish per aquaria. Due to the likelihood that the observed natural density underrepresents the true natural density, I used 1 crayfish per tank as the low density treatment and 3 as the high.

*Animal conditioning:*

Mountain Madtoms (*N. eleutherus*) were selected as the surrogate species for the Chucky Madtom because they are native to the same watershed and have a similar life history (Burr et al. 2005; Starnes and Starnes 1985). Mountain Madtoms were collected by backpack electroshocking and seining from the French Broad River 2.9 km NE of Newport in Cocke County, TN (N 35.98268; W 83.16161) and 5.1 km E of Marbledale in Knox County, TN (N 35.95826; W 83.76248). The madtoms were housed in a recirculating aquaculture system on a long-term basis at CFI in Knoxville, TN in 190-L aquaria following the same protocols as described in Part I. The total lengths of the fish ranged from 40 mm to 89 mm.
Kentucky River Crayfish and Virile Crayfish were collected by backpack electroshocking and D-frame dipnetting from Little Chucky Creek 11.2 km SW of Mosheim, TN in Greene County (N 36.12439; W 83.05320) and Bent Creek, also a tributary to the Nolichucky River, 7.6 km SxSE of Russellville, TN in Hamblen County (N 36.19883; W 83.15488). Due to the similar morphology of the native Surgeon Crayfish (*O. forceps*) and the difficulty in keying females and Form II male crayfish to species, this native may also have been inadvertently collected and used in behavioral trials in addition to the two invasives. The crayfish were maintained in a flow-through aquaculture system using dechlorinated municipal water at the Johnson Animal Research and Teaching Unit (JARTU) facility at the University of Tennessee in a 300-L tank oxygenated with bubbling air stones and fed frozen blood worms and commercial fish pellets *ad libitum*. Light regime was controlled daily by an automatic timer set to switch on at 0700 h and off at 1900 h each day. Crayfish lengths (TL) ranged from 23 mm to 94 mm. All organisms were acclimated to captivity for at least one week before being used in experimental trials, and no individual was used for more than one trial to ensure independence of replication.

*Acute experiment:*

The acute experiment was designed to test the immediate impact invasive crayfish would have on the ability of the test madtoms to take cover under a shelter object under varying crayfish densities, size differences, and prior establishment of the cover object. A series of 9 trials consisting of 6 independent replicates were run. Each replicate occurred in a single 75-L glass aquarium equipped with a bubbling air stone, a layer of sand-gravel substrate, and screens covering the top to prevent escape (Appendix F). Animals were fed 24 h prior to trials
to minimize foraging, but not fed during experimental trials to minimize confounding effects of
competition for food. At the start of a trial, an opaque plastic divider was placed in the middle
of each tank. A randomly selected madtom was introduced into one side of the tank, and either
1, 2, or 3 crayfish into the other. Species were kept separate for a 24-h acclimation period in the
experimental aquarium.

To determine the effect of crayfish density, 3 trials each were conducted with 1, 2, or 3
crayfish per tank. Control trials were conducted with each tank containing only a cover object
and either a single madtom or a single crayfish. The control trials were carried out to ensure
that individual madtoms or crayfish would utilize the cover object in the absence of
competition.

The recovery plan for Chucky Madtoms recommends propagation as a recovery method
should an extant population be rediscovered (Kuhajda 2016b). My study assessed whether
reintroduction of an endangered madtom species would be a viable option in a stream already
heavily infested with invasive crayfish by determining the ability of a congener to outcompete
crayfish already established in their preferred shelter habitat. Each tank was randomly assigned
to one of three treatments to determine the effect of territorial “establishment.” Establishment
is defined here as the habitation of an available cover object at the start of the 24-h acclimation
period to either 1) the madtom, 2) the crayfish, or 3) neither. The cover object was a piece of
slate tile measuring 200 mm x 200 mm, propped up on one side to height of 9 mm with a small
piece of plastic. These are approximately the dimensions of preferred cover rocks by Chucky
Madtoms and Mountain Madtoms (pers. comm., P. Rakes, CFI). In each trial, there were 2
replicates in which the madtom was given the cover object first, 2 in which the crayfish was
given the cover object first, and 2 in which neither were given the cover object during the
acclimation period.

The dividers were removed at the end of the 24-h acclimation period, and the
competition phase began. In those replicates where neither species was given a cover object,
one was placed in the tank at this time. The madtoms and crayfish were allowed to move freely
about the tank and interact with each other. After 24 h and 48 h from the start of the
competition phase, the position of the madtom was recorded as being either “out” (i.e., not
under the tile) or “under” (i.e., under the tile). A madtom was considered successful if it was
under the tile. To minimize the effect human activity might have on the experimental animals, a
tarp was hung in front of the experimental tanks, and data was recorded quickly and discreetly.
All records were taken during the daytime to avoid the effects of nocturnal foraging behavior
exhibited by both madtoms and crayfish. At the end of the 48-h competition phase, the total
lengths of all test animals were measured, and size difference between the madtom and
crayfish in each replicate was recorded ([total length of madtom] – [total length of crayfish]).
When multiple crayfish were present in a replicate, the average size difference was used. A
negative value represents the madtom being smaller than the crayfish on average.

Fisher’s Exact Test was also run on a contingency table formed of the counts of the
madtom “under” or “out” during the 24-h data and the 48-h data to ensure there was not a
significant effect of time. There was a slight increase in the frequency of madtoms occurring
under shelter (Figure 3), but this was not significant ($P =0.14$). Thus, the following statistical
analyses were performed on the competition phase data as a whole.
The effect of average size difference on the percent frequency the madtom was “under” was analyzed by binomial logistic regression. Contingency tables were created for the variables of density and establishment treatment, and Fisher’s Exact Test was used to determine significance of each variable. All analyses were carried out using R Studio v. 0.99.491.

_Chronic experiment:_

The chronic experiment tested how the madtoms’ ability to maintain territory under a cover object changed with long-term exposure to invasive crayfish competition. Four trials with 6 independent replicates each were run in aquaria as described in the acute experiment. Each replicate contained one madtom and two crayfish. Half of the replicates were designed with each crayfish larger than the madtom, and half with each crayfish smaller than the madtom. After a 24-h acclimation period in which crayfish and madtom were separated by dividers, a cover object was placed into each tank, and the madtoms’ position relative to the cover object was recorded every 24 h for 5 days. Two controls of a single madtom and a cover object were run in parallel. At the end of each trial, the total lengths of crayfish and fish were measured, and the average size difference between crayfish and madtom in each replicate calculated. A negative value represented when the madtom was smaller than the crayfish in a tank with it. The condition of each fish was assessed and scored on a scale of 1 to 5 (Table 5).

The effect of average size difference on the frequency a madtom was found under the cover object and on its health score were analyzed separately using analysis of variance (ANOVA). To determine whether the data fit the assumption of normality, a Shapiro-Wilk test
was performed and quantile-quantile plots created for each variable. All analyses were carried out in R Studio v. 0.99.491.

Table 5: Description of health scores assigned to each madtom at the end of each chronic trial.

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dead</td>
</tr>
<tr>
<td>2</td>
<td>Injury on all fins; heavily diseased; behavior abnormal</td>
</tr>
<tr>
<td>3</td>
<td>Injury on more than two fins; mild sign of disease; behavior normal</td>
</tr>
<tr>
<td>4</td>
<td>Injury on one to two fins; behavior normal</td>
</tr>
<tr>
<td>5</td>
<td>Healthy</td>
</tr>
</tbody>
</table>

Results

Controls:

All control trials yielded 100% occupancy of the cover object for both the acute and chronic trials. This demonstrates that in the absence of competition, madtoms and crayfish will occupy the cavity beneath the tile. Observations taken after dark revealed that both crayfish and madtoms emerge from cover at night to forage for food, but activity is at a minimum when the lights are on, and both species appear to seek shelter during the day. For this reason, l
determined that single daily observation was representative of the occupancy of the cover object throughout the daytime, when shelter is most necessary and desired.

**Acute trials:**

Rates of occupancy for the madtom were very high in every acute trial (Table 6). In the face of increasing crayfish densities, the frequency of the madtom under the cover object dropped slightly (Figure 4), but not significantly ($P=0.37$). The most common observation was co-habitation of the madtom and a single crayfish, sometimes two. When an animal was excluded from the cover object, it was almost always another crayfish, not the fish. In addition, crayfish densities of 3 often resulted in crayfish mortality.

While those treatments when the madtom established its territory under the cover object during the acclimation phase had the highest occupancy rates after the competition phase (Figure 5), there was still no signification relationship between this variable and the frequency of madtom occupancy ($P=0.24$).

**Average size difference** was the only variable that had a significant effect on madtom occupancy ($P=0.01$). Madtom size relative to the crayfish in each replicate was positively correlated with the frequency with which the madtom was observed under the shelter (Figure 6). As crayfish total length increased, madtoms were less successful at competing for the cover object. In 3 cases, small, juvenile madtoms less than 55 mm total length were killed and consumed by the much larger crayfish in the tank with it.
Figure 3: Madtom occupancy of cover object 24 h & 48 h after the start of the competition phase.

Table 6: The frequency of observation when the madtom was successful at taking shelter under the cover object ("under") and when it was not ("out") for: (1) crayfish density and (2) establishment of cover.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Under</th>
<th>Out</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Crayfish (Control)</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>1 Crayfish</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>2 Crayfish</td>
<td>26</td>
<td>10</td>
</tr>
<tr>
<td>3 Crayfish</td>
<td>23</td>
<td>7</td>
</tr>
<tr>
<td>Madtom Establishes</td>
<td>28</td>
<td>4</td>
</tr>
<tr>
<td>Crayfish Establishes</td>
<td>21</td>
<td>9</td>
</tr>
<tr>
<td>Neither Establish</td>
<td>26</td>
<td>8</td>
</tr>
</tbody>
</table>
Figure 4: Madtom occupancy under cover object by increasing density of crayfish (P=0.37).

Figure 5: Madtom occupancy under cover object by prior territory establishment (P=0.24).
Figure 6: Percent occupancy of shelter given average size difference between madtom and crayfish; negative values indicate the madtom is smaller than the crayfish ($P=0.01; r^2=0.1$).

Chronic experiment:

When madtoms had to compete for a cover object for 5 days, the overall success of the fish fell drastically (Figure 7). The percent frequency of madtom success was significantly lower ($P=0.004$) after the chronic trials than during the acute trials, implying that there is a long-term effect of invasive crayfish exposure when shelter habitat is limited. For this experiment, crayfish and madtoms were not randomly selected, but were selected for each replicate based on size. Madtom occupancy was positively correlated with madtom size relative to the crayfish ($P=0.001$; Figure 8). As crayfish became increasingly larger than the madtom, the madtom was
unable to gain access to the cavity under the cover object as frequently as when it was larger than both crayfish. Madtom juveniles (TL < 55 mm) were especially outcompeted by large crayfish.

In addition to habitat occupancy, the fitness of the fish was significantly affected by interactions with the invasive crayfishes. Madtom health was inversely correlated with the average size difference ($P= 0.001$; Figure 9). Crayfish were seen grabbing at the fish and tearing their fins with their claws, and often the stress of avoiding crayfish and/or habitat exclusion resulted in an outbreak of *Ichthyophthirius multifiliis* (ich), a common parasitic protozoan that colonizes immunocompromised fish. On several occasions, crayfish competition was lethal. While the majority of the time a madtom was discovered to have died and been consumed during the course of a trial, the death was unobserved. But there was one occasion when a live madtom was witnessed being captured, killed, and eaten by a large crayfish. Thus, there is evidence that madtom mortalities were not a result of independent factors but a direct result of crayfish predation. Extended exposure and the resulting reduced fitness may have enabled crayfish to prey on the fish more easily than they could have on healthy ones.

**Discussion**

These results support the hypothesis that large invasive crayfish will outcompete smaller Mountain Madtoms from limited shelter habitat, especially over an extended amount of time. However, increasing crayfish density and territory establishment did not result in a significantly higher rate of madtom exclusion as predicted. While large adult madtoms were overwhelmingly successful at outcompeting crayfish for shelter over a short period of time,
Figure 7: Madtom occupancy of cover object after 48 h and after 5 d of competition with invasive crayfishes \((P=0.0004)\).

Figure 8: Madtom occupancy of cover object as average relative size difference increases \((P<0.001; r^2=0.43)\).
chronic exposure to large crayfish impacted the ability of the madtom to take shelter, decreased the overall fitness of the fish, and at times, resulted in death.

In the acute experiment, the single significant factor was relative size. When the average size of the crayfish was larger than the total length of the madtom, the madtom suffered from habitat exclusion. But when the madtom had the size advantage, it had a very high success rate. When size difference was specifically manipulated in the chronic experiment, the effect became even clearer. This is consistent with the findings of many ecological studies suggesting that competition in general often boils down to the rule “bigger is better” (Kingsolver and Raymond 2008) and supports the common theme of size-specific competitive ability in several crayfish competition studies (Figler et al. 1999; Rabeni 1985; Vorburger and Ribi 1999). While several species of native crayfish occur in Little Chucky Creek, both *O. virilis* and *O. juvenilis* are noted.
for the relatively large body size they may attain, which would exacerbate competitive interactions with madtoms assuming cover habitat is limited.

Habitat exclusion would have a profound impact on madtoms in a natural setting. Prior studies have documented a decrease in growth rate and an increase in the mortality of cavity-dwelling fish who have been evicted from shelter (Bishop et al. 2008; Light 2005; Rahel and Stein 1988). Thus, an exposed madtom is vulnerable to predation by larger, sight-feeding piscivorous fishes e.g. black basses (*Micropterus* spp.) (Emmett and Cochran 2010). Mortality can be expected to increase where invasive crayfish exclude madtoms from critical refugia.

Going beyond habitat exclusion, the overall health and fitness of madtoms significantly decreased after long-term exposure to large crayfish (Fig. 8). Madtoms were observed being actively pursued around the tank by crayfish. At the end of the competition period, many fish had multiple injuries on their fins consistent with aggressive interactions from crayfish. In addition, several madtoms were heavily infested with ich, a disease known to affect fish immunocompromised from stress (Fairfield 2000). Constant agonistic interactions with crayfish, extended periods of pursuit, and habitat exclusion are all factors that would induce a stress response from madtoms. Diseased or injured fish cannot expend as much energy on feeding or reproducing, and are more susceptible to predation (Light 2005). In fact, large crayfish actively preyed on small fish in this study, and not a single madtom under 50 mm TL survived a 5-day trial. Because nonindigenous crayfish such as *O. juvenilis* and *O. virilis* exhibit greater agonistic tendencies than their native counterparts (Gherardi and Cioni 2004; Pintor et al. 2008), it stands to reason that Chucky Madtoms would have suffered from more negative interactions with the invasives, resulting in compromised fitness and a greater vulnerability to predation,
were access to refugia limited due to an increase in overall crayfish abundance. This also indicates that one mechanism by which invasive crayfish could have initiated or exacerbated the decline of the Chucky Madtom could be through the elimination of juveniles and thus recruitment.

Kuhlmann (2016) found that rising overall crayfish density, regardless of status as nonnative or native species, had a significant effect on stream ecology, and my study did reveal a noticeable, albeit insignificant, negative correlation between crayfish density and madtom occupancy (Figure 4). Interestingly, the most common observation was the madtom cohabitating the cavity under the cover object with one or two crayfish, rather than total exclusion. This is presumably not a natural occurrence, based on reports that crayfish and madtoms rarely share shelter spaces (Dinkins; 1984; Guan and Wiles 1997). Cohabitation in this study is likely due to the very limited nature of shelter in the experimental setting, and hence a last resort for both organisms.

When densities reached 2 and above, crayfish were most often excluded from shelter by other crayfish. On a number of occasions, crayfish were killed by other crayfish, thereby reducing the functional density in that particular replicate. The highly aggressive predilection of the crayfish may have increased negative intraspecific interactions, resulting in larger effects of density on the crayfish rather than the relatively placid fish and impairing the ability to detect significance on interspecific competition.

Where establishment was concerned, madtoms did have high occupancy rates when they were allowed to establish territory underneath the tile prior to crayfish exposure. Occupancy dropped when neither animal was allowed to establish, and fell even more when
crayfish colonized the cover object first (Figure 5). While this pattern is consistent with the original prediction, establishment did not have a significant effect. Madtoms and crayfish were both observed to vacate the cavity after dark to forage just as they would in a natural environment, in a way resetting the establishment factor on a diel cycle. Because madtoms were able to regain access to the cavity under the tile by daylight in nearly every trial regardless of treatment, establishment does not appear to play a role in invasive crayfish competition. Therefore, prior establishment of invasive crayfish in Little Chucky Creek alone would likely not be a hindrance to madtom stocking success in Little Chucky Creek, or in other areas where crayfish have caused the decline or extirpation of madtom populations. However, stocking size of propagated Chucky Madtoms may need to be of a sufficient length to prevent predation and stress-related habitat exclusion by larger-bodied crayfish.

Overall, this study demonstrates that the large-bodied invasive Kentucky River and Virile crayfishes have a detrimental impact on madtoms when habitat availability is limited. Through habitat exclusion, fitness reduction, and predation, the crayfish were able to outcompete small and juvenile fish for shelter. Madtoms are dependent on cover for protection and reproduction, so in a natural setting, such habitat exclusion would likely result in a population decline. Yet from the natural densities of invasive crayfish in Little Chucky Creek reported here, encounters with large invasive crayfish alone may not be common enough to explain the extirpation of the Chucky Madtom. Better sampling methods for the abundance and composition of crayfish species is needed before determining whether the conditions present in the laboratory accurately reflect those in the wild.
Other considerations not tested here should be taken into account when implicating nonindigenous crayfish as a factor in the disappearance of the Chucky Madtom or other madtom species, particularly the well-documented predation of crayfish on benthic fish eggs and larvae (Dorn and Wojdak 2004; Holdich 1999; Rahel 1989). The reduction of egg and larval survival would seriously cripple such an extremely endemic species with relatively low fecundity. Although investigating the potential of Chucky Madtom egg consumption by Kentucky River Crayfish and Virile Crayfish is not currently feasible, a study using a surrogate species as performed here would benefit management efforts regarding invasive crayfish. Furthermore, there are many indirect mechanisms through which the introduction of the crayfish may have impacted the Chucky Madtom, including an increase in stream turbidity, reduction of the benthic aquatic insects the madtom relies upon for food, or a complete shift in the trophic organization of the stream through macrophyte elimination and increased detrital processing rates (Freeman et al. 2010; Twardochleb et al. 2013). Lastly, it is important to recognize that abiotic factors e.g. declining water quality and agricultural runoff may have catalyzed the madtom’s population crash, and the crayfish simply filled a newly-vacated niche. Nonetheless, successful invasions of the Kentucky River and Virile crayfish in countless and varied regions on multiple continents and the biodiversity decline resulting therein, plus the outcomes of this study demonstrating their ability to outcompete and harm madtoms, indicate it is probable that the two crayfish species were a component in the Chucky Madtom’s decline. Moreover, other madtom populations currently – and some in the future most certainly will – face a similar fate if crayfish are allowed to spread unchecked. Complete eradication of
nonindigenous crayfish from Little Chucky Creek and other invaded streams is recommended for the recovery protocols of impacted madtom species.
Concluding Remarks

Madtoms as a clade are under more threats at this moment than at any other point in history. Anthropogenic activities in the form of irresponsible agricultural practices, human-induced climate change, riparian zone eradication, and introduction of invasive species like the crayfish discussed above have made much of the streams madtoms evolved and exist in nearly uninhabitable. Time is of the essence if we are to preserve the great biodiversity they represent. Only by deepening our understanding of their specialized adaptations to their unique life history as well as the natural and anthropogenic factors affecting their community dynamics can we hope to save them from extreme reduction or complete extinction. While it may be too late for the Chucky Madtom, recognizing the factors that sent it to the brink could be pivotal in applying recovery strategies to other species. I hope that this study has shed a little light on the plight that America’s miniature catfishes are facing, and will help guide future studies and management strategies to better protect these invaluable creatures.
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Appendices
Appendix A: Recirculating aquaculture system at CFI used to maintain and captively spawn the madtoms, front (left) and back (right).
Appendix B: Cover objects available for spawning habitat in each aquarium. Bottom photo shows the pot plate sealed with substrate by the breeding male.
Appendix C: Mountain Madtom egg clutch 1 consisted of 55 yellow, spherical eggs that adhered to one another in a single aggregation, with some grains of substrate attached. This clutch was isolated and incubated in an opaque plastic tray with a bubbling airstone and covered with an opaque black plastic cover to prevent light penetration.
Appendix D: Clutch 3 hours after spawning with male (left) and female (right) above. The parents consumed all the eggs within 24 h.
Appendix E: Clutch 4 with male guardian.
Appendix F: Experimental aquarium set up during acclimation phase.
Vita

Meredith Hayes Harris grew up in Murfreesboro, TN before moving the foothills of the Smokies to attend the University of Tennessee where she got her B.S. in Ecology and Evolutionary Biology in 2013. Fascinated by the amazing biodiversity of freshwater fish in East Tennessee mountain streams, she began volunteering then working for Conservation Fisheries, Inc. After a couple of years of working to propagate and restore rare and endangered fish, she began to pursue her M.S. in Fisheries Science and explore the ecology of the mysterious madtoms. She lives in Knoxville, TN with her husband Mark and their beloved dog Dixie.