# CONSERVATION OF AQUATIC BIODIVERSITY THROUGH MOLECULAR ECOLOGY: ADVANCEMENTS IN SPECIES IDENTIFICATION, HOST-PARASITE RELATIONSHIPS, AND GENETIC RESPONSE TO DISEASE

by

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(Under the Direction of John P. Wares)

ABSTRACT

Globally, aquatic biodiversity is in decline. Expanding demand for natural resources, habitat modification, and global climate change stress aquatic systems and as a result, many taxa are in decline. To slow the loss of biodiversity and to maintain current species richness, understanding how populations respond to environmental changes directly imposed by human activity is key. For this, *molecular ecology* is a useful tool and becoming the frontline in management. Using methods centered on information gathered through DNA- and RNA-based markers, the diversity and connectivity of populations – as well as inter-species dependencies – can be assessed. Additionally, by analyzing an individual's gene expression patterns, their response to environmental stressors can be determined.

I investigated imperiled taxa from two different aquatic systems. The first study investigates how freshwater mussels respond to human induced shifts in fish communities. Freshwater mussels require a fish host to complete their life cycle. Some mussels are generalists (using many different hosts) while others are specialists (using very few hosts). Identifying mussel larvae

(glochidia) embedded within the gills of their fish hosts is difficult. Thus, I developed a mussel specific genetic barcode for identifying glochidia in the presence of overwhelming host DNA. Using genetic barcodes, I identify glochidia attached to fish to determine what mussels are using each fish species as a host, if any fish were an ecologically important host, and the environmental factors that promote mussel larvae finding a host. I found that generalist mussels use native fish species indiscriminately, but shifts in fish communities through habitat modification can cause generalist mussels to primarily associate with fish species previously absent before habitat modification occurred. In addition to freshwater mussels, the response of corals to stressors was assessed. Reef-building corals are critically imperiled due to diseases induced by rising sea-surface temperatures. In the US Virgin Islands, some *Acropora palmata* colonies are less susceptible to disease than others. To determine if there is a heritable basis for disease resistance, I used RNA-Seq to assess how corals differentially respond to disease. I found significant variation in gene expression across corals, but no differential expression that correlates to disease resilience.

INDEX WORDS: Molecular Ecology, Unionid Freshwater Mussels, *Acropora palmata*, Genetic Barcode, RNA-Seq, Glochidia

# Conservation of Aquatic Biodiversity Through Molecular Ecology: Advancements in Species Identification, Host-parasite Relationships, and Genetic Response to Disease

by

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## DEDICATION

I would like to dedicate my dissertation to my advisors who were a fundamental aspect of my training and development. I am immensely appreciative of their guidance and moral support.

And

To the state of Florida. I know your kinda crazy, but exploring your dense biodiversity made an impression upon me that carries through today.

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# CHAPTER 1

## INTRODUCTION AND LITERATURE REVIEW

Aquatic ecosystems are strongly impacted by human activities (Dudgeon, Artington et al. 2006, Halpern, Walbridge et al. 2008). Biodiversity in aquatic environments is declining faster than biodiversity in terrestrial environments (Sala, Chapin et al. 2000). Freshwater mussels (Bivalvia: Unionoida: Unionidae) are one of the most biodiverse groups in North America. Freshwater mussels are ecosystem engineers and provide important services including water filtration and nutrient cycling (Vaughn 2010, Allen and Vaughn 2011), as well as providing habitat for other organisms (Mills and Reynolds 2003, Wisniewski, Bockrath et al. 2013). Freshwater mussels are also sensitive to environmental changes, thus are great indicators of ecosystem health. Many factors have contributed to the reduction of mussel biodiversity including overexploitation, flow modification, water pollution, habitat degradation, and species invasion (Dudgeon, Artington et al. 2006) and the combined effects of each.

Throughout the 1800's pearls found in freshwater mussels were highly sought after, but because very few mussels actually have pearls, large populations of mussels would crash due to extensive harvesting. After the pearl rush, freshwater mussels were collected to support the pearl button industry, where millions of mussels were turned into buttons (Anthony and Downing 2001). Around the same time, a huge effort was being made to channelize and impound many rivers to improve navigation, produce electricity, and to store water (Haag 2012). The new impoundments restricted water flow, either drying up whole stretches of habitat or drowning it. In areas where

mussels were not immediately killed, the impoundments restricted host fish migration. This prevented glochidia – the larvae of unionids that must transform on fish gills – from completing their life cycle and effectively dispersing. Alteration of waterways also removed important riparian buffers which increased the amount of sediment that eroded into rivers, smothering mussel beds.

In addition to altering physical habitat, pollution has severely impacted aquatic systems. For instance, zinc and lead mining almost completely eliminated mussel populations in Missouri, Kansas, and Oklahoma and the contamination from mining efforts continues to impact streams today (Angelo, Cringan et al. 2007) The button industry, impoundments, channelization, and the overall habitat degradation resulted in large losses in mussel population size and species diversity. The largest current threat to freshwater mussels continues to be habitat degradation, including impoundments. With approximately 300 species of freshwater mussel in North America, mussel species diversity remains high, yet over 70% of those are threatened, endangered, or extinct (Williams 1993). In order to better understand what management efforts are required to maintain current mussel populations and to promote the development of new ones, better understanding of the basics of mussel-fish interactions is key to bolstering the survival of new mussel recruits.

**Research not included in, but applicable to the formation of this dissertation:**

The goal for my dissertation is to become familiar with the many methods of molecular ecology used in the conservation of aquatic biodiversity. In addition to the questions addressed in my dissertation, I also used principles of population genetics to investigate population fragmentation, as well as utilized genetic barcoding to identify fish eggs found within the mantle tissue of mussels in natural populations. These findings not only expand our understanding of how current populations are influenced by human activities, they also expand our understanding of basic life history and species interactions.

*Population structure in freshwater mussels (Small, Bockrath et al. 2012; all authors contributed equally):*

Habitat fragmentation is a serious problem in conservation biology. It restricts gene flow between sexually reproducing populations, thus decreasing genetic diversity and inbreeding depression with each isolated populations. Along the Altamaha River, there is a power plant that uses water from the river to cool their system and the hot water is released back into the river, causing water temperatures and sediment load to be significantly higher than normal. Freshwater mussels are present upstream and down stream of this power plant, but the area around the power plant itself is absent of mussels. We wanted to know if each population, upstream and downstream, were isolated from one another. Using Cytochrome oxidase subunit I and microsatellite markers, we assessed the genetic variation within each population and the genetic variation shared between populations to assess the potential for genetic isolation. We found that freshwater mussels in the genus *Elliptio* have high genetic diversity and little evidence of being restricted by the presence of hot water. Because mussels use fish for migration, it is likely that

fish are moving mussels past the heated water to other locations, thus fish movement and distribution is likely just as responsible for the distribution of mussels as the presence of suitable habitat of for mussels(Rashleigh and DeAngelis 2007). Additionally, because mussels are long-lived species, the genetic diversity we observed in these populations may not reflect current situations and all evidence of genetic isolation may be hidden by historic gene flow and ancestral diversity that occurred before the power plant was built.

*Mutualistic relationships in shad and Elliptio (Wisniewski, Bockrath et al. 2013):*

Freshwater mussels have an obligate parasitic larval stage and require a fish host to complete their life cycle. Many studies have looked into the host requirements for mussel species of interest, but few have looked at the benefits fish may gain out of this host-parasite relationship. During field surveys, fish eggs with live embryos in various stages of development were observed within the mantle cavity of multiple freshwater mussels in multiple sites along the Altamaha River Drainage. Using a mitochondrial marker commonly used for fish identification, Cytochrome B, each egg collected was genetically identified as American shad (*Alosa sapidissima*). American shad, along with other *Alosa* species are anadromous fish. They spend their adult lives in the ocean but migrate up freshwater rivers to reproduce. When breeding, American shad prefer to lay their eggs in rocky substrates, prevent them from being swept downstream. Because these eggs were in various stages of development during collection, we believe the American shad are purposely spawning over mussel beds because of the substrate they provide. With this newly recognized potential mutualistic relationship between mussels and fish, new management practices, where the restoration of one group facilitates the restoration of the other.

*How allowing for anadromous fish migration improve mussel populations (In preparation):*

With the construction multiple impoundments across most rivers in the Southeastern United States, the movement of fish have been restricted. Anadromous fish live in the ocean but migrate up freshwater rivers to reproduce. The many impoundments on large rivers such as the Mississippi and the Apalachicola have prevented anadromous fishes from making their yearly migrations upstream. Because of this, their numbers have drastically reduced along with the freshwater mussels that use them as hosts. Since 2005, the Jim Woodruff Lock and Dam, on the Apalachicola River, has been opened during the annual Alabama shad (*Alosa alabamae*) run. As a result, their numbers have drastically improved. Here, we wanted to know what mussels were attaching to and being carried by Alabama shad to the upper reaches of the Apalachicola-Chattahoochee-Flint (ACF) River Basin. Using the genetic barcode developed in Chapter 2 of this dissertation (FORF), I identified the glochidia recovered from Alabama shad gills as *Elliptio crassidens*. Though it is common in the ACF, *E. crassidens* is locally endangered and nearly extirpated in the upper reaches of the Mississippi, where their known fish host – Alabama shad and skipjack herring – are also extirpated. With these findings, we have additional support that opening large impoundments to allow anadromous fish to migrate upstream not only improves their numbers, but would allow for the repopulation of locally extinct mussel species.

Here, I extend on my understanding of freshwater ecosystems and conservation genetics methods through utilization of eDNA principals for identification of mussel larvae, assessing species abundance and habitat preferences, using Next-generation sequencing to investigate genetic response to disease. Every method used in each of the following three chapters can be used in concert with one another in the conservation of aquatic biodiversity and my understanding of

each of these methods, in conjunction with my previous experience, has prepared me for a career as a conservation biologist with a focus in management.

**General Information on Unionid Freshwater Mussels:**

North America is home to the world's largest diversity of freshwater mussels, with nearly half of worlds species (Haag 2012). Freshwater mussels in the family Unionidae are subdivided into five tribes, Amblemini, Anodontini, Lampsilini, Pleurobemini, and Quadrulini. A single genus, *Gonidae*, falls outside of these tribes into its own group (Campbell, Serb et al. 2005, Graf and Cummings 2007). In North America, the most species rich tribe is Lampsilini with 22 genera, characterized by their elaborate means of fish host attraction (Zanatta and Murphy 2006). Freshwater mussels have an obligate parasitic larval stage, where larvae (glochidia) attach to a suitable fish host, metamorphosing from a glochidium to a juvenile, in order to complete their life cycle. Male mussels release their sperm in spherical packets called spermatozeugmata (Waller and Lasse 1997). Female mussels will take in the sperm while they siphon water and will fertilize eggs stored in their gills (Figure 1b). Females will brood their eggs in specialized gill pouches. Some mussel species utilize modified mantle tissue and gill pouches to resemble prey items, such as fish and bundles of eggs (Figure 1a). These modified tissue structures are designed to attract specific fish hosts (Zanatta and Murphy 2006, Haag 2012). When a fish recognizes these modified structures as prey, they attack the mussel, rupturing their gill pouches and releasing glochidia. Glochidia will then attach to the fish's gills (and sometimes fins) where they become encysted and complete metamorphosis. Not all mussels have modified structures to attract fish hosts. Some release clusters of glochidia and unfertilized eggs – called conglutinates – that resemble worms and insect larvae, while other mussels will simply release their glochidia

into strands of mucus (Haag 2012). Mussels who produce conglutinates are still attracting a specific type of host (e.g.: insectivorous vs. piscivorous fish), while mussels who release mucus strands aim to get their glochidia on any available fish (Haag and Warren 2003). Once metamorphosis is complete, juvenile mussels drop off their fish host, settle to the sediment, and continue to grow into adults. Because mussels are sessile as adults, they relay on their fish host for migration, thus the history of species distribution and diversification is closely tied to the movement and diversification of fish (Zanatta and Wilson 2011).

A)

B)

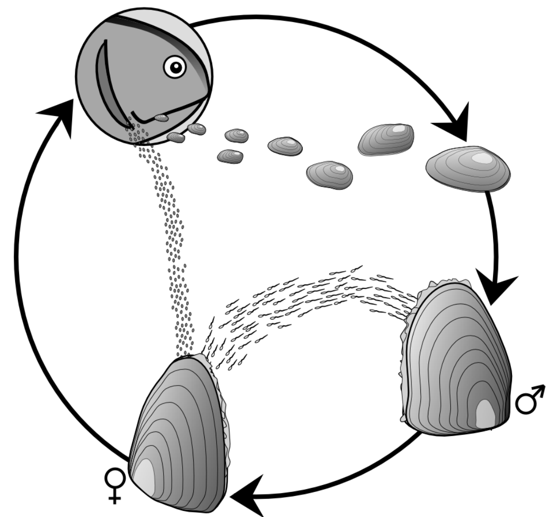


Figure 1.1: Description of freshwater mussel lifecycle and infestation strategy. Figure 1a shows how a freshwater mussel, *Lampsilis* species, attracts a bass using modified mantle tissue (Havasy 2013). Figure 1b depicts the complete freshwater mussel life cycle (Lindgren 2007).

Once settled, freshwater mussels grow quickly adding layers of nacre, or proteinaceous calcium carbonate, to their shells much like rings on a tree (Day 1984, Lutz and Clark II 1984). Though there is variation in the amount of nacre they deposit due to habitat and available nutrients, counting these rings allows for the approximation of a mussel's age (Day 1984, Lutz and Clark II 1984). Freshwater mussels have large variations in their lifespans with some species living as little as 3 years while others live over 150 years (ref). Mussels also have large variation in their shell morphology and size, some as small as a fingernail and others as large as your hand (Haag 2012). The large mussels tend to be in rivers and lakes, while the small mussels are generally located in streams and headwaters of river drainages. The species diversity of fish host and method used to attract them vary across mussels and are largely dependent on a mussel's general location. Mussels who live in smaller streams and headwaters are mostly specialists who utilize lures to attract fish hosts, while mussels who live in large rivers and lakes tend to be generalists and release their glochidia in mucus nets and strands. Mussels in smaller streams tend to be specialists because the abundance and biodiversity of fishes is less than those in large rivers. Because of this, mussels have a greater need to attract a fish to ensure that their glochidia make it to a suitable host. In large rivers, generalist mussels can afford to let their glochidia drift in the current because species abundance is much greater and they will have a decent chance of encountering a fish host (Vaughn and Taylor 2000, Rashleigh and DeAngelis 2007). With these two host strategies, mussels who use lures have specifically adapted toward attraction and metamorphosis on a specific fish host. In many cases, non-host fish will reject the glochidia of a host specialist. Host generalists, on the other hand, can apparently successfully metamorphose on a variety of host fish (Haag and Warren 2003).

**Background Information on Elkhorn Coral, *Acropora palmata*:**

The foundation of coral reefs is established primarily by large, stony corals (Scleractinia). These reef-building corals provided the rocky substrate for sponges and other coral species to attach to as well as habitat for countless organisms from every phylum. Historically, the dominant reef building coral in the Caribbean was *Acropora palmata* (Pandolfi 2002, Porter, Kosmynin et al. 2002) but due to rising sea surface temperatures and multitude of white syndrome diseases, *Acropora palmata* has severely been reducing by over 90% (Patterson 2002, Porter, Kosmynin et al. 2002). Without these important reef-building corals, key habitat for one of the most bio-diverse marine ecosystems on Earth is being decimated.

Corals are large, long-lived colonial organisms that have complex interactions with endosymbiotic algae (zooxanthellae) as well as the microbial community that lives within the mucus corals produce. Zooxanthellae can be free living, but mostly live within the tissue of coral, giving them their color. Zooxanthellae over produce glucose, supplying food to the coral, while the coral provides shelter for the zooxanthellae (Trench 1993, Rohwer and Youle 2010). There are multiple different groups of zooxanthellae, all varying in their sensitivity to light and water temperatures. Corals who live in deeper water will have a different zooxanthallae group from another coral that lives at the top of a reef in intense sunlight.

Acropora palmata reproduces sexually and asexually through fragmentation. When branches break off, they can resettle and continue to grow independent of their parent colony (Bak and Engel 1979). In some reefs at least 40% of colonies are a result of fragmentation (Lirman 2000). *A. palmata* also reproduces  through sexual reproduction. They synchronously release sperm and

egg into the water column in warm summer months (August) coinciding with the full moon (Kojis 1986). Distinctly isolated populations occur in east Caribbean and west Caribbean with west Caribbean populations exhibiting has more sexual reproduction than the east Caribbean (Baums 2005).

The largest current threat to reef-building corals is increasing climate change. Sea surface temperatures (SST) have been rising steadily over the last century (Huang et al. 2015) and the occurrence of WSD have risen along with it. When SST gets high, corals become very stressed and are more susceptible to disease (Harvell, Mitchell et al. 2002, Randall, Jordan-Garza et al. 2014, Randall and van Woesik 2015). Rising SST cause bleaching events, which leave corals weak and slow to recover. With increasing incidents of bleaching, corals are becoming less able to recover from previous bleaching events, growing weaker with every subsequent event (Szmant and Gassman 1990, Baird and Marshal 2002). Some zooxanthellae are more tolerant to high water temperatures and light intensity, but there is a significant trade off with using them. The thermo-tolerant zooxanthellae cause coral to grow more slowly than less thermo-tolerant zooxanthellae and because coral reefs are continually eroded, lags in coral grown would, over time, resulting in a loss of reef supporting corals (Ortiz, González-Rivero et al. 2013).

Through coral and freshwater mussels are very different phylogenetically and are not closely related, they are comparable in the role they serve within their ecosystems. Both are foundational species in their respective high-biodiversity ecosystems and provide important ecosystem functions that allow for biodiversity to be maintained. Because both coral and mussels are imperiled, a loss of either would critically impair ecosystem health and the maintenance of

biodiversity. The application of conservation genetics methods to both coral and mussels reflects my understanding that management and conservation of biodiversity requires understanding diverse ways of documenting and analyzing genetic variation, community interactions, and adaption to quickly changing environmental conditions.

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# CHAPTER 2

# GENETIC BARCODE-BASED SPECIES IDENTIFICATION OF FRESHWATER MUSSEL GLOCHIDIA RECOVERED FROM NATURALLY-INFESTED FISHES [1]

[1]Bockrath, K.D., N.A. Johnson, J.P. Wares. *To be submitted to Molecular Ecology Resources*

## Abstract

Freshwater mussels in North America are species rich and vastly diverse in morphology and habitat use. Due to habitat destruction, many mussel populations are in decline. Because mussels have an obligate parasitic larval stage, recruitment is dependent on host availability. Some mussel species have adapted methods of attracting fish hosts with lures and host use is best understood for these taxa. Conversely, there is little known about host use in mussel taxa that lack conspicuous lures. Genetic data provide insights into host use in mussel taxa that lack lures because genetic markers can be used to identify mussel glochidia on naturally infested fish. An ideal genetic marker would be unique to mussel genomes yet capable of distinguishing between mussel species. Here, we show that Female-specific Open Reading Frame (FORF) is an ideal genetic marker for mussel species identification on naturally infested fish and present a FORF barcode database specific to mussel taxa in the Apalachicola-Chattahoochee-Flint (ACF) and Altamaha River Basins. Representatives of all extant mussel taxa were collected from the ACF and, using tissue samples, a FORF genetic barcode database was generated. Using mussel glochidia recovered from naturally-infested Alabama shad, FORF was used to identify the mussel species.

## Introduction

Freshwater mussels in North America are species rich and vary in morphology and habitat use. Because of this diversity, the ecosystem functions that mussels provide – for example water filtration and nutrient cycling (Vaughn 2010, Allen and Vaughn 2011) - are likely to occur across freshwater systems. Due to habitat destruction, many mussel populations are in decline (Williams, Warren Jr. et al. 1993). To sustain these populations, mussel larvae (glochidia) must

successfully recruit to replenish populations as well as establish new populations. Because mussels have an obligate parasitic larval stage, recruitment is dependent on host availability and hosts may play a major role in glochidia dispersal. Some mussel species have developed methods of attracting fish hosts with lures: either modified mantel tissue, or packages of glochidia and unfertilized eggs (conglutinates), that resemble insects and worms. Mussels with lures attract specific groups of fish, many times specializing on one or a few closely related fish species. Other mussel species lack lures and release glochidia via mucus nets, where glochidia are free to attach to any fish they come into contact with. Because of this generalist approach, host use is poorly understood in these mussel taxa (Haag 2012).

Recently, genetic data have provided insights into fish host use in mussel taxa that lack lures by using genetic markers to identify mussel glochidia on infested fish hosts. The genetic markers typically used in these studies, such as mitochondrial cytochrome oxidase subunit I (COI) and ribosomal internal transcribed spacer (ITS), are found in all metazoans and certainly in both mussels and fish (Boyer, Howe et al. 2011, Zieritz, Gum et al. 2012). Because mussel glochidia encyst in fish tissue, the risk of contamination of isolated DNA from host tissue is high when removing glochidia attached to the gills or fins of hosts. An ideal genetic marker would be unique to mussel genomes yet capable of distinguishing between mussel species. Here, we show that Female-specific Open Reading Frame (FORF) is an ideal genetic marker for mussel species identification of mussel glochidia recovered from fish host tissue.

Mussels exhibit an interesting mode of mitochondrial inheritance. Typically, all metazoan offspring inherit mitochondria maternally, but in mussels, male offspring inherit mitochondria

both maternally and paternally, a phenomenon known as Double Uniparental Inheritance (DUI) (Zouros, Oberhouser-Ball et al. 1994). The mechanism behind DUI is still unresolved, but recently identified sex-specific open reading frames in the Control Region of the mitochondrial may be involved in facilitating DUI (Breton, Stewart et al. 2011). Under Double Uniparental Inheritance, all mussels will have the Female-specific open reading frame (FORF), while males will only harbor FORF in cells of the somatic tissue, and male-specific open reading frame in cells of gonadal tissue. Because Male-specific and Female-specific Open Reading Frame are so tightly associated with sex, these sex-specific ORFs are thought to not only be involved in maintaining DUI, but sex determination as well. Thus, it may be under selection to maintain its functionality (absence of stop codons) despite being located within the highly variable control region. While other mitochondrial markers are functionally constrained, promoting amino acid conservation (Ballard and Melvin 2010), FORF appears to be far less constrained (Breton et al. 2011a) and may freely accumulate mutations other than those leading to stop codons. Over time, these mutations can become fixed within populations or species. As this locus is specific to mussel mitochondrial genomes and able to evolve rapidly, FORF may be an ideal genetic barcode suitable for mussel species identification.

Here we have developed a set of primers to amplify the FORF portion of the mitochondrial control region, and first show that these primers do not amplify control region DNA from the host fish tissue. We then generated sequence data for representatives of all known Unionid species in the Apalachicola-Chattahoochee-Flint and Altamaha river systems of Georgia. As the southeastern United States harbors the highest diversity of freshwater mussels, we want to add to what is known of host-glochidia relationships for future management efforts. With these data,

and comparisons of within- and between-species divergence at FORF, we explicitly show that this marker is suitable for mussel species identification from host tissues and test this on some naturally infested fish hosts.

## Methods

### *Biological Material: Tissue Collection of Freshwater Mussels*

DNA aliquots were obtained from previous studies (Small 2009) to represent the 29 extant species currently recognized to occur in the Apalachicola River Basin (Williams et al. 2014) and 14 extant species in the Altamaha River Basin (Table 2.1).

### *FORF Barcode Database for ACF Freshwater Mussels*

To determine how much genetic diversity is present at FORF, a target of 5 adult individuals were used to generate the database. Some individuals were rare in our collections, thus fewer of these individuals were included in the study (Table 2.1). DNA was extracted using a Proteinase K extraction method following Small (2009). In the case of *Elliptio spinosa*, DNA was extracted from the periostracum using a CTAB extraction protocol (Small 2009). From all individuals both COI and FORF were PCR amplified and sequenced on an ABI 3730XL machine. Cytochrome oxidase subunit I (COI) was PCR amplified and sequenced following Johnson (2014) (ACF mussels) and Small (2009) (Altamaha mussels), while FORF was PCR amplified and sequenced following Breton (2011a). Sequences were visualized and edited using CodonCode Aligner (v.4.2.5). The ends of each sequence were clipped using default read quality settings within CodonCode Aligner and ambiguities were assessed through comparison across multiple reads from the same individual. Initial FORF sequences from ACF mussels were used to design an

additional primer set that increased amplification success, ACF_Vvil_FORF-for: TCCTCAAGGAAGGTAGCCGA and Vvil_FORF-rev: ATGTCGGTAGAGGCAGGACT. The PCR amplification protocol was as follows: an initial denaturation step of 94°C for 3 minutes, followed by 45 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 minute, and a final extension time of 7 minutes at 72°C. Each reaction contained 8.3µl water, 4µl Promega GoTaq Green 5X PCR buffer, 3µl 25 mM $MgCl_2$, 1.5µl 10 mM dNTPs (at 2.5 mM each), 1µl BSA, 0.5µl of each primer, and 0.2µl (1 unit) of Promega GoTaq DNA polymerase. To each PCR reaction, 1µl of template DNA (at 20-50ng/µl) was added for total volume of 20µl. Sequences were visually inspected and edited using CodonCode Aligner (v.4.2.5).

*Barcode Gap Analysis*

To determine if FORF is a suitable genetic marker for mussel species identification we did a barcode gap analysis by comparing intra- and interspecific genetic divergence at FORF and COI. FORF and COI sequences were aligned using Muscle in Geneious v.8.1.6. Intra- and interspecific genetic divergence was compared using a p-distance model using the R package SPIDER. The p-distance model assumes no molecular model of genetic evolution, but simply measures the proportion of nucleotide differences for each pairwise comparison, appropriate for non-phylogenetic pairwise comparisons. Because a previous study shows that mitochondrial markers were typically unable to distinguish between multiple *Elliptio* species (Small et al. 2012), in a separate analysis *Elliptio* species were lumped into one taxonomic group and intra- and inter-specific pairwise comparisons were calculated. A barcode gap analysis was completed for COI using the same procedure as FORF. The distributions of intra- and interspecific pairwise comparisons were plotted against the frequency of the proportion of pairwise differences.

*Phylogenetic Analysis of FORF and COI*

A Jukes-Cantor distance, neighbor-joining tree was generated in Geneious v. 8.1.6 for both FORF and COI sequence alignments. Each neighbor-joining tree was used as "starter trees" to generate Maximum likelihood phylogenies in PhyML with 100 bootstrap replicates. To generate each phylogeny in PhyML, a suitable model of molecular evolution was needed; FORF and COI alignments were submitted to ModelTest in the Phangorn R package. Within each gene tree, the Species Delimitation plugin for Geneious vs. 8.1.6 was used to test if each species was reciprocally monophyletic (Rosenberg 2007). The Species Delimitation plugin also summarized the probability that an unknown specimen would be correctly identified given the phylogenetic tree and user specified species identities.

*Biological Material: Recovery of Glochidia from Naturally Infested Fish*

From April to June of 2012, 133 Alabama shad (*Alosa alabamae*) were collected downstream of Jim Woodruff Lock and Dam on the Apalachicola River. Gills were removed and placed into individual jars and filled with isopropanol. All gill collections were performed by the Georgia Department of Natural Resources. Gill arches were individually separated from each gill set and visually inspected for glochidia under a dissecting scope. Freshwater mussel glochidia were removed from infested gill arches and DNA was extracted following Casquet et al. (2011).

*Identification of Glochidia Recovered from Naturally Infested Fish*

For each glochidium, COI was PCR amplified following (Small et al. 2009) and FORF was PCR amplified using the Vvil_FORF primers adapted from Breton et al. (2011a). All PCR products were sequenced using an ABI 3730XL Sanger sequencer. Sequences were visualized and edited

in CodonCode Aligner (v.4.2.5). Both glochidia COI and FORF sequences were BLASTed to confirm that each sequence was a mussel sequence. Glochidia species were identified by BLASTing both COI and FORF sequences to the ACF COI and ACF FORF databases (Table 2.9).

## Results

### *FORF and COI Sequencing and Barcode Gap Analysis*

This study generated 313 COI and 232 FORF barcode sequences for 29 species of Unionid freshwater mussel from the ACF and 15 species from the Altamaha River basin. Per drainage, 126 COI and 104 FORF sequences from the ACF, and 187 COI and 128 FORF from Altamaha were collected (Table 2.1). COI sequences averaged 572 bp across all species while FORF sequence length varies by genus. FORF in most taxa is approximately 350 bps (240-400 bps), while FORF in the genus *Elliptio* is approximately 180 bps (160-200 bps). Significant variation was observed within and between species at FORF (Figure 2.1). Roughly twice as much intraspecific variation was seen in FORF when compared to COI (Figure 2.1 and 2.2). Variation at FORF ranged between 0-20% pairwise genetic difference within species and 20-80% between species (Figure 2.1). In both the ACF and the Altamaha, a few genera exhibit a remarkable ~70% pairwise genetic difference within species. More taxa in the ACF show this high level of intraspecific genetic difference, producing an overlapping distribution in the intra- and interspecific genetic difference (Figure 2.1A-B). Variation at COI ranged between 0-10% within species and most interspecific comparisons ranged between 10-20%. The only genus with overlapping pairwise intra- and interspecific genetic differences was *Elliptio* (Figure 2.1G and 2.2G). In *Elliptio*, both intra- and interspecific pairwise genetic difference at FORF was 0-15%

with completely overlapping distributions. COI shows much less variation in comparison to FORF with 0-5% pairwise genetic difference within species and 0-8% between species. Though FORF possess more intra- and interspecific difference, COI has less of an overlap in the distributions. Once *Elliptio* was grouped into a single taxon, overlap in the distribution of the inter- and intraspecific genetic differences was reduced (Figures 2.1 and 2.2). However, these raw distributions of pairwise differences do not lead to ambiguity in taxon assignment.

*Phylogenetic analysis of FORF and COI*

The maximum likelihood gene trees for the COI and FORF are very similar in that each mitochondrial maker produces a phylogenetic tree with monophyletic species, with the exception of those in the genus *Elliptio*, who overall grouped monophyletically as a genus (Figures 2.5 and 2.6). In the FORF phylogeny, some species are reconstructed as polyphyletic. While many species maintained monophyletic clades (Table 2.3), those in the genus *Villosa* and *Lampsilis* showed multiple and distinct clustering patterns where each genus consistently clustered together, but not always showing the same relationships between species (Figure 2.3). For instance, *L. splendida* and *L. dolabraeformis*, both species from the Altamaha, are sister to each other and nested within other *Lampsilis* species from the ACF. In a separate clade, *L. splendida* is basal to *L. dolabraeformis*. Interestingly, *Lampsilis* species from the Altamaha formed two distinct clades in both COI and FORF. Each species formed two monophyletic groups. Additionally, *Pyganodon grandis* was polyphyletic, clustering with *Toxolasma paulum* and *Anodonta heardi,* species that coincide with *P. grandis* in the ACF. *Elliptio* was also polyphyletic, clustering more closely together by genus but still forming multiple clades. Bootstrap values across each tree are poor in the deep nodes but toward the tips, where OTUs

grouped by nominal species, bootstrap values significantly increased, as is typical for such rapidly-evolving markers (Wares et al. 2009). With both COI and FORF, there is no obvious division between the ACF and the Altamaha River basins with the exception of *Utterbackia imbecillis*, which is present in both the ACF and Altamaha, clustered by drainage. Three *Elliptio* species, *E. spinosa*, *E. shepardiana*, and *E. arctata*, consistently clustered distinctly apart from each other and other *Elliptio* species both at COI and FORF. Most taxa in the COI phylogeny were monophyletic and there was a high probability that an unknown sample would be correctly identified (probability of identification = P ID) given the phylogeny and user specified species identities (Table2. 4). Even though 30% of taxa were not monophyletic, the P ID value remained very high.  In the *Elliptio* COI phylogeny, only *E. spinosa* was monophyletic (100% P ID). Additionally, for six *Elliptio* species there is less than a 60% probability for which an unknown samples would be correctly identified (Table 2.5). While most species were monophyletic and had high P ID values at COI, the FORF phylogeny is not as clear-cut. Approximately half (53%) of the species are monophyletic at FORF but 70% of taxa had P ID values higher than 60%. Though many species are not monophyletic, there is still enough variation to correctly identify a large portion of taxa evaluated to at least the genus level (Table 2.6).

*Identification of glochidia Recovered from Naturally Infested Fish*

Fourteen COI and FORF sequences were successfully collected from glochidia recovered from Alabama shad gills. Sixty percent of COI sequences from these samples BLASTed to *Alosa* sequences in Genbank (American shad) COI while all FORF sequences were identified as a freshwater mussel species (Genbank ID: *Fusconaia flava*, the closest taxa available on Genbank)

(Table 2.2). When compared to the FORF database, all of the sampled glochidia were identified as an *Elliptio* species, most of which were *Elliptio crassidens* (Table 2.9)

**Discussion**

When using genetic markers to identify taxa from mixed-DNA samples – as in some studies of gut contents (eg: Jo et al. 2014), tissue-related microbiome (eg: Leray and Knowlton 2015), or host-parasite interactions (eg: Tedersoo et al. 2008) – two things are important when selecting a gene region. The first is to select a gene region that is specific to the target species or genera, effectively eliminating the surrounding cells or tissues from the non-focal organism. The second is to identify a gene region (or regions) that is genetically diverse enough to allow for identification to the species level. Traditionally used genetic markers - we specifically tested COI - consistently amplify host fish DNA in attempts to identify freshwater mussel larvae. As a result, the ability to identify glochidia is lost in the mix of host and glochidia. Here we have shown that Female-specific Open Reading Frame (FORF) consistently amplifies glochidia DNA in the presence of fish host contamination, reducing the need for tedious dissections and the risk of losing data due to host contamination. FORF is a highly diverse open reading frame in the Control Region of the mitochondria and has approximately twice the genetic diversity of COI. The high pair-wise genetic diversity observed in this study is consistent with previous descriptions of FORF and the control region of the mitochondria (Burzynski et al. 2003; Cao et al. 2004; Breton et al 2011a; Breton et al. 2009). Interestingly, there is over 100 bp length difference between species in the genus *Elliptio* and all other Unionid taxa. Because this is consistent, there may be a biologically significant difference in the length of the ORF within the control region of the mitochondria and because the control region is highly variable, it is

plausible that large sections of the control region were deleted in the lineage leading to *Elliptio*. FORF in *Elliptio* is free of stop codons, suggesting that it remains functional despite its shorter length, but because there is little to no genetic difference in *Elliptio* across coding regions of the mitochondria, the potential role of FORF in reproduction may have been altered when coding sequence length was lost.

Like traditionally used genetic markers, FORF is able to identify most freshwater mussels to species, with the exception of those in the genus *Elliptio*. *Elliptio* is a taxonomically problematic group and each species is polyphyletic within the genus *Elliptio* (Campbell et al. 2005). It has been previously observed that species in the genus *Elliptio* group monophyletically within their tribe, but polyphyletically within the genus (Campbell et al. 2005). With the high level of plasticity in *Elliptio* morphology, misidentification is common (Shea et al. 2011), potentially contributing to the polyphyly seen across phylogenies and the overlap observed in the barcode gap analysis within the genus. With many described species endemic to individual drainages, it is possible that *Elliptio* went through a recent radiation, allowing for rapid speciation. With rapid speciation in a short time frame, there might not have been enough time for genetic variation in the mitochondrial genome to become fixed between species, further contributing to poor taxonomic resolution. Additionally, many *Elliptio* species release glochidia during the same time period suggesting conspecific males release sperm simultaneously. Female mussels take in sperm while they siphon water, encountering sperm from multiple species. With the potential for introgression, there may be fewer true “species” than what is currently accepted. Because it is unclear as to whether every described species within the genus *Elliptio* is indeed a distinct species (evident by the lack of genetic distance and plastic morphology), with additional nuclear

data, further genetic analysis of the genus *Elliptio* is required for its taxonomic resolution. In the future, utilization of microsatellite markers, which typically evolve faster than mitochondrial markers, to genetically distinguish *Elliptio* species should be pursued.

The FORF database constructed in this study is specific to the species found in the ACF and the Altamaha river basins but, like other commonly used genetic markers, it can be expanded to include other mussel species. FORF is located in the control region of the mitochondria, making it more challenging than COI to PCR amplify. Additionally, FORF is not as accurate as COI at identifying samples to the species level. The high genetic variation at FORF and the lack of homology across taxa make sequence alignment difficult, thus FORF may not be a suitable marker for phylogenetic analysis. With some of these challenges, it is unlikely that FORF will replace traditionally used genetic markers for adult mussel species identification, but it provides reliable identification of glochidia recovered from infested fish while avoiding the risk of host contamination. With no host contamination, less time can be spent carefully dissecting glochidia from fish tissues. Because *Elliptio* produces a shorter PCR product then other taxa, rapid identification of *Elliptio* versus non-*Elliptio* is possible with simple visualization on an agarose gel. Additionally, it is worth noting that amplification of FORF from species in the Altamaha was less difficult than species from the ACF. With the combination of different amplification success and the significant difference in FORF base pair length between *Elliptio* and non-*Elliptio* species, whole mitochondrial genome sequencing may be informative regarding these two observations. The control region of the Unionid mitochondria is not always in the same place across species (Breton et al. 2009), which would influence amplification success between

species, but having whole drainages with different amplification success is interesting and should be investigated further as it will have an impact conservation efforts through genetic methods.

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**Data Accessibility**

FORF DNA sequence will be available on Genbank and accession numbers will be available in the publication and COI sequences are available in both Genbank and BOLD. BLAST databases for FORF and COI will be available in a Dryad Digital Repository. Voucher specimens for the ACF are available through the Florida Museum of Natural History.

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## Tables and Figures

Table 1: List of species included in the study. All 20 extant species were included from the Apalachicola River Drainage (ACF) as well as all 14 extant species from the Altamaha River Drainage (Alt). Numbers in parenthesis represent the number of individuals successfully sequenced, FORF and COI, respectively.

| ACF Species | Alt Species |
|---|---|
| *Alasmidonta triangulata* (2, 3) | *Alasmidonta arcula* (5, 15) |
| *Amblema neislerii* (6, 4) | *Elliptio angustata* (3, 5) |
| *Anodonta heardi* (2, 3) | *Elliptio complanata* (16, 15) |
| *Anodontoides radiatus* (1, 1) | *Elliptio dariensis* (14, 15) |
| *Elliptio arctata* (1, 7) | *Elliptio hopetonensis* (16, 15) |
| *Elliptio crassidens* (4, 3) | *Elliptio icterina* (15, 15) |
| *Elliptio fraterna* (5, 4) | *Elliptio shepardiana* (16, 15) |
| *Elliptio fumata* (12, 11) | *Elliptio spinosa* (2, 15) |
| *Elliptio nigella* (5, 6) | *Lampsilis dolabraeformis* (13, 15) |
| *Elliptio pullata* (10, 8) | *Lampsilis splendida* (14, 15) |
| *Elliptoideus sloatianus* (1, 1) | *Pyganodon gibbosa* (5, 15) |
| *Ellptio purpurella* (4, 3) | *Toxolasma parvus* (1, 2) |
| *Glebula rotundata* (5, 10) | *Uniomerus carolinas* (1,1) |
| *Hamiota subangulata* (3, 3) | *Utterbackia imbecillis* (2, 15) |
| *Lampsilis floridensis* (3, 3) | *Villosa delumbis* (6, 15) |
| *Lampsilis straminea* (2, 4) | |
| *Medionidus penicillatus* (4, 4) | |
| *Megalonaias floridensis* (2, 2) | |
| *Megalonaias nervosa* (1, 1) | |
| *Pleurobema pyriforme* (2, 2) | |
| *Pyganodon grandis* (2, 6) | |
| *Quadrula infucata* (4, 4) | |
| *Toxolasma paulum* (4, 4) | |
| *Uniomerus columbensis* (2, 3) | |
| *Utterbackia imbecillis* (2, 2) | |
| *Utterbackia pegyae* (3, 2) | |
| *Villosa lienosa* (7, 9) | |
| *Villosa vibex* (4, 9) | |
| *Villosa villosa* (1, 4) | |

Table 2: Blast ID results of Glochidia recovered from *Alosa alabamae* gill tissue. COI and FORF sequences was sequenced from each glochidium

| Gene Region | Number of Glochidia | Blast ID of Fish | Blast ID of Mussel | Percent Correct ID | E-value Fish ID | E-value Mussel ID |
|---|---|---|---|---|---|---|
| COI | 14 | 9 | 5 | 35.71 | 1.6014-7 | 1.2857-28 |
| FORF | 14 | 0 | 14 | 100 | NA | 1.9319-3 |

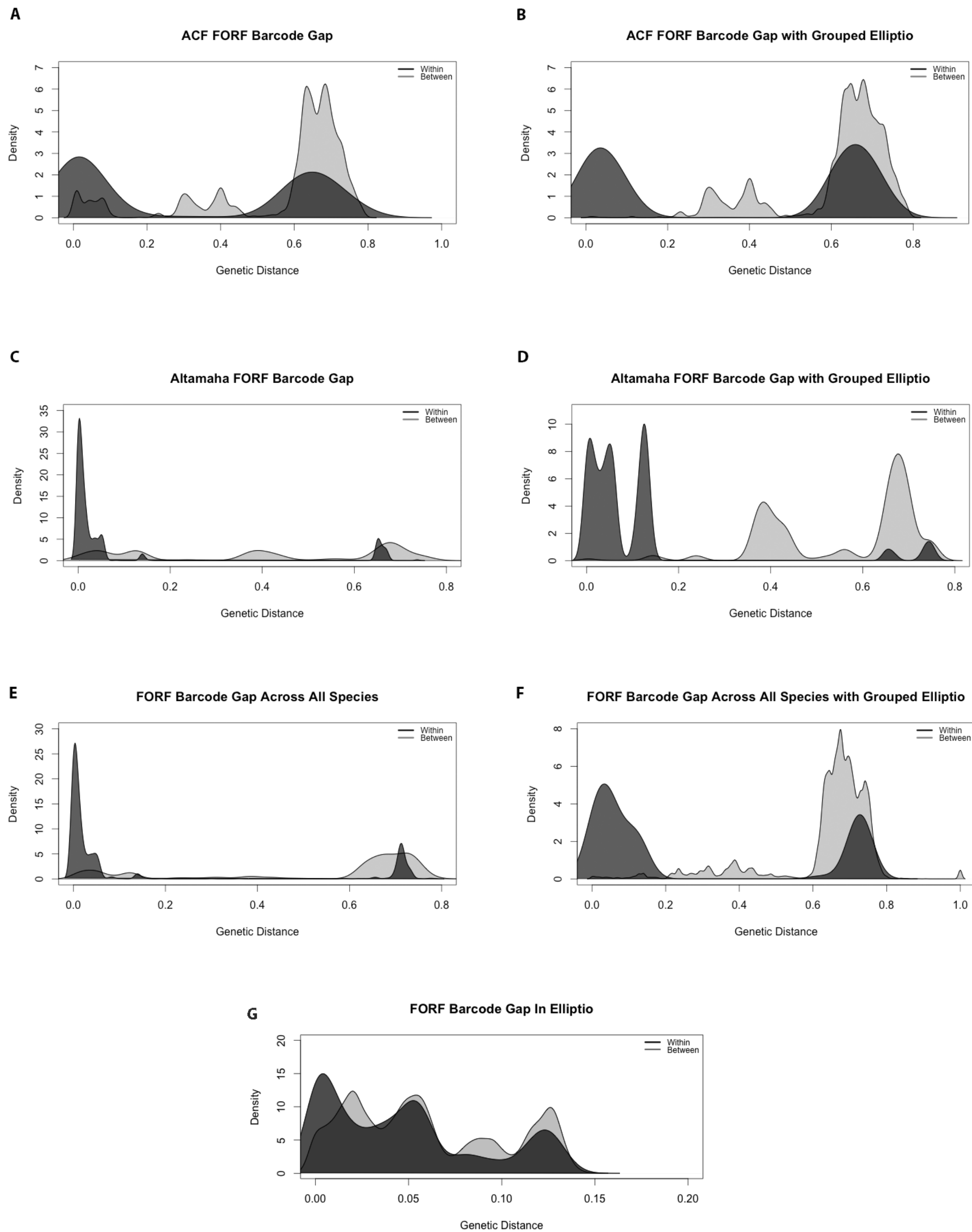


Figure 2.1. Distribution of intra- and interspecific genetic difference at FORF. Distribution of the proportion of pairwise genetic differences within and between freshwater mussel species plotted against the frequencies of the proportion of pairwise genetic differences observed. A smoothing kernel was applied to the distribution. A) Pairwise comparison of Unionid mussels in the Apalachicola River Basin (ACF), B) ACF with species of the genus

Elliptio grouped together, C) Altamaha River basin, D) Altamaha River basin with species of the genus Elliptio grouped together, E) pair-wise comparison of all species across both the ACF and Altamaha River basins, F) pair-wise comparison of all species across both the ACF and Altamaha River basins with species in the genus Elliptio grouped together. G) A barcode gap analysis of species within the genus *Elliptio*.

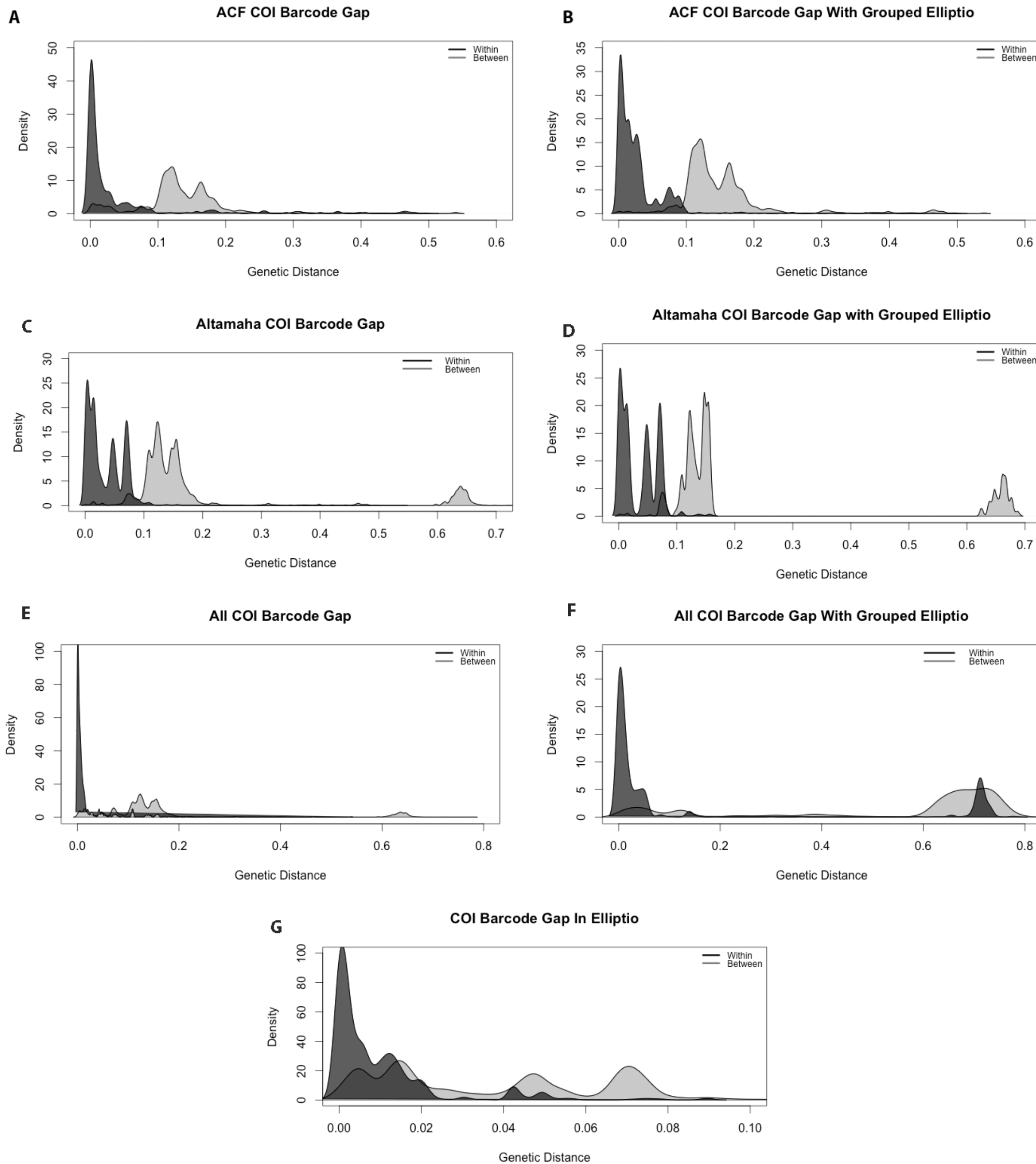


Figure 2.2. Distribution of intra- and interspecific genetic difference at COI. Distribution of the proportion of pairwise genetic differences within and between freshwater mussel species plotted against the frequencies of the proportion of pairwise genetic differences

observed. A) The pairwise comparison of every species within the ACF and Alt drainages. B) Pairwise comparisons between all species in the ACF and Alt drainages are compared with species of the genus *Elliptio* grouped together. A) Pairwise comparison of Unionid mussels in the Apalachicola River Basin (ACF), B) ACF with species of the genus Elliptio grouped together, C) Altamaha River basin, D) Altamaha River basin with species of the genus Elliptio grouped together, E) pair-wise comparison of all species across both the ACF and Altamaha River basins, F) pair-wise comparison of all species across both the ACF and Altamaha River basins with species in the genus Elliptio grouped together. G) A barcode gap analysis of species within the genus *Elliptio*.

Figure 2.3. FORF maximum likelihood gene tree of all species across the ACF and Alt. A GTR model of molecular evolution was used and the data was bootstrapped 100 times. Monophyletic clades are collapsed for simplicity.

Figure 2.4. Maximum likelihood FORF gene tree of all *Elliptio* species across the ACF and Altamaha River drainage. A GTR model of molecular evolution was used and the data was bootstrapped 100 times. Monophyletic clades are collapsed for simplicity.

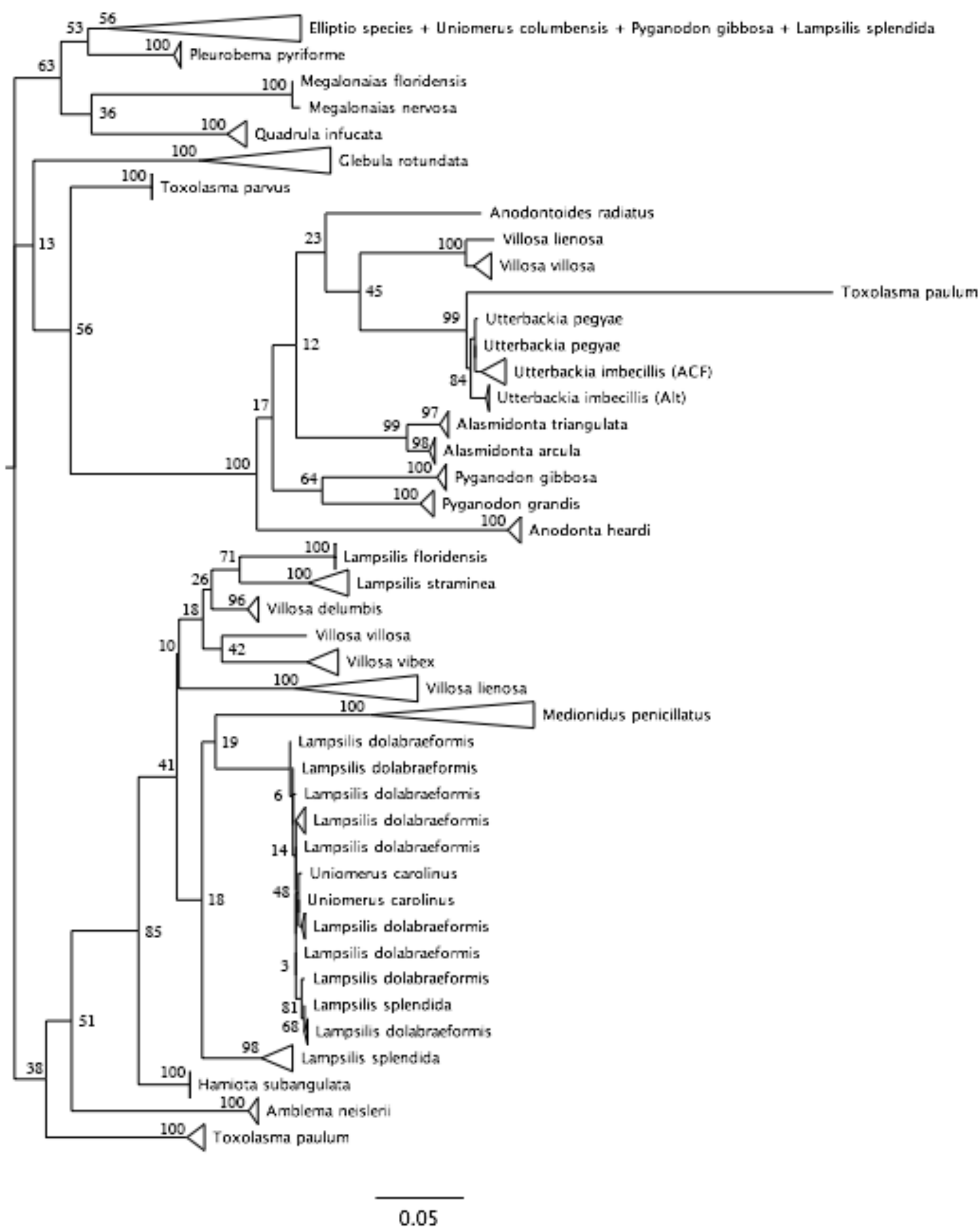


Figure 2.5: Maximum likelihood COI gene tree of all species across the ACF and the Altamaha River drainage. A GTR model of molecular evolution was used and the data was bootstrapped 100 times. Monophyletic clades and species in the genus *Elliptio* are condensed for simplicity. See figure 7 and 8 for the expanded Elliptio clade.

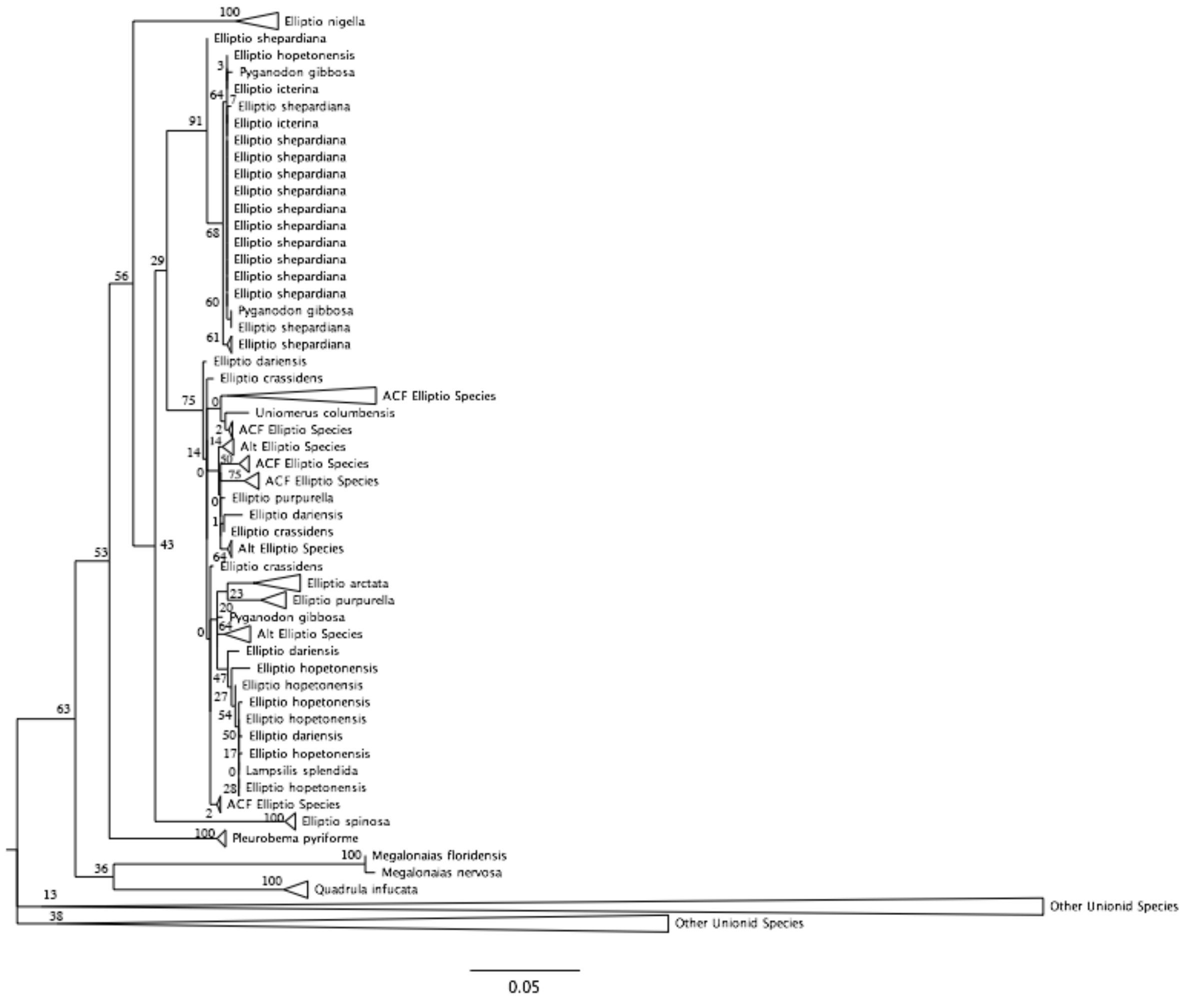


Figure 2.6: Maximum likelihood COI gene tree (from Figure 5) with the *Elliptio* clade expanded to show polyphyly across taxa. The rest of the tree condensed for simplicity.  A GTR model of molecular evolution was used and the data was bootstrapped 100 times.

Figure 2.7: Elliptio COI maximum likelihood tree.

Figure 2.8. FORF Neighbor-joining tree with glochidia recovered from Alabama shad. Same as Figure 3, just *Elliptio* is expanded.

Table 2.3: FORF Species Delimitation. Species: focal species being considered, Closest Species: closest species to the focal species, Monophyletic: whether the focal species is monophyletic or not. Intra Dist: the average pairwise distance among individuals of the focal species. The larger the value, the more diverse the focal species is. Inter Dist: the average pairwise distance between the focal species and the next closest species. The larger the value, the more distinct the species are. Intra/Inter: the ratio of Intra Dist and Inter Dist is a measure of genetic differentiation between the focal species and its closest species. Small values indicate that the genetic differences of the focal species are small relative to the differences between the focal species and closest species. P ID: the probability, with 95% confidence intervals, of making a correct identification of an unknown specimen of the focal species.

| Species | Closest Species | Monophyletic | Intra Dist | Inter Dist | Intra/Inter | P ID |
|---|---|---|---|---|---|---|
| *Elliptio* | *A. neislerii* | no | 0.592 | 0.796 | 0.74 | 0.93 (0.90, 0.96) |
| *L. straminea* | *A. neislerii* | no | 2.342 | 1.654 | 1.42 | 0.21 (0.05, 0.36) |
| *L. splendida* | *U. imbecillis* (Alt) | no | 1.382 | 1.235 | 1.12 | 0.71 (0.67, 0.75) |
| *A. arcula* | *U. imbecillis* (Alt) | yes | 0.062 | 1.634 | 0.04 | 0.98 (0.87, 1.0) |
| *P. grandis* | *Elliptio* | no | 2.815 | 2.008 | 1.4 | 0.12 (0.00, 0.28) |
| *U. carolinas* | *U. columbensis* | yes | 0.00 | 0.248 | 0.00 | 0.96 (0.83, 1.0) |
| *T. paulum* | *Elliptio* | no | 3.107 | 2.142 | 1.45 | 0.19 (0.03, 0.34) |
| *M. floridensis* | *U. imbecillis* (Alt) | no | 2.661 | 1.401 | 1.9 | 0.00 (0.00, 0.00) |
| *U. columbensis* | *U. carolinas* | yes | 0.011 | 0.248 | 0.05 | 0.95 (0.80, 1.0) |
| *V. delumbis* | *M. penicillatus* | yes | 0.02 | 0.432 | 0.05 | 0.97 (0.87, 1.0) |
| *V. lienosa* | *V. vibex* | no | 1.194 | 1.027 | 1.16 | 0.52 (0.46, 0.59) |
| *P. gibbosa* | *U. imbecillis* (Alt) | no | 1.104 | 1.506 | 0.73 | 0.76 (0.65, 0.86) |
| *M. penicillatus* | *V. delumbis* | yes | 0.004 | 0.432 | 0.01 | 0.98 (0.87, 1.0) |
| *U. imbecillis* (Alt) | *V. vibex* | no | 0.027 | 0.745 | 0.04 | 0.96 (0.81, 1.0) |
| *L. dolabraeformis* | *U. imbecillis* (Alt) | no | 1.411 | 1.298 | 1.09 | 0.74 (0.69, 0.78) |
| *V. vibex* | *U. imbecillis* (Alt) | yes | 0.002 | 0.745 | 2.63E-03 | 1.00 (0.86, 1.0) |
| *A. neislerii* | *G. rotundata* | yes | 0.012 | 0.454 | 0.03 | 0.99 (0.85, 1.0) |
| *A. heardi* | *U. imbecillis* (Alt) | yes | 0.025 | 1.692 | 0.01 | 0.97 (0.82, 1.0) |
| *G. rotundata* | *A. neislerii* | yes | 0.00 | 0.454 | 0.00 | 0.98 (0.87, 1.0) |
| *H. subangulata* | *U. imbecillis* (Alt) | no | 2.607 | 1.374 | 1.9 | 0.00 (0.00, 0.00) |
| *A. radiatus* | *Elliptio* | yes | 0.00 | 1.647 | 0.00 | 0.96 (0.83, 1.0) |
| *L. floridensis* | *M. penicillatus* | no | 1.37 | 0.794 | 1.73 | 0.03 (0.00, 0.19) |
| *Q. infucata* | *Elliptio* | yes | 0.00 | 1.565 | 0.00 | 0.96 (0.83, 1.0) |
| *V. villosa* | *U. imbecillis* (Alt) | no | 2.56 | 1.35 | 1.9 | 0.00 (0.00, 0.00) |
| *U. pegyae* | *U. imbecillis* (Alt) | yes | 0.959 | 1.557 | 0.62 | 0.60 (0.45, 0.76) |
| *P. pyriforme* | *Elliptio* | no | 3.375 | 2.188 | 1.54 | 0.03 (0.00, 0.20) |
| *E. sloatianus* | *A. neislerii* | yes | 0.00 | 0.63 | 0.00 | 0.96 (0.83, 1.0) |
| *U. imbecillis* (ACF) | *U. imbecillis* (Alt) | yes | 0.00 | 1.253 | 0.00 | 0.98 (0.83, 1.0) |
| *A. triangulata* | *A. neislerii* | yes | 0.007 | 0.956 | 0.01 | 0.98 (0.83, 1.0) |
| *T. parvus* | *Elliptio* | yes | 0.00 | 1.467 | 0.00 | 0.96 (0.83, 1.0) |

Table 2.4: Elliptio FORF Species Delimitation. Table details are the same as those in Table 3.

| Species | Closest Species | Monophyletic | Intra Dist | Inter Dist | Intra/Inter | P ID |
|---|---|---|---|---|---|---|
| *E. hopetonenis* | *E. dariensis* | no | 0.024 | 0.046 | 0.52 | 0.96 (0.93, 0.99) |
| *E. fumata* | *E. nigella* | no | 0.019 | 0.018 | 1.05 | 0.77 (0.72, 0.81) |
| *E. icterina* | *E. crassidens* | no | 0.002 | 0.016 | 0.15 | 0.98 (0.93, 1.0) |
| *E. dariensis* | *E.crassidens* | no | 0.024 | 0.019 | 1.31 | 0.50 (0.46, 0.55) |
| *E. crassidens* | *E. nigella* | no | 0.007 | 0.015 | 0.44 | 0.85 (0.74, 0.95) |
| *E. fraterna* | *E. fumata* | no | 0.039 | 0.029 | 1.33 | 0.22 (0.10, 0.34) |
| *E. shepardiana* | *E. angustata* | no | 0.018 | 0.069 | 0.25 | 0.97 (0.94, 1.00) |
| *E. complanata* | *E. icterina* | no | 0.028 | 0.019 | 1.46 | 0.31 (0.28, 0.34) |
| *E. pullata* | *E. crassidens* | no | 0.037 | 0.026 | 1.41 | 0.27 (0.21, 0.33) |
| *E. spinosa* | *E. icterina* | no | 0.064 | 0.033 | 1.93 | 0.00 (0.00, 0.00) |
| *E. purpurella* | *E. fumata* | no | 0.052 | 0.051 | 1.02 | 0.43 (0.28, 0.58) |
| *E. nigella* | *E. crassidens* | no | 0.02 | 0.015 | 1.3 | 0.27 (0.12, 0.43) |
| *E. arctata* | *E. fraterna* | no | 0.033 | 0.074 | 0.45 | 0.95 (0.91, 0.99) |
| *E. angustata* | *E. shepardiana* | yes | 0.106 | 0.069 | 1.54 | 0.03 (0.00, 0.20) |

Table 2.5: COI Species Delimitation. Table details are the same as those in Table 3.

| Species | Closest Species | Monophyletic | Intra Dist | Inter Dist | Intra/Inter | P ID |
|---|---|---|---|---|---|---|
| *Elliptio* | *U. columbensis* | no | 0.05 | 0.05 | 1.04 | 0.81 (0.78, 0.83) |
| *L. dolabraeformis* | *U. carolinas* | no | 0.01 | 0.01 | 1.02 | 0.79 (0.74, 0.83) |
| *V. delumbis* | *L. straminea* | yes | 0.01 | 0.09 | 0.07 | 0.99 (0.95, 1.0) |
| *V. lienosa* | *H. subangulata* | no | 0.20 | 0.19 | 1.10 | 0.51 (0.40, 0.61) |
| *P. gibbosa* | *P. grandis* | no | 0.15 | 0.19 | 0.83 | 0.83 (0.78, 0.89) |
| *H. subangulata* | *V. delumbis* | yes | 0.00 | 0.10 | 0.00 | 1.00 (0.86, 1.0) |
| *A. arcula* | *A. triangulata* | yes | 0.00 | 0.04 | 0.04 | 1.00 (0.96, 1.0) |
| *P. pyriforme* | *Elliptio* | yes | 0.01 | 0.11 | 0.04 | 0.96 (0.80, 1.0) |
| *U. imbecillis* (Alt) | *U. pegyae* | yes | 0.00 | 0.01 | 0.12 | 0.98 (0.94, 1.0) |
| *A. triangulata* | *A. arcula* | yes | 0.00 | 0.04 | 0.09 | 0.96 (0.81, 1.0) |
| *M. penicillatus* | *U. carolinas* | yes | 0.05 | 0.17 | 0.33 | 0.89 (0.78, 1.0) |
| *L. splendida* | *L. dolabraeformis* | no | 0.06 | 0.11 | 0.51 | 0.94 (0.90, 0.99) |
| *G. roduntata* | *T. parvus* | yes | 0.02 | 0.18 | 0.11 | 0.98 (0.92, 1.0) |
| *P. grandis* | *A. arcula* | yes | 0.01 | 0.18 | 0.03 | 0.98 (0.87, 1.0) |
| *L. straminea* | *V. delumbis* | yes | 0.01 | 0.09 | 0.16 | 0.95 (0.84, 1.0) |
| *V. vibex* | *V. delumbis* | yes | 0.01 | 0.09 | 0.08 | 0.98 (0.92, 1.0) |
| *A. neislerii* | *H. subangulata* | yes | 0.01 | 0.17 | 0.04 | 0.98 (0.84, 1.0) |
| *U. imbecillis* (ACF) | *U. pegyae* | yes | 0.01 | 0.01 | 1.25 | 0.21 (0.05, 0.37) |
| *T. paulum* | *T. parvus* | no | 0.39 | 0.29 | 1.36 | 0.24 (0.08, 0.39) |
| *V. villosa* | *U. pegyae* | no | 0.30 | 0.24 | 1.25 | 0.30 (0.15, 0.45) |
| *A. heardi* | *P. grandis* | yes | 0.01 | 0.25 | 0.02 | 1.00 (0.85, 1.0) |
| *T. parvus* | *P. pyriforme* | yes | 0.00 | 0.17 | 0.00 | 0.98 (0.83, 1.0) |
| *L. floridensis* | *V. delumbis* | yes | 0.00 | 0.10 | 0.00 | 1.00 (0.86, 1.0) |
| *U. columbensis* | *Elliptio* | yes | 0.00 | 0.05 | 0.00 | 0.96 (0.83, 1.0) |
| *Q. infucata* | *P. pyriforme* | yes | 0.01 | 0.17 | 0.04 | 0.97 (0.86, 1.0) |
| *U. pegyae* | *U. imbecillis* (ACF) | no | 0.00 | 0.01 | 0.13 | 0.90 (0.75, 1.0) |
| *A. radiatus* | *U. pegyae* | yes | 0.00 | 0.18 | 0.00 | 0.96 (0.83, 1.0) |
| *U. carolinas* | *L. dolabraeformis* | no | 0.00 | 0.01 | 0.30 | 0.80 (0.64, 0.95) |
| *M. nervosua* | *M. floridensis* | yes | 0.00 | 0.01 | 0.00 | 0.96 (0.83, 1.0) |
| *M. floridensis* | *M. nervosua* | yes | 0.00 | 0.01 | 0.00 | 0.96 (0.83, 1.0) |

Table 2.6: Elliptio COI Species Delimitation. Table details are the same as those in Table 3.

| Species | Closest Species | Monophyletic | Intra Dist | Inter Dist | Intra/Inter | P ID |
|---|---|---|---|---|---|---|
| *E. shepardiana* | *E. icterina* | no | 0.00 | 0.05 | 0.06 | 0.99 (0.95, 1.0) |
| *E. hopetonensis* | *E. complanata* | no | 0.02 | 0.02 | 1.00 | 0.80 (0.76, 0.84) |
| *E. nigella* | *E. crassidens* | no | 0.07 | 0.05 | 1.28 | 0.35 (0.24, 0.46) |
| *E. arctata* | *E. crassidens* | no | 0.03 | 0.03 | 1.02 | 0.67 (0.60, 0.73) |
| *E. fraterna* | *E. crassidens* | no | 0.02 | 0.02 | 0.96 | 0.54 (0.43, 0.65) |
| *E. dariensis* | *E. crassidens* | no | 0.01 | 0.01 | 1.22 | 0.61 (0.57, 0.66) |
| *E. icterina* | *E. complanata* | no | 0.02 | 0.01 | 1.31 | 0.50 (0.46, 0.55) |
| *E. spinosa* | *E. crassidens* | yes | 0.00 | 0.10 | 0.02 | 1.00 (0.96, 1.0) |
| *E. complanata* | *E. crassidens* | no | 0.01 | 0.01 | 0.91 | 0.85 (0.81, 0.89) |
| *E. fumata* | *E. crassidens* | no | 0.03 | 0.02 | 1.56 | 0.00 (0.00, 0.02) |
| *E. pullata* | *E. crassidens* | no | 0.03 | 0.02 | 1.57 | 0.00 (0.00, 0.00) |
| *E. crassidens* | *E. complanata* | no | 0.01 | 0.01 | 0.68 | 0.62 (0.48, 0.77) |
| *E. purpurella* | *E. crassidens* | no | 0.03 | 0.03 | 1.13 | 0.37 (0.22, 0.52) |

Table 2.7: FORF Species Delimitation Table by Genus. Table details are the same as those in Table 2.3.

| Genus | Closest Genus | Monophyletic | Intra Dist | Inter Dist | Intra/Inter | P ID |
|---|---|---|---|---|---|---|
| *Elliptio* | *Amblema* | no | 0.59 | 0.80 | 0.74 | 0.93 (0.90, 0.96) |
| *Uniomerus* | *Amblema* | yes | 0.17 | 0.82 | 0.21 | 0.89 (0.75, 1.0) |
| *Toxolasma* | *Elliptio* | no | 3.06 | 1.97 | 1.55 | 0.00 (0.00, 0.12) |
| *Megalonaias* | *Elliptio* | no | 2.66 | 1.48 | 1.80 | 0.00 (0.00, 0.04) |
| *Pyganodon* | *Elliptio* | no | 1.84 | 1.79 | 1.03 | 0.66 (0.59, 0.73) |
| *Medionidus* | *Amblema* | yes | 0.00 | 0.61 | 0.01 | 0.98 (0.87, 1.0) |
| *Utterbackia* | *Elliptio* | no | 1.38 | 1.87 | 0.74 | 0.75 (0.65, 0.85) |
| *Lampsilis* | *Elliptio* | no | 1.60 | 1.46 | 1.10 | 0.76 (0.74, 0.79) |
| *Amblema* | *Glebula* | yes | 0.01 | 0.45 | 0.03 | 0.99 (0.85, 1.0) |
| *Anodonta* | *Elliptio* | yes | 0.03 | 1.88 | 0.01 | 0.97 (0.82, 1.0) |
| *Glebula* | *Amblema* | yes | 0.00 | 0.45 | 0.00 | 0.98 (0.87, 1.0) |
| *Hamiota* | *Elliptio* | no | 2.61 | 1.45 | 1.80 | 0.00 (0.00, 0.04) |
| *Anodontoides* | *Elliptio* | yes | 0.00 | 1.65 | 0.00 | 0.96 (0.83, 1.0) |
| *Quadrula* | *Elliptio* | yes | 0.00 | 1.56 | 0.00 | 0.96 (0.83, 1.0) |
| *Villosa* | *Elliptio* | no | 1.47 | 1.42 | 1.03 | 0.81 (0.78, 0.84) |
| *Pleurobema* | *Elliptio* | no | 3.38 | 2.19 | 1.54 | 0.03 (0.00, 0.20) |
| *Elliptoideus* | *Amblema* | yes | 0.00 | 0.63 | 0.00 | 0.96 (0.83, 1.0) |
| *Alasmidonta* | *Elliptio* | no | 1.62 | 2.09 | 0.77 | 0.82 (0.75, 0.88) |

Table 2.8: Alosa glochidia Blast ID. Glochidia recovered from *Alosa alabamae* gills were blast against the FORF database. Top Blast species identifications are reported along with the e-value and percent coverage of the reference sequence. Multiple species identifications are reported if equality ranked species IDs were generated.

| Alosa Glochidia FORF Sequence | FORF BLAST ID | E-Value | % Identity |
|---|---|---|---|
| Alosa17 | | | |
| | *E.crassidens* | 3e-74 | 99 |
| Alosa40 | | | |
| | *E.crassidens* | 7e-44 | 100 |
| | *E.fumata* | 7e-44 | 100 |
| | *E.arctata* | 7e-44 | 100 |
| Alosa29 | | | |
| | *E.crassidens* | 4e-73 | 99 |
| Alosa46 | | | |
| | *E.crassidens* | 1e-73 | 99 |
| Alosa05 | | | |
| | *E.crassidens* | 2e-75 | 100 |
| Alosa03 | | | |
| | *E.crassidens* | 2e-75 | 100 |
| Alosa24 | | | |
| | *E.crassidens* | 9e-75 | 100 |
| Alosa35 | | | |
| | *E.crassidens* | 4e-73 | 99 |
| | *E.nigella* | 4e-73 | 99 |
| | *E.pullata* | 4e-73 | 99 |
| Alosa38 | | | |
| | *E.fumata* | 6e-45 | 98 |
| | *E.arctata* | 6e-45 | 98 |
| | *E.pullata* | 6e-45 | 98 |
| Alosa44 | | | |
| | *E.crassidens* | 1e-72 | 99 |
| Alosa47 | | | |
| | *E.crassidens* | 2e-71 | 99 |
| Alosa08 | | | |
| | *E.fumata* | 2e-47 | 90 |
| | *E.crassidens* | 2e-47 | 90 |
| | *E.arctata* | 2e-47 | 90 |
| | *E.nigella* | 2e-47 | 90 |
| Alosa22 | | | |
| | *E.crassidens* | 2e-60 | 98 |
| | *E.fumata* | 2e-60 | 98 |
| | *E.nigella* | 2e-60 | 98 |
| Alosa32 | | | |
| | *E.fumata* | 5e-36 | 91 |
| | *E.crassidens* | 5e-36 | 91 |
| | *E.arctata* | 5e-36 | 91 |
| | *E.nigella* | 5e-36 | 91 |

# CHAPTER 3

# EVALUATING FISH COMMUNITY PHENOTYPES THAT PROMOTE GLOCHIDIA INFESTATIONS OF GENERALIST FRESHWATER MUSSELS (FAMILY: UNIONIDAE)[1]

[1]Bockrath, K.D., J.P. Wares, R. Bringolf, M.C. Freeman. *To be submitted to Hydrobiologia*

## Abstract

Freshwater mussels are a species rich but threatened group of aquatic invertebrates. Their parasitic larval stage makes them dependent on a suitable fish host in order to complete their life cycle. With over 70% of mussel species threatened, endangered, or extinct, it is important to understand the interactions mussels have with their fish hosts. These interactions are better understood in mussel taxa that utilize lures to attract host fish (specialists) compared to mussels taxa that do not (generalists). Using naturally infested fish, we identified the fish species that are suitable hosts for generalist mussels. Using physical and behavioral fish traits along habitat preferences, we determined what factors promote mussel infestations. Fish that interact with vegetation and debris are more likely to become infested than those who lived in open water. Additionally, generalist mussels can metamorphose to juveniles on many different fishes species, but use a single fish species as a primary host.

## Introduction

North America is home to the world's largest diversity of freshwater mussel, most of which is located in the Southeastern United States. Freshwater mussels are ecosystem engineers and provide important services including water filtration and nutrient cycling (Allen and Vaughn 2011), as well as providing habitat for other organisms (Wisniewski et al. 2013). Freshwater mussels were once prevalent but are now in sharp decline. With approximately 300 species of freshwater mussel in North America, over 70% of those are threatened, endangered, or extinct (Williams 1993). Freshwater mussels have an obligate parasitic larval stage, where larvae (glochidia) attach to a suitable fish host, metamorphosing from a glochidium to a juvenile, in order to complete its life cycle. Once metamorphosis is complete, juvenile mussels drop off their

fish host, settle to the sediment, and continue to grow into adults. Thus, the diversity of freshwater mussels is linked closely to interactions with the fish community.

Freshwater mussels use two methods of getting their glochidia to a suitable fish host. They can directly attract a fish host by producing lures filled with glochidia, or they can take more of an opportunistic approach by releasing their glochidia in mucus nets that indiscriminately come into contact with anything in the environment. There are tradeoffs between using each method. Most mussel species that use lures are host specialists and can typically only metamorphose on a small number of fish species, but because they densely package their glochidia into lures, the fish that come into contact with these lures become heavily infested (Williams, Warren Jr. et al. 1993, Baird 2000). On the other hand, mussels that utilize mucus nets are typically host generalists and their glochidia can metamorphose on a large number of species (Neves and Widlak 1988). In contrast to specialist mussels, generalist mussels loosely disperse their glochidia across mucus nets, thus any fish that comes into contact with a mucus net will have a far lighter infestation compared to specialist mussels (Martel and Lauzon-Guay 2005; Kneeland and Rhymer 2008; Klunzinger et al. 2012).

The mussels for which host use is most thoroughly understood are those in the Lampsilini tribe. These mussels use a direct method of attracting fish hosts by using conspicuous lures and tend to be host specialists (Haag et al. 1999; Haag and Warren 1999; Haag and Warren 2003) . Though the Lampsilini tribe is species rich, comprising approximately 30% of Unionid mussel diversity, the remaining 60% of Unionids are "generalist" mussels. Because it is difficult to directly observe host infestations of generalist mussels in the field, many mussel-fish interactions are

poorly characterized. In addition to not knowing which fish are suitable hosts, little is known about the mussel-fish interactions in a fish community. Across infestation strategies (but especially generalist), little is known about the breadth of a fish community that is infested at a given time, as well as if multiple mussel species are infesting the same fish species or the same individual simultaneously. In a stream community, there may be multiple factors that affect mussel infestation success and because different mussel species will use different infestation strategies in the same stream, there may be competition between mussels to infest the same community of fish. Because specialist mussels approach host infestation in an "all-or-none" strategy and heavily infest their fish hosts, little physical space may be available for other mussel species (both specialist and generalists) to infest the same fish. Conversely, generalist mussels should impose a much lighter glochidia load on each individual fish, allowing for the opportunity for multiple mussels to infest the same individual fish.

In addition to competition for physical space on a fish host, environmental factors and fish behavior may also play a significant role in mussel infestation success. Generalist mussels rely on their mucus nets to passively interact with potential fish hosts, thus there may be some factors that make different fish species more or less likely to become infested. Though fish species overlap in aspects of their habitat, fish are quite variable in their body plan. Physical differences, such as mouth position, gape size, and gill area may make a fish more likely to become infested and/or to carry more glochidia. Likewise, environmental preferences or behavioral differences between fish species may promote interactions with mucus nets. For instance, if a generalist mussel releases a mucus net into swift moving riffles, glochidia may not come into contact with a fish and if they do, there is likely little time for glochidia to physically attach to a fish.

Conversely, if a mucus net were to drift in slower currents or get snagged by vegetation, there will be more opportunity for glochidia to interact with fish.

One way we can study host use in generalist mussels is to identify the glochidia that are encysted on wild caught, naturally infested fish (Kneeland and Rhymer 2008; Boyer et all. 2011; Zieritz et al. 2012). This method can tell us which fish are possible hosts, but does not confirm whether or not glochidia can successfully metamorphose on each fish species inspected. Another application of this method is that infestation rates and infestation densities of naturally infested fish can be determined. We take this method one step further by bringing fish back to a lab setting and allowing glochidia to complete metamorphosis. Metamorphosis success can be directly tested in a lab setting by exposing a suspected fish host to thousands of glochidia (Fritts et al. 2012; Johnson et al. 2012; Klunzinger et al. 2012), but with forced exposure we know nothing about natural infestation densities. By taking potentially infested fish back to a lab setting, we can not only identify what mussel glochidia are present on which fish species, we can determine natural infestation densities, metamorphosis success on a wide breadth of fish species, as well as identify any fish host characteristics that may make a fish more susceptible to infestation. Here, we investigate these four aspects of mussel infestation across a fish community. We hypothesize that 1) generalist mussel species will be found on many different fish species at low densities and that specialist mussels will be recovered from very few fish taxa but at higher densities; 2) Because larger fish have often more “habitat” (gill area) for multiple mussels, generalist mussels will be denser on larger fish and that larger fish will have multiple mussel species on them simultaneously; 3) lastly, because mucus nets may have a higher probability of interacting with

fish in less volatile environments, fish who primarily inhabit areas rich in vegetation and debris will be more likely to become infested.

## Methods

### *Field collections*

Collections were completed in two consecutive years (June 2014-2015) in an approximately 800m stretch of Line Creek (A tributary of the Flint River) in Peachtree City, GA. The habitat along Line Creek is heterogeneous with stretches of deep and slow moving water, interrupted by stretches of rocky shoal habitat with faster moving water. Some small deep pools are found alongside rocky shoals. Fish were collected using seine net, cast net, and minnow traps; each effort was designed to collect ~150 fish, limited by facility capacity. Fish were transported in aerated coolers back to the University of Georgia and placed in an Aquatic Habitat (AHAB) unit (Pentair Aquatic Eco-Systems). If possible, fish were individually placed in separate holding tanks, but when space was limited, multiple individuals of the same fish species were placed in a single holding tank. Mesh cups (150um) were placed under the overflow of each holding tank in the AHAB unit. Every other day, holding tanks were flushed, allowing any rejected glochidia or transformed juvenile mussels to be collected in the mesh cups. Each cup was then backwashed with deionized water in 50 ml conical tubes and inspected under 10X magnification using a WILD Heerbrugg dissecting microscope. Glochidia and juveniles were scored and individually collected for DNA analyses. To aid in mussel identification, glochidia and juveniles were roughly binned into groups via relative size, color, and shell morphology. Digital images of a subset of glochidia and juveniles were taken under 40X magnification using a Celestron Pentaview LCD digital microscope (Model # 44348). Mussels were scored as glochidia if they

were open, had no visible shell growth or mantle tissue, and lacked a foot. Mussels were scored as juveniles if they had a moving foot. Water sampling was concluded when no mussels were collected after 3 consecutive sampling days. If there were multiple fish in a holding tank, the number of mussels (glochidia and juveniles) of each taxon collected was divided by the number of individual fish in the tank. After water sampling was complete, the fish were euthanized using a lethal dose of MS-222 and their fins and gills were visually inspected for glochidia using the same dissecting microscope described above.

*Genetic Identification of Glochidia and Juveniles*

DNA was extracted from each individual glochidium and juvenile mussel recovered using a Chelex DNA extraction method following Casquet et al. (2011). Mitochondrial cytochrome oxidase subunit I (COI) was PCR amplified using universal COI primers (Folmer et al. 1994) following Small (2009). PCR reactions consisted of 4µl 5X GoTaq PCR buffer, 3µl 25mM magnesium chloride, 1.5µl 10Mm dNTPs, 1µl 10mg/ml Bovine serum albumin (BSA), 0.5µl each primer, 0.2µl GoTaq polymerase, 7.3µl water, 1µl of template DNA (20-50ng/µl) for a total volume of 20µl. PCR reactions went through the following thermocycler amplification steps: an initial denaturation step at 94°C for 2 minutes followed by 36 cycles of 94°C for 30seconds, 45°C for 30seconds, 72°C for 45seconds, then a final extension step of 72°C for 2 minutes. PCR products were sequenced on an ABI Sequencer and edited and aligned using CodonCode Aligner v.4.2.5 using default settings. Contigs were compared to a COI blast database specific to freshwater mussels in the Apalachicola-Chattahoochee-Flint River Basin (ACF) (Bockrath et al. 2015, in preparation) and the highest ranking BLAST hits by E-value were recorded. Sequences within each contig were randomly selected and individually BLASTed against the ACF COI

database to confirm species identity. Any sequences that did not match the ACF COI database were submitted to NCBI Blast for sequence identification.

*Determining mussel species diversity and their ecologically suitable hosts*

Simpson's Diversity Index (D) (Simpson 1949) is a measure of species diversity that takes into account the total number of species present in a sample, as well as the relative abundance of each species. The value of D ranges of 0 to1 with 0 meaning no diversity and 1 meaning infinite diversity. To determine the species diversity of freshwater mussel collected from each fish species, the Simpson's Diversity Index was calculated for each fish species using the following equation:

$$D = 1 - \Sigma \left(\frac{n}{N}\right)^2$$

$$n = the\ total\ number\ of\ individuals\ of\ a\ fish\ species\ in\ a\ sample$$

$$N = the\ total\ number\ of\ individals\ of\ all\ fish\ species\ in\ a\ sample$$

The Inverse Simpson's Diversity Index (ISDI) provides an easily interpretable measure of the effective number of species in a sample and is calculated by taking the inverse of D. For any fish where mussels were recovered, the minimum number of species in a sample is 1 (ISDI = 1) while the maximum number of species is N, the total number of species sampled. If all species are evenly distributed across a sample, the ISDI will return all N species (ISDI = N). Conversely, if a few species are common while the remaining species are rare, the ISDI will show that there are effectively fewer species.

For each mussel species, fish that are acting as hosts in natural infestations, or ecologically suitable hosts, were determined by taking the product of the total number of each fish species collected, the number of each fish species infested, and the infestation density of each fish species. The products were divided by the largest product and multiplied by 100 to give an Ecological Host Suitability Index (EHSI) (Baird 2009). This calculation takes into account the most commonly collected fish, the most readily infested fish, and the heaviest infestations. Thus, even if a fish species were common, but not heavily infested, it would not be described as the most ecologically suitable host fish, while a more rare fish species that is heavily infested would be described as a suitable fish host. Only the fish species that carried multiple mussel species were included.

*Determining Environmental and Physical Factors Correlated with Mussel Infestations*

For each fish collected, standard length, jaw length, and jaw width was measured. The area of the mouth, or "gape size", was calculated using jaw length and the width between each jaw (Figure 1). Life history data for each fish species was downloaded from the Virginia Tech FishTraits Database (fishtraits.info). The dataset was divided by presence or absence of glochidia, and by the number of mussels that were recovered. Each dataset was identical except for the infestation information and were identically analyzed for significant variables associated with the presence of glochidia on fish and heavy glochidia infestations, respectively. Pearson's correlation tests were run for each variable and if any variable significantly correlated with another, it was removed from consideration. Variables included in all analyses are as follows: *Infestation status* (presence/absence), *infestation density*, *fish species*, *body length*, *gape size*, benthic dwelling (*benthic*), surface or column dwelling (*surwcol*), diet consisting of vegetation (*macvascu*), diet

consisting of detritus (*detritus*), diet consisting of fish and crabs (*fshcrcrb*), proportion of time spent breeding in May (*may*), living in/around muck (*muck*), living in/around cobble (*cobble*), living in vegetation (*vegetat*), living in debris/detritus (*debrdetr*). *Infestation density*, *body length*, *gape size*, and *may* are continuous variables (ranging from 0-1), while the remaining variables are binomial (either 0 or 1). Generalized linear models were built using uncorrelated variables in R. Models were built, compared, and ranked for best of fit using Akaike Information Criterion (AIC) in the R package "AICcmodavg". AIC provides a means of model selection by measuring the relative quality of all proposed models for a given dataset. Each model is ranked in relation to all other proposed models in how well it preserves the information provided in a given dataset.

## Results

### *Fish Collection Effort and Mussel Infestation*

A total of 391 individual fish were collected from two consecutive seasons with 148 individuals collected in 2014 and 200 individuals collected in 2015. Over the two-year sampling effort, thirteen different fish species were collected. The abundance of each species was consistent across both years and were grouped in this analysis. Spottail shiner (*Notropis hudsonius*) and bluegill (*Lepomis macrochirus*) were by far the most common species collected at 27.59% and 23.56% of the total catch. Despite being the most prevalent species, less than 30% of spottail shiners were infested. Bluegill, on the other hand, had the highest rate of infestation at 97% followed by golden shiner (*Notemigonus crysoleucas*) at 82% (Figure 3.2, Table 3.1). Sample sizes for the remaining fish taxa varied from 48 individuals (13.79% of catch) to a single

individual (0.29% of catch) but for many fish species, over 50% carried at least a single glochidium.

During the binning process while all glochidia/juveniles were recovered from their fish hosts, four distinct juvenile morphologies were observed: Large, orange, and pointed (LOP) (Figure 3.3A-B); large, white, and long (LWL) (Figure3. 3H); large, white, gills (LWG) (Figure 3.3G); small, while, rounded (SWR) (Figure3. 3C-D); small, white, "D"-shaped (WSD) (Figure 3.3E-F). Species were initially identified by shell morphology and release time (Williams et al. 2014; Brim Box and Williams 2000). A subset of the recovered mussels were sequenced at COI to confirm species identification. After DNA barcoding using COI, LOP was identified as *Utterbackia imbecilis* (E-value = 0.0) and LWL was identified as *Villosa vibex* (E-value = 0.00). LWL were observed in very high densities on a single largemouth bass (*Micropterous salmoides*) and were absent from all other infested fish. Most SWR individuals were identified as *Elliptio pullata* (E-value = 0.0), while a small subset was identified as *Toxoplasma pullus* (E-value = 0.0). LWG individuals were not Unionid mussels and were identified as *Pisidium casertanum* (E-value = 0.0). Similarly, WSD were identified as the clam, *Corbicula fluminea* (E-value = 0.0). A total of 1,437 mussels were recovered (724 mussels collected in 2014 and 735 mussels collected in 2015). Of the mussels recovered from the 2014 collection, 21% were *Elliptio pullata*, 16% were *Utterbackia imbecillis* and 62% were *Villosa vibex*. Of the successfully sequenced mussels from the 2015 collection, 88% were *Elliptio pullata*, 12% were *Utterbackia imbecillis*, 0.82% were *Toxolasma pullus* (Table 2). Of the 2015 collection, 1.34% were *Corbicula fluminea* and 0.27% awere*Pisidium casertanum*. Across both year and all fish species, *Elliptio pullata* was the most commonly recovered Unionid mussel species (53.32%, N=

816), followed by *Villosa vibex* (28.06%, N=414), *Utterbackia imbecillis* (16.2%, N=239), and *Toxolasma pullus* (0.40%, N=6).

*Inverse Simpson's Diversity Index and Ecological Host Suitability Index*

*Lepomis macrochirus* was the most ecologically suitable host for both *Elliptio pullata* and *Utterbackia imbecilis* (EHSI = 1) (Table 3.4). All other fish species were either clearly not a suitable host, or were significantly less suitable than *L. macrochirus*. The next most suitable host for *U. imbecilis* is *Notemigonus crysoleucas* (EHSI = 0.22). All other fish species have significantly lower EHSI values. Inverse Simpson's Diversity Index supported the Ecological Host Suitability Index with *L. macrochirus* being the prominent host for both *E. pullata* and *U. imbecilis* and *N. crysoleucas* being a secondary host for *U. imbecilis* (Figure 3.4).

*Factors Associated with Infestation*

Different environmental variables were significantly correlated with the presence of glochidia and the abundance of glochidia, but because of the high variance in sample numbers across fish species, these trends were skewed to reflect the traits of the most common fish species (Table 3.5 and 3.6). As noted above, bluegill were the most commonly infested fish, but golden shiner and bass were the most heavily infested. Because some fish species were more abundant in the collection than others, which can potentially to skew the results to reflect the characteristics of the most common species, each species was randomly subsampled for 30 individuals. If less than 30 individuals were collected, all individuals were included in the dataset. After normalizing for fish species, the factors associated with being infested (at least on glochidia or juvenile) stayed the same (Table 3.7). Gape size and a diet of aquatic vegetation and detritus remained

significantly positively correlated with infestation (Table 3.7 and Figure 3.5), while being a column feeder and living in muck or cobble were negatively associated with infestation. After normalization, the factors associated with being more heavily infested changed (Table 3.6 vs. Table 3.8). Factors positively associated with higher density of infestation were somewhat contradictory to infestation alone. Associating with muck was positively correlated to higher infestations while eating aquatic vascular plants was negatively associated (Figure 3.6).

**Discussion**

Understanding how mussels can successfully infest their fish hosts is an important factor to consider when conserving freshwater mussel biodiversity. We wanted to know more about host use in generalist mussels as well as the dynamics of natural infestations. Here, we 1) identified what mussels were attached to and successfully metamorphosing on fishes in a stream community, 2) investigated the possibility of competition between mussels for host use by assessing the infestation densities that occur on naturally infested fish for both specialist and generalist mussels, and 3) determine what physical, environmental, and/or behavior fish traits may promote mussel infestation.

Both *Elliptio* (Lellis et al. 2013) and *Utterbackia* (Watters and O'Dee 1998) release mucus nets and are considered host generalist. Glochidia from both of these generalist mussel genera were recovered from most fish species collected, and with the exception of blackbanded darters (*Percina nigrofaciata*), both mussel species successfully metamorphosed on all fish species it infested. For most fish species, *Elliptio pullata* dominated the infestations, with the exception of *Micropterous salmoides*, which was dominated by *Villosa vibex*. Three fish species, *N.*

*crysoleucas*, *P. nigrofaciata*, and *P. nigromaculatus*, were equally infested by each generalist mussel species, *Elliptio pullata* and *Utterbackia imbecillis*. Additionally, metamorphosis success for both generalist mussels was similar across all fish species, including the three that are equally infested by both generalist mussels. This suggests that there is a lack of competition across generalist mussels for available fish species. Interestingly, the largemouth bass carried very few generalist mussels in comparison to all other fish species inspected. Sunfish (*Lepomis* sp.), crappie (*Pomoxis* sp.), and bass (*Micropterous* sp.) have similar life histories, similar morphologies (i.e.: large gapes), and all interact regularly with vegetation and other debris in their habitat (Casterlin and Reynolds 1978, Lazur and Chapman 1996, Gautreau and Curry 2012, Lellis, White et al. 2013), but while the bass was overwhelmingly infested with the specialist species, *H. subangulata* , it was lightly infested by both generalist mussel glochidia. This suggests that there may be competition for physical space on an already heavily infested fish. Though specialist mussels may infest fewer fish than generalist mussels, they do appear to get a large number of their offspring to a host without having to compete for physical space. On the other hand, generalist mussels who come into contact with an already infested fish run the risk of not being able to effectively attach to a fish host.

Sunfish, crappie, and bass all make spawning nests in the sandy, gravelly river bottom, which would all potentially exposes them to specialist mussels (Klunzinger et al. 2012) . All sunfish and crappie collected were fairly small and their diets would most likely not include many larger prey items such as small fish and earthworms. Thus, they may not attack a mussel lure until they are large adults. Most sunfish collected may not have been in breeding condition at collection time and would be mostly exposed to mucus nets before mussel lures.

Golden shiners (Carp family) were frequently infested but did not appear to be as suitable of a host as bluegill. Even across sunfish species, bluegill was the most ecologically suitable host by a large margin. The other fish species appeared to be functionally redundant for generalist mussel glochidia. This suggests that even though generalist mussels have the ability to successfully infest and metamorphose on many different fish species, there may be a primary host with many functionally redundant backup hosts. In Line Creek, bluegill appears to be the primary host with other sunfish species and golden shiner being the primary redundant backup hosts. An analogous situation occurs among herbivorous reef fish. There are many reef fish that eat macroalgae, but a very small subset of those fishes will account for the vast majority of macroalgal consumption. Though there appears to be a wide range of fish species that graze macroalgae and functional redundancy amongst species, in reality, there are very few primary grazers and removing these grazers would likely lead to macroalgae overgrowth in a coral reef system (Rasher et al. 2013).

The same may hold true for the generalist mussels in Line Creek. If the primary host is removed, the functional redundancy of the other fish species may not be enough to maintain generalist mussel populations. Though the extirpation of bluegill is unlikely – it is representative of degraded and human-influenced stream systems (Moyle, Smith et al. 1982) – the same patterns of host use observed in Line Creek may be consistent for generalist mussels across river drainages where fish assemblages are different. Though glochidia prefer native host fish, they will attach to and metamorphose on introduced species (Moyle and Nichols 1973). Bluegill may not be the ideal host and are not typically found in stream communities, but they are apt to living

in disturbed habitats, thus may become a suitable substitute for primary hosts during habitat degradation and modification. Line Creek is a heavily urbanized stream with two impoundments that may be the source of bluegill. Through the modification of Line Creek, fish diversity was likely modified and the primary host of many mussel species may have been extirpated. As a result, generalist mussels may have been forced to shift to bluegill when their primary hosts are lost.

Overall, to maintain freshwater mussel biodiversity, it is important to maintain habitat heterogeneity and fish species diversity. The presence of vegetation and debris in the water column allow the mucus nets of generalist mussel to interact with and attach to potential fish hosts to a greater extent if these elements were missing. Additionally, maintenance of variable habitats promote fish species diversity, increasing the potential for the presence of suitable host fish for specialist mussels, as well as primary and functionally redundant host fish for generalist mussels (Miller et al. 2009; Muotka et al. 2012).

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**Tables and Figures**

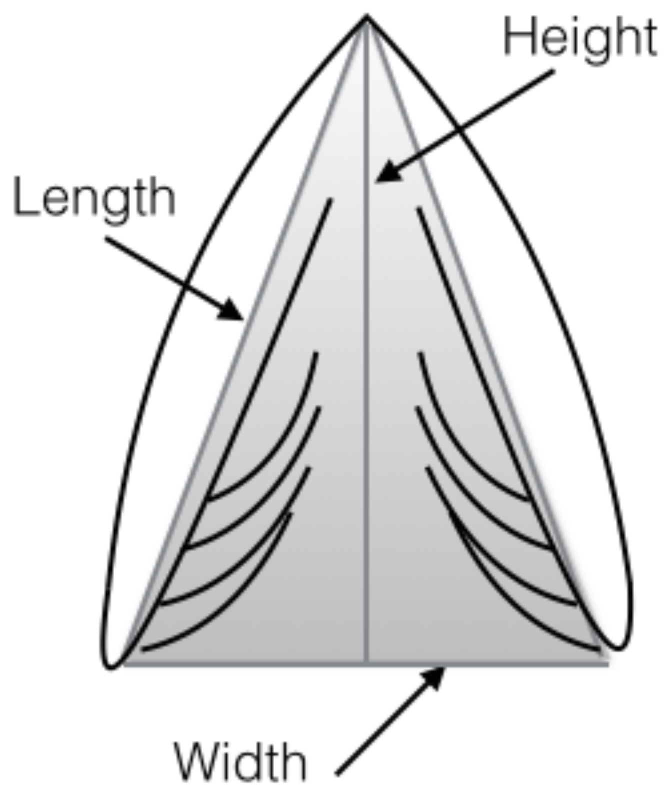


Figure 3.1. Gape Measurements. Illustrated dorsal view of a fish mouth. Solid gray triangle is the ears that was measured (Gape Size). $Height = SQRT((0.5Width)^2 + Length^2)$. $Gape\ Size = 0.5Width\ X\ Height$.

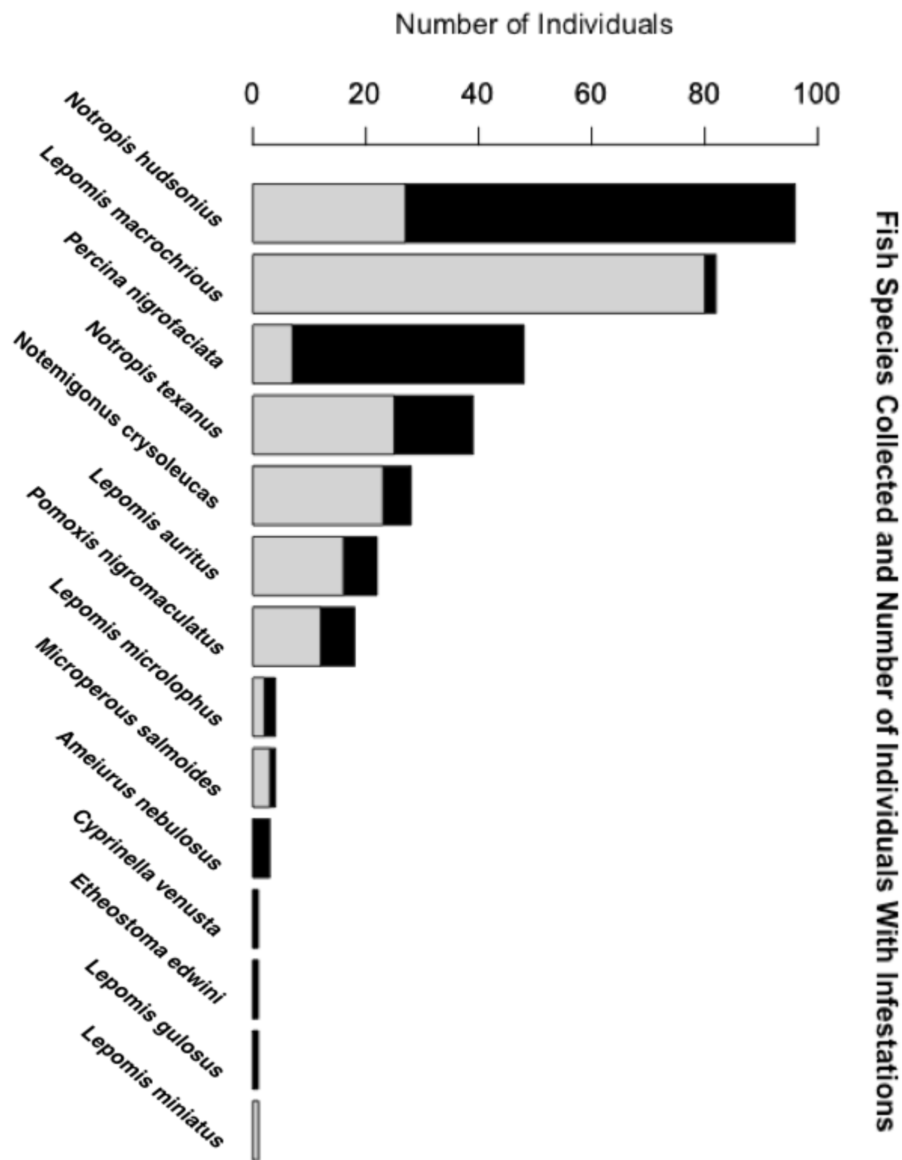


Figure 3.2. Fish Species Collection Effort at Line Creek. Black bars are the total number of individuals of each fish species collected. Grey bars represent the number of individuals infested.

Table 3.1. Line Creek Mussel Infestations

| Fish Species | Sample Size | Infested | Percent Infested | Mussels Recovered | Mussels/Fish |
|---|---|---|---|---|---|
| *Ameriurus nebulosus* | 3 | 1 | 33.33 | 1 | 1 |
| *Cyprinella venusta* | 1 | 0 | 0 | 1 | 0 |
| *Etheostoma edwini* | 1 | 0 | 0 | 0 | 0 |
| *Lepomis auritus* | 22 | 16 | 72.73 | 123 | 7.69 |
| *Lepomis gulosus* | 1 | 0 | 0 | 0 | 0 |
| *Lepomis macrochirus* | 82 | 80 | 97.56 | 371 | 4.64 |
| *Lepomis microlophus* | 4 | 2 | 50 | 28 | 14 |
| *Lepomis miniatus* | 1 | 1 | 100 | 8 | 8 |
| *Micropterous salmoides* | 4 | 3 | 75 | 431 | 143.67 |
| *Notemigonus crysoleucas* | 28 | 23 | 82.14 | 346 | 15.04 |
| *Notropis hudsonious* | 96 | 27 | 28.13 | 56 | 2.07 |
| *Notropis texanus* | 39 | 25 | 64.10 | 75 | 3 |
| *Percina nigrofaciata* | 48 | 7 | 14.58 | 8 | 1.14 |
| *Pomoxis nigromaculatus* | 18 | 12 | 66.67 | 29 | 2.42 |

Table 3.2. Fish Infestation by Mussel Species. *Elliptio pullata* glochidia (EG), juveniles (EJ), total infestation (ET), and percent metamorphosis (EM); *Utterbackia imbecillis* glochidia (UG), juveniles (UJ), total infestation (UT), and percent metamorphosis (UM), *Toxolasma pullus* glochidia (TG), juveniles (TJ), total infestation (TT), and percent metamorphosis (TM); *Villosa vibex* glochidia (HG), juveniles (HJ), total infestation (TT), and percent metamorphosis (HM). All *M. salmoides* died in transit and glochidia were unable to complete metamorphoses. The lack of information on juveniles is denoted by "-".

| Fish.Species | EG | EJ | ET | EM | UG | UJ | UT | UM | TG | TJ | TT | TM | VG | VJ | VT | VM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *A. nebulosus* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *C. venusta* | 0 | 1 | 1 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *E. edwini* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *L. auritus* | 57 | 62 | 119 | 52.1 | 3 | 1 | 4 | 33.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *L. gulosus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *L. macrochirus* | 89 | 208 | 297 | 70 | 33 | 30 | 63 | 47.6 | 5 | 0 | 5 | 0 | 0 | 0 | 0 | 0 |
| *L. microlophus* | 14 | 7 | 21 | 33.3 | 5 | 2 | 7 | 28.6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *L. miniatus* | 0 | 0 | 0 | 0 | 3 | 5 | 8 | 62.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *M. salmoides* | 8 | - | 8 | - | 9 | - | 9 | - | 0 | - | 0 | - | 414 | - | 414 | - |
| *N. crysoleucas* | 83 | 141 | 224 | 63 | 55 | 67 | 122 | 54.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *N. hudsonious* | 38 | 7 | 45 | 15.5 | 9 | 2 | 11 | 18.2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *N. texanus* | 43 | 22 | 65 | 33.8 | 8 | 2 | 10 | 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *P. nigrofaciata* | 4 | 0 | 4 | 0 | 2 | 2 | 4 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *P. nigromaculatus* | 1 | 15 | 16 | 93.7 | 1 | 12 | 13 | 92.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Table 3.3: Recovered Mussel Species Identity. ACF COI Database results.

| Mussel Species | Number Sequenced | % Identity | % Coverage | E-Value |
|---|---|---|---|---|
| *Elliptio pullata* | 284 | 99 | 82 | 0.00 |
| *Villosa vibex* | 11 | 92 | 93 | 0.00 |
| *Toxolasma paulus* | 6 | 99 | 95 | 0.00 |
| *Utterbackia imbecillis* | 45 | 100 | 100 | 0.00 |
| *Corbicula fluminea* | 4 | 99 | 95 | 0.00 |
| *Pisidium Casertanum* | 2 | 94 | 98 | 0.00 |

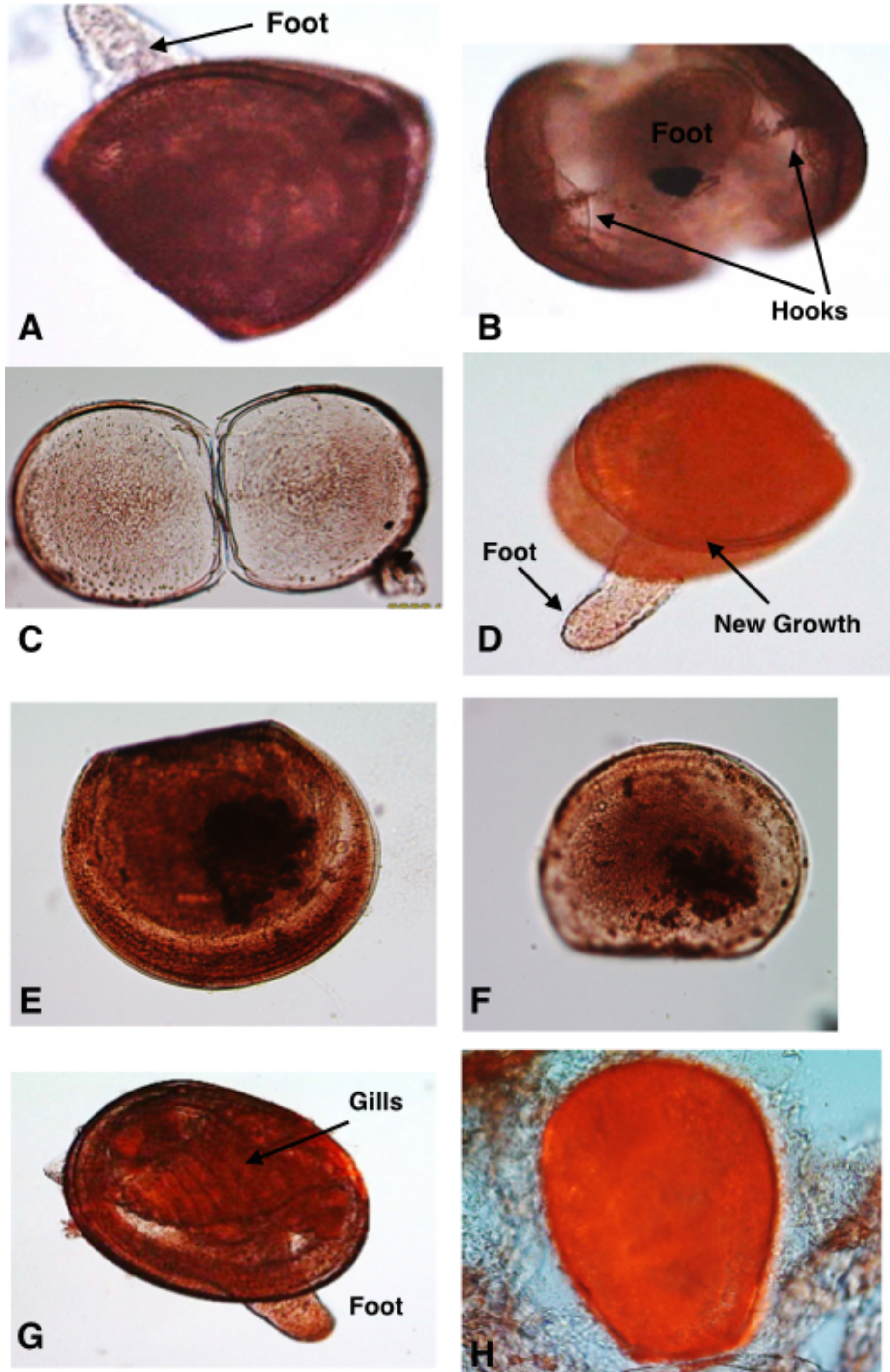


Figure 3.3. Observed Mussel Morphology. *Elliptio pullata* and *Toxolasma pullus* were not differentiated morphologically, but were identified genetically using COI. A) *U. imbecillis* with protruding ciliated foot, B) *U. imbecillis* showing hooked shell morphology, C) *E. complanata* or *T. paulum* glochidia, D) *E. complanata* or *T. paulum* juvenile with protruding ciliated foot and new growth on shell, E and F) *Corbicula fluminea*, G) Adult *Pisidium* species, H) *Villosa vibex*.

Table 3.4. Ecological Host Index Table: Ecological host suitability was assessed for all fish species from which mussels were recovered. Ecological host suitability was assessed for *Elliptio*, *Utterbackia*, and *Toxolasma* juveniles. Because *Micropterous salmoides* died in transit, only glochidia were successfully recovered for *Villosa*.

| Fish Host Species | *Elliptio* | *Utterbackia* | *Toxolasma* | *Villosa* |
|---|---|---|---|---|
| **Centrarchidae** | | | | |
| *Lepomis auritus* | 0.02 | 0 | 0 | 0 |
| *Lepomis gulosus* | 0 | 0 | 0 | 0 |
| *Lepomis macrochirus* | 1 | 1 | 1 | 0 |
| *Lepomis microlophus* | 0 | 0 | 0 | 0 |
| *Lepomis miniatus* | 0 | 0 | 0 | 0 |
| *Micropterous salmoides* | 0 | 0 | 0 | 1 |
| *Pomoxis nigromaculatus* | 0 | 0.01 | 0 | 0 |
| **Cyprinidae** | | | | |
| *Cyprinella venusta* | 0 | 0 | 0 | 0 |
| *Notemigonus crysoleucas* | 0.07 | 0.22 | 0 | 0 |
| *Notropis hudsonius* | 0.01 | 0.03 | 0 | 0 |
| *Notropis texanus* | 0.02 | 0.01 | 0 | 0 |
| **Ictaluridae** | | | | |
| *Ameiurus nebulosus* | 0 | 0 | 0 | 0 |
| **Percidae** | | | | |
| *Etheostoma edwini* | 0 | 0 | 0 | 0 |
| *Percina nigrofaciata* | 0 | 0 | 0 | 0 |

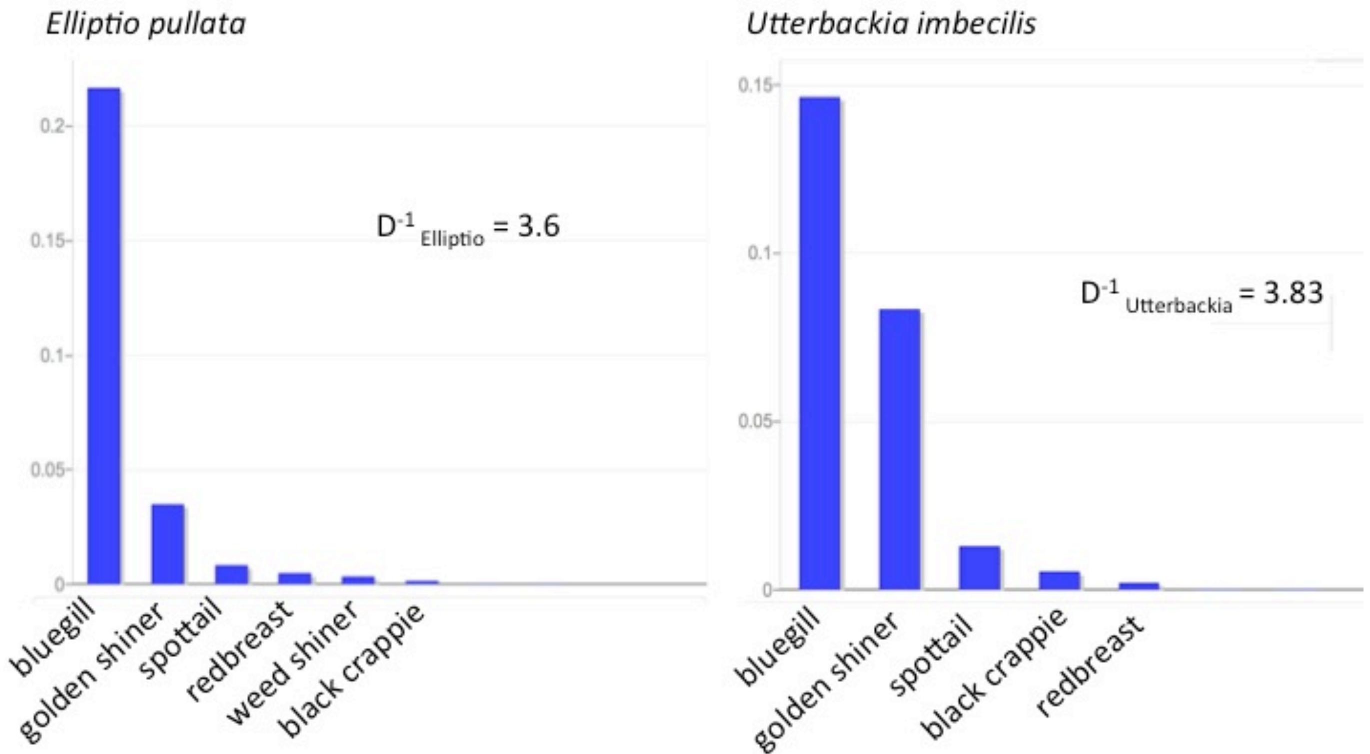


Figure 3.4 Inverse Simpson's Diversity Index of Ecologically Functional Hosts.

Table 3.5. Non-Normalized AIC Model of Glochidia Infestation.

| | Estimate | Std. Error | t value | Pr(>\|t\|) | Significance |
|---|---|---|---|---|---|
| Intercept | -0.2183783 | 0.099415 | -2.197 | 0.0287 | * |
| gape | 0.0006465 | 0.0002045 | 3.162 | 0.0017 | ** |
| surwcol | 0.473573 | 0.0895764 | 5.287 | 2.14e-07 | *** |
| macvascu | 0.5907487 | 0.539154 | 10.957 | <2e16 | *** |
| detritus | -4.8196 | 0.0924636 | -5.212 | 3.11e-07 | *** |
| muck | 0.142817 | 0.72385 | 1.973 | 0.0492 | * |
| cobble | 0.1631022 | 0.735424 | 2.218 | 2.72e-02 | * |

Notes: Null deviance: 93.637 on 374 degrees of freedom, Residual deviance: 62.962 on 368 degrees of freedom, ***, ** and * denote significance at the 0.1, 1 and 5% levels, respectively.

Table3.6. Non-normalized AIC Model of Glochidia Infestation Density

| | Estimate | Std. Error | t value | Pr(>\|t\|) | Significance |
|---|---|---|---|---|---|
| Intercept | 16.95126 | 9.78811 | 1.732 | 0.08416 | . |
| fish | -1.43833 | 0.56742 | 2.535 | 0.01167 | * |
| gape | 0.06922 | 0.01005 | 6.887 | 2.55e-11 | *** |
| surwcol | 10.51936 | 4.90858 | 2.143 | 0.03278 | * |
| macvascu | -36.99303 | 6.42148 | 5.761 | 1.79e-08 | *** |
| fshcrcrb | -11.89189 | 4.26023 | 2,791 | 0.00553 | ** |
| muck | -32.36417 | 5.93722 | 5.451 | 9.30e-08 | *** |
| vegetat | 32.36288 | 5.56047 | 5.82 | 1.30e-08 | *** |

Notes: Null deviance: 93.637 on 374 degrees of freedom, Residual deviance: 62.962 on 368 degrees of freedom, ***, **, *, and . denote significance at the 0.1, 1, 5, and 10% levels, respectively.

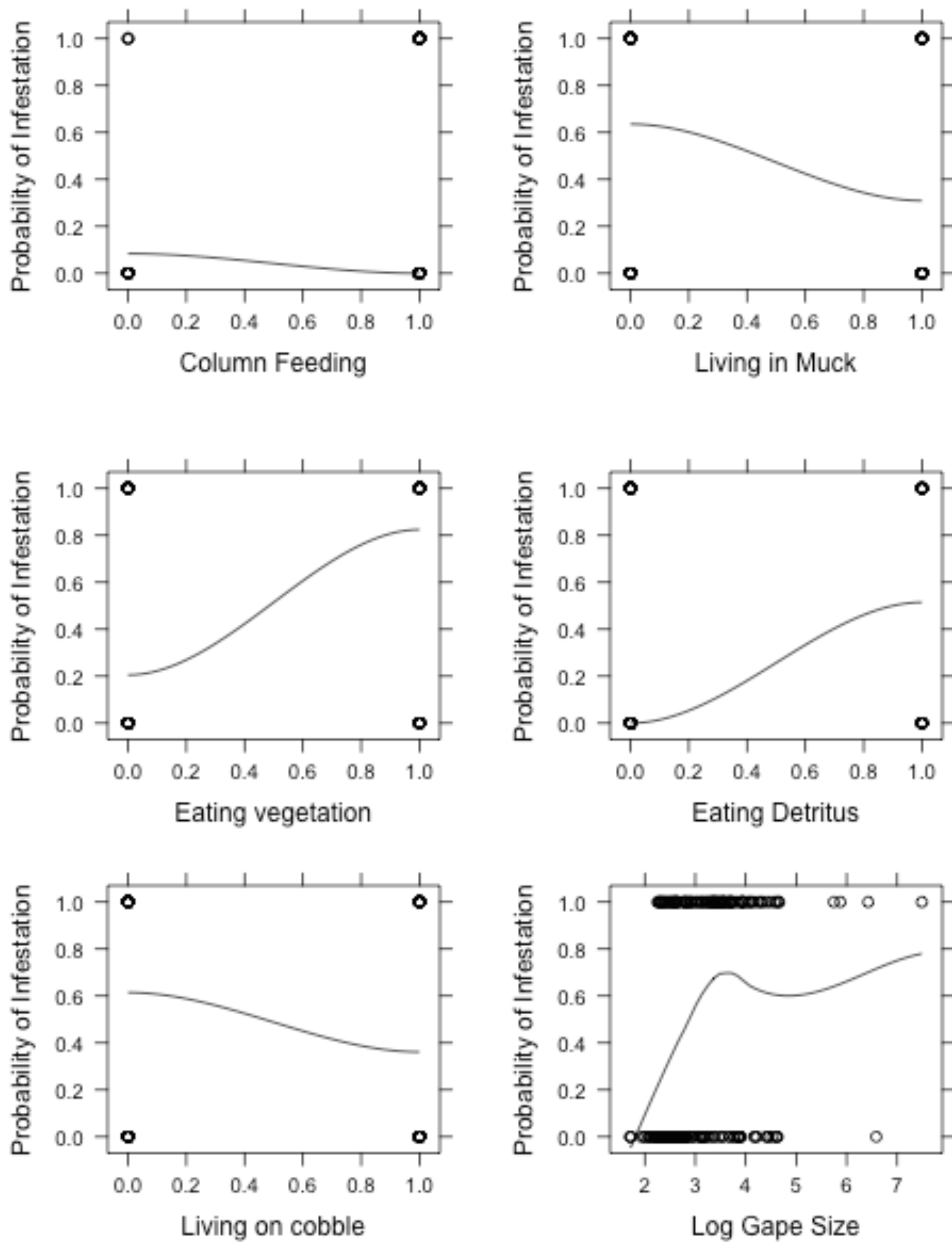


Figure 3.5. Probability of Infestation by Environmental or Behavioral Trait. All environmental and behavioral traits are binomial variables except Gape Size, which is continuous. Density of each binomial variable determines the shape of the curve.

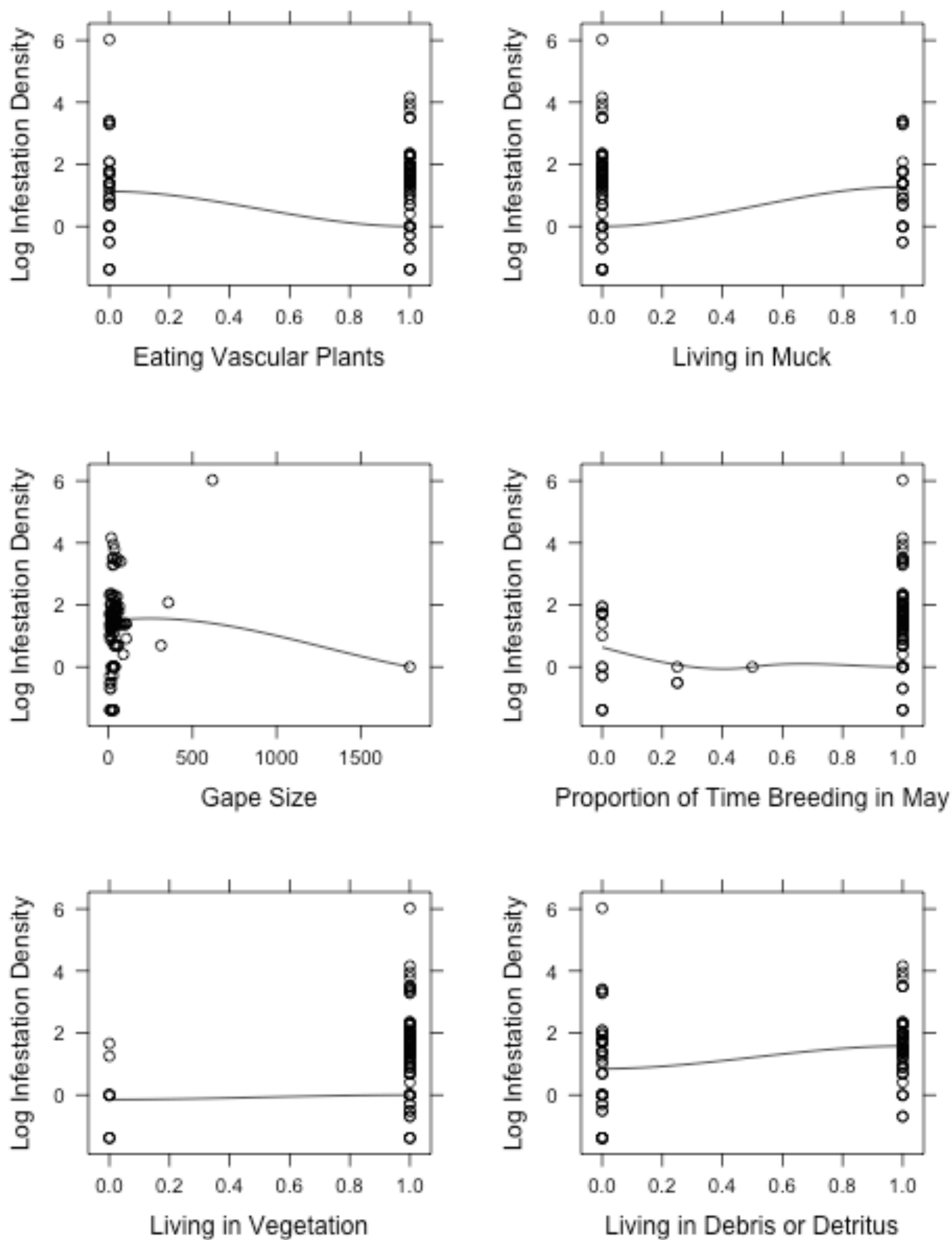


Figure 3.6. Higher infestation density with environmental and behavioral traits. Infestation density is a logarithmic distribution, so infestation density was log transformed. All variables on the x-axis are binomial, except “Gape Size” and “Proportion of Time Breeding in May”.

Table 3.7. Normalized AIC Model of Presence of Glochidia Infestation

| | Estimate | Std. Error | t value | Pr(>\|t\|) | Significance |
|---|---|---|---|---|---|
| Intercept | -0.2669 | 0.1329 | -2.01 | 0.0459 | * |
| gape | 0.0005 | 0.0002 | 2.28 | 0.0238 | * |
| surwcol | 0.5109 | 0.1017 | 5.02 | 0.0000 | *** |
| macvascu | 0.5699 | 0.0916 | 6.22 | 0.0000 | *** |
| detritus | -0.4541 | 0.1087 | -4.18 | 0.0000 | *** |
| muck | 0.1762 | 0.0886 | 1.99 | 0.0479 | * |
| cobble | 0.1529 | 0.0822 | 1.86 | 0.0644 | . |

Notes:Null deviance: 51.495 on 205 degrees of freedom, Residual deviance: 36.109 on 199 degrees of freedom, ., *, **, *** denote significance at the 10, 5, 1, 0.1% levels, respectively.

Table 3.8. Normalized AIC Model of Glochidia Infestation Density

| | Estimate | Std. Error | t value | Pr(>\|t\|) | Significance |
|---|---|---|---|---|---|
| Intercept | -13.8417 | 8.6489 | -1.60 | 0.1111 | |
| gape | 0.0570 | 0.0137 | 4.17 | 0.0000 | *** |
| macvascu | -23.2609 | 7.6911 | -3.02 | 0.0028 | ** |
| may | 16.3179 | 7.3369 | 2.22 | 0.0273 | * |
| muck | -32.7162 | 7.4678 | -4.38 | 0.0000 | *** |
| vegetat | 40.6559 | 9.1980 | 4.42 | 0.0000 | *** |
| debrdetr | -13.7498 | 6.8341 | -2.01 | 0.0456 | * |

Notes: Null deviance: 182358 on 204 degrees of freedom, Residual deviance: 149929 on 198 degrees of freedom, ***, ** and * denote significance at the 0.1, 1 and 5% levels, respectively.

# CHAPTER 4

# COMPARATIVE TRANSCRIPTOMICS BETWEEN DISEASE-RESISTANT AND SUSCEPTIBLE *ACROPORA PALMATA* IN THE U.S. VIRGIN ISLANDS[1]

[1]Bockrath, K.D., E.M Muller, J.P. Wares. *To be submitted to PeerJ*

## Abstract

White Syndrome Disease (WSD) has severely reduced the number of elkhorn coral (*Acropora palmata*) in the Florida Keys. With so few *A. palmata* reefs remaining, studying their response to disease is difficult. However, *A. palmata* in the US Virgin Islands (USVI) have, until recently, been relatively unaffected by WSD. The USVI population thus provides a dynamic means of studying *A. palmata* response to WSD. Sixteen USVI *A. palmata* colonies were followed over 10 years and appear to show differences in disease tolerance, where some colonies have fewer occurrences of WSD than others and suffer less tissue loss if they become infected. To determine if an underlying genetic basis is contributing to disease response, coral nubbins were collected from these 16 *A. palmata* colonies; 8 from disease resilient corals and 8 from disease susceptible corals. Total RNA was sequenced and differential expression between coral colonies was compared and assessed in relation to their disease history and health status at time of collection. We find no apparent difference in constitutive expression patterns between these two groups.


## Introduction

The foundation of coral reefs is established primarily by large, stony corals (Scleractinia). One of the dominant reef building corals in the Caribbean is *Acropora palmata* (Porter et al. 2002; Pandolfi 2002). *Acropora palmata* has severely declined due to a series of white syndrome diseases (WSD), such as white band and white pox disease, reducing *A. palmata* numbers by over 90% (Patterson et al. 2002; Porter et al. 2002). Without these important reef-building corals, key habitat for one of the most bio-diverse marine ecosystems on Earth is being decimated.

Corals are large, long-lived colonial organisms that have complex interactions between each individual within the colony as well as the microbial community that lives within the mucus corals produce. The microbial community within coral mucus functions as both a nutrient resource (ie: nitrogen fixation) as well as a probable support of innate immunity through antimicrobial interactions within the community (Sutherland et al. 2010). Though it appears that the microbial community is mostly beneficial, there are some community members that can – under certain conditions – act as disease agents or associates (Sutherland 2010, Gignoux-Wolfsohn 2015). Each of these potential pathogens are normally found in the microbiome of healthy corals and remain benign under normal environmental conditions, but when these conditions change their densities may increase to pathogenic levels (Lesser et al. 2007; Muller and Woesik 2012). Thus each of these microbes may indeed cause WSD but are opportunistic pathogens when a corals immune system is suppressed during times of stress (Harvell, Mitchell et al. 2002, Muller 2012).

Sea surface temperatures (SST) have been raising steadily over the last century (Huang et al. 2015) and the occurrence of WSD have risen along with it. When SST gets high, corals become very stressed and are more susceptible to disease (Harvell, Mitchell et al. 2002, Randall, Jordan-Garza et al. 2014, Sweet and Bythell 2015). Not all *Acropora* corals are affected by increased SST in the same way – even those of the same species. In a recent study, variation in heat stress susceptibility was assessed in two populations of *Acropora* coral. One population consistently experienced high SST but had very few bleaching events. The other population did not regularly experience extreme SST but when they did, they went through widespread bleaching events. Through assessing differential gene expression between thermotolerant and susceptible corals, it

was determined that the thermotolerant corals consistently expressed a suite of genes associated with heat stress, even when SST was in normal ranges (Barshis et al. 2013). If front-loading of these genes allow the thermotolerant corals to resist bleaching events, and high SST are directly associated with coral disease, then corals that show disease resilience my also be front-loading the expression of genes that allow them to be less susceptible to disease.

While *A. palmata* populations in most of the Caribbean have been decimated by WSD, *A. palmata* in the US Virgin Islands (USVI) have just started to suffer from WSD. Interestingly, *A. palmata* in the USVI infected with WSD do not often die (Muller and van Woesik 2014). They show variation in the severity of infection and the rate in which they become infected (ie: some get infected every year, while others do not). Because these colonies are all under the same environmental conditions and equally susceptible to developing WSD, the difference in disease occurrence within the USVI colonies may be due to the genetic differences between *A. palmata* colonies. Here, we aim to test if the variation in observed White Syndrome infections is due to genetic differences between corals – specifically, whether there are constitutive differences in how expression of the host genome may influence the outcome of interactions that lead to WSD. We utilize whole transcriptome data to compare gene expression patterns between coral colonies in the USVI and attempt to correlate variation across genetic sites to long-term health trends.

**Methods**

*Coral Tissue Collections*

Sixteen *Acropora palmata* colonies in the US Virgin Islands, St. John were followed over 7 years. Twice a year (March and September) colonies were observed for the presence or absence

of disease. Over this time period, disease instances were recorded along with the disease(s) they exhibited. Colonies that were “Resilient” to disease were defined as exhibiting 5 or less incidences of white pox disease over the 7-year period and having less than 10% tissue loss per disease incident. Colonies were considered “Susceptible” if they exhibited 10 or more incidences of white pox disease over the 7-year observation period with no restriction of the amount of tissue lost per disease incident (Table 4.1). Coral fragments were collected during each biannual observation. Fragments were stored on ice immediately after arriving to the boat and frozen to -80°C once on land.

*Genetic Data Collection and Analysis*

Samples from 16 genetically distinct *A. palmata* colonies (Rogers et al. 2012) that showed either resilience or susceptibility to infection (Table 4.1) were selected for total RNA sequencing. These samples were collected in September 2013. Total RNA extractions were completed using Qiazol RNA extraction method modified from Barshis et al. (2013). TrueSeq RNA PE-75 libraries were generated by the Georgia Genomics Facility and library quality was assessing using an Agilent 2100 Bioanalyzer. All 16 libraries were run on two Illumina Next-Seq 500 High Flow Cell lanes at the Georgia Genomics Facility. Sequences were quality checked using FastQC (Barshis 2013); reads were checked for low quality scores (< 30) and any adapter sequences that were not removed from each library were identified. Low quality reads with a quality score less than 30 were removed and adapter sequences were clipped using FastqMcf (Aronesty 2011). The Tuxedo Suite (Trapnell et al. 2012) was used to assess differential trancriptomics between all pairs of coral colonies as well as between those sets of colonies that show resistance versus susceptibility to white pox infections. In order to isolate the coral RNA sequence fragments from

any RNA from associated symbionts or the microbial community, all 16 *A. palmata* libraries were aligned to 454 data from *Acropora palmata* (Polato et al. 2011) and the *Acropora digitifera* genome (Shinzato et al. 2011) using default parameters in Tophat (Trapnell et al. 2012). In addition to assembling reads to a genome or reference, Tophat will also identify splice sites. Post transcription, mRNA is clipped to remove all introns, transforming premature RNA to mature mRNA, which only consists of exons. Tophat will identify the transcripts that map to the same gene region, but contain different exons. These are identified as potential splice sites. Cufflinks was then used to assemble transcripts. Cufflinks reports a parsimonious transcriptome assembly by reporting the fewest full-length transcript fragments need to describe all splice variants. Cufflinks uses a model of RNA-Seq to assign reads to transcripts and to calculate expression levels. The probability that each rad was a result of a transcript – or expressed gene – is relative to its abundance and its length. Because the expression of a transcript is proportional to its length, transcripts are normalized by the average length of all expressed transcripts. Assembled transcripts were compared using Cuffcompare and then cuffdiff was used to identify differentially expressed genes, transcripts and isoforms (transcripts from paralogous genes), as well as identify differential splicing (same transcript with different exons) and any differential promoter use. Transcripts are normalized by their length and the expression of a transcript is proportional to the number of reads sequenced from that transcript after normalization and expression levels are defined as the Fragments per kilobase of transcript per million fragments mapped (FPKM). The expression quality of each library as well as differential gene expression, splice variants, and differences in transcriptional start sites (TSS) and promoter use were visualized in the R-project package cummeRbund (Trapnell et al. 2012).

## Results

*Differential Gene Expression Assay*

Over 520 million reads were sequenced for all 16 RNA-seq libraries, giving an average of 32,545,492 reads were sequenced per library (Table 4.2). RNA quality was not ideal (RIN values between 2.5 and 3.5), but were consistent across all libraries allowing gene expression differences to be observable without significant bias across libraries (Romero et al. 2014). Sequence coverage varied between alignments to *A. palmata* and *A. digitifera* with more consistent but lower coverage in the *A. palmata* alignment, but more variable and greater coverage in the *A. digitifera* alignment (Tables 4.2-3). RNA libraries were of equivalent quality and gene density was consistent (Figure 4.1).

There is high variation between all 16 USVI *A. palmata* libraries with 317 genes significantly ($P<0.05$) differentially expressed across all libraries when aligned to *A. digitifera* and 6418 when aligned to *A. palmata*. There were no significantly differentially expressed isoforms or TSS when aligned to the *A. digitifera* genome, but when libraries were aligned to the *A. palmata* 454 data, there were 6298 TSS and 6152 differentially expressed isoforms. There were no differences in promoter use and no detected splice variants.

When libraries were grouped as "Susceptible" and "Resiliant", there initially appeared to be a suite of genes that were significantly upregulated in susceptible corals (Figure 4.2), but under further inspection, there are not significantly differentially expressed genes between the groups of "Susceptible" and "Resilient" colonies, but there are significant differences between colony 15 (in which group) and all other colonies (Figure 4.3). There are 6 isoforms that were more

highly expressed in colony 15 than any other colony (Figure 4.4). The higher expression profile of colony 15 was not due to difference in relative expression levels because colony 15 shared identical expression profiles with three other colonies (Figure 4.5), which did not show any significant difference in expression levels of any gene or isoforms. The isoforms that were significantly up regulated in colony 15 were ribosomal genes. Once colony 15 was removed from analysis, there were no significant differentially expressed genes between “Resilient” or “Susceptible” corals or “Healthy” and “Diseased” (Figure 4.6).

**Discussion**

Corals are complex systems of interacting microbes, algae, and metazoans. To fully understand how corals become diseased and respond to disease, the entire coral complex needs to be studied. Here we investigated the potential for constant underlying gene expression differences in corals that, over years, have show resilience or susceptibility to WSD. Barshis et al. (2013) compared genome wide gene expression of thermally sensitive and resilient corals and found that under control conditions, thermally resilient corals up-regulated 60 genes associated with thermal stress while thermally sensitive corals did not. Up-regulating these 60 genes while not under heat stress may allow thermally resilient corals to prevent bleaching events when water temperatures rise. Using a similar approach to Barshis et al. (2013), we assess gene expression in 16 USVI *Acropora palmata* colonies with varying disease histories, but interestingly did not see any differentially regulated genes between coral colonies, regardless of health (healthy or diseased) or disease status (resilient or susceptible). In concordance with Granados-Cifuentes et al. (2013), there was considerable variation in gene expression between coral colonies when health or disease status was not designated, showing that reef-building corals seem to naturally have high

variance in gene expression despite being collected from the same reef. Interestingly, in Granados-Cifuentes et al. (2013), the genes that were differentially expressed are those involved in innate immunity, apoptosis, response to stimulus, as well as heat stress, but the variation in expression did not correspond to the colony's genotype. Unlike Granados-Cifuentes et al. (2013), the 16 USVI corals examined here show no significant gene expression patterns between resilient and susceptible colonies. One colony, Colony 15, shows higher expression of ribosomal RNA such as 28S and ITS1. With high levels of gene expression variation there should be the potential for variation in response to stress and adaptation to environmental changes, thus the variation we see in disease occurrence between colonies my have little to do with a consistently expressed suit of genes, but the plasticity of response each coral has to its environment.

Corals are colonial organisms and even though each polyp is genetically identical and are connected to each other, each polyp is its own organism and may have a different gene expression profile from a polyp 10 cm away. All nubbins collected from each of the 16 USVI colonies were healthy tissue, even from colonies that were disease at the time of collection. Because of this, we can only really determine if the coral, overall, has some underlying constant gene expression that may make it more or less susceptible to infection. Wright et al. (2015) investigated gene expression patterns in diseased and healthy corals. They found that significant expression differences in diseased corals were at the site of infection and that the rest of the coral colony was not significantly different from healthy, unaffected colonies. Alternatively, each colony may express a suite of genes once initially infected and because gene expression quickly returns to normal after a stress response (Seneca and Palumbi 2015), each coral nubbin may have

been collected too late after initial disease occurrence, which would have prevented the detection of expression differences.

A gene expression assay across tissues at the onset of disease would tell us more about how each of the 16 colonies are responding to infection. Both “Resilient” and “Susceptible” *A. palmata* colonies have had white pox at some point over the 7-year survey and each coral may respond to stress and disease differently. Each colony may express the same genes at relatively the same level, but corals that are more “Susceptible” to disease may have a delay in gene expression, causing the disease to progress further than those that are more “Resilient” to infection, thus causing them to lose more tissue. One factor that may be important in WSD susceptibility is the expression of stress related proteins, including those associated with thermal stress. Allene oxide synthase and lipoxygenase (AOS-LOX), a fusion protein involved in coral stress response, is more sensitive to moderate temperature changes than heat shock proteins, where heat shock proteins are responsive to sever temperature changes (Lõhelaid et al. 2015). Resilient corals may over express, or are faster at expressing, AOX-LOX in response to stress, making them less susceptible to rises in SST, thus less susceptible to disease. Another potential factor is variation in regulatory networks responsible for gene expression pathways which may lead to rapid or delayed downstream gene expression (Li et al. 2006; Yeange and Jaakkola 2003).

Because coral nubbins were not immediately frozen or preserved in RNAlater in the field, RIN degradation occurred, but all libraries were equally degraded. Even with severe RNA degradation, comparisons of gene expression between samples is not compromised as long as the RIN values are similar and the degradation in RIN value is not associated with a specific

treatment (e.g.: different tissue types) (Romero et al. 2014). RNA degradation would most likely reduce our ability to detect rare transcripts or minor differences in gene expression patterns, but because we are searching for “front loaded” genes, RNA degradation should not have drastically hindered our study. A factor that would have a larger effect on our ability to detect gene expression differences would be the initial alignment step. Each USVI *A. palmata* library was aligned to two different references: 454 data from *A. palmata* (Polato et al. 2011) and an *A. digitifera* genome (Shinzato et al. 2011). Aligning reads to 454 data limits our downstream analyses to what genes were captured while generating the 454 data. Similarly, aligning our data to the *A. digitifera* genome will exclude any reads that are too divergent from the reference. With *A. digitifiera* being approximately 12 million years divergent from *A. palmata* (van Oppen et al. 2001), though not a massive time scale for corals, it is significant. Genes involved with innate immunity, including heat shock proteins, can evolve quickly (Fares et al. 2002; Harpur and Zayed 2013), and when enough time, the ability to successfully align reads to a highly divergent region would be significantly impaired.

In conclusion, coral disease is a complicated issue that requires the investigation of interacting taxa along with changing environmental conditions. Gene expression assess and shifts in microbial relationships provide important insights to the potential causes of WSD as well as potential genetic factors that may make a colony more or less susceptible to succumbing to thermal stress and disease. With most coral reefs in sharp decline due to rising sea surface temperatures and WSD, further investigation into the adaptive potential remaining reefs have is warranted.

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**Figures and Figures**

Table 4.1. Disease Status and Condition of USVI St. John *Acropora palmata*. Disease resilient was defined as having 5 incidences of White Pox (WPX) disease or less over the 7 year period, also <10% of tissue lost to disease per incident. Disease susceptible was defined as having 10 or more incidences of WPX disease over the 7-year period, with no restriction on amount of tissue lost per incident.

| Sample ID | Disease Type | Health Status | WPX incidences Feb 2003-Dec 2009 |
|---|---|---|---|
| 1 | Resilient | Healthy | 4 |
| 2 | Resilient | Healthy | 1 |
| 3 | Susceptible | Healthy | 10 |
| 4 | Resilient | Healthy | 0 |
| 5 | Susceptible | Healthy | 11 |
| 6 | Susceptible | WSD | 16 |
| 7 | Susceptible | Healthy | 12 |
| 8 | Resilient | Healthy | 2 |
| 9 | Resilient | WSD | 5 |
| 10 | Resilient | WSD | 5 |
| 11 | Resilient | Healthy | 2 |
| 12 | Susceptible | WDS | 13 |
| 13 | Resilient | WSD | 3 |
| 14 | Susceptible | WSD | 11 |
| 15 | Susceptible | Healthy | 15 |
| 16 | Susceptible | WSD | 12 |

Table 4.2. Acrpora palmata RNA-Seq Transcriptome Coverage: FPKM values after alignment to the *Acropora palmata* 454 reference.

| | Overall FPKM | | | Mean per Gene | | | Median per Gene | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Library | FPKM | Low | High | FPKM | Low | High | FPKM | Low | High | Trans/Gene |
| 1 | 4532594 | 2885113 | 6180092 | 225.67 | 143.64 | 307.70 | 25.13 | 17.36 | 32.58 | 20086 |
| 2 | 2173230 | 1633517 | 2713049 | 146.93 | 110.44 | 183.43 | 42.87 | 29.32 | 55.67 | 14792 |
| 3 | 4662862 | 3008031 | 6317704 | 228.87 | 147.65 | 310.10 | 25.60 | 17.82 | 33.07 | 20374 |
| 4 | 5126421 | 3228462 | 7024407 | 258.69 | 162.91 | 354.46 | 26.45 | 18.55 | 34.23 | 19818 |
| 5 | 4085066 | 2656599 | 5513608 | 202.38 | 131.61 | 273.15 | 24.13 | 16.51 | 31.67 | 20186 |
| 6 | 4339858 | 2869162 | 5810571 | 220.40 | 145.71 | 295.08 | 24.71 | 17.13 | 32.07 | 19692 |
| 7 | 4336484 | 2945234 | 5727792 | 221.47 | 150.42 | 292.53 | 23.20 | 16.03 | 30.03 | 19581 |
| 8 | 5525101 | 3507818 | 7542417 | 254.17 | 161.37 | 346.97 | 21.54 | 14.61 | 28.01 | 21739 |
| 9 | 3409944 | 2275724 | 4544175 | 172.50 | 115.12 | 229.89 | 21.99 | 15.08 | 28.57 | 19768 |
| 10 | 3588681 | 2346430 | 4830946 | 167.56 | 109.56 | 225.56 | 20.89 | 14.46 | 27.15 | 21418 |
| 11 | 3882326 | 2563455 | 5201226 | 166.27 | 109.78 | 222.76 | 19.58 | 13.76 | 25.13 | 23350 |
| 12 | 5126884 | 3360896 | 6892879 | 253.59 | 166.24 | 340.94 | 25.15 | 17.61 | 32.45 | 20218 |
| 13 | 5384601 | 3535514 | 7233694 | 263.48 | 173.00 | 353.96 | 24.07 | 17.07 | 30.81 | 20437 |
| 14 | 6483344 | 4102227 | 8864516 | 328.14 | 207.62 | 448.65 | 24.33 | 16.56 | 31.62 | 19759 |
| 15 | 2237798 | 1771398 | 2704199 | 148.95 | 117.91 | 180.00 | 34.44 | 22.91 | 45.62 | 15024 |
| 16 | 4738781 | 3201027 | 6276648 | 200.67 | 135.55 | 265.79 | 18.39 | 13.01 | 23.47 | 23616 |
| Total | 69633975 | 45890607 | 93377923 | 3459.74 | 2288.53 | 4630.97 | 402.47 | 277.79 | 522.15 | 319858 |
| Average | 4352123 | 2868162 | 5836120 | 216.23 | 143.03 | 289.44 | 25.15 | 17.36 | 32.63 | 19991.13 |
| Median | 4436226 | 2915173 | 5995331 | 220.94 | 144.68 | 293.81 | 24.23 | 16.82 | 31.65 | 20136 |

Table 4.3. Acrpora palmata RNA-Seq Transcriptome Coverage: FPKM values after alignment to *Acropora digitifera* genome reference.

| | Overall FPKM | | | Mean per Gene | | | Median per Gene | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Library | FPKM | Low | High | FPKM | Low | High | FPKM | Low | High | Trans per Gene |
| 1 | 2607540 | 1999387 | 3215964 | 140.88 | 108.02 | 173.75 | 53.06 | 32.41 | 72.98 | 18510 |
| 2 | 9900615 | 7099312 | 12701951 | 524.53 | 376.12 | 672.95 | 102.44 | 65.94 | 135.89 | 18876 |
| 3 | 52691621 | 34090160 | 71293149 | 2371.25 | 1534.14 | 3208.36 | 55.99 | 36.83 | 74.41 | 22222 |
| 4 | 48498680 | 29747029 | 67250404 | 2157.03 | 1323.03 | 2991.03 | 58.58 | 38.03 | 78.42 | 22485 |
| 5 | 42377191 | 27304397 | 57450068 | 1966.18 | 1266.85 | 2665.52 | 55.28 | 35.82 | 74.31 | 21554 |
| 6 | 34142168 | 22835238 | 45449135 | 1557.72 | 1041.84 | 2073.59 | 57.86 | 37.02 | 76.78 | 21919 |
| 7 | 45659373 | 29541753 | 61777045 | 2154.35 | 1393.87 | 2914.83 | 53.72 | 35.05 | 71.15 | 21195 |
| 8 | 39339331 | 24064750 | 54613950 | 1632.68 | 998.74 | 2266.61 | 46.61 | 30.07 | 62.17 | 24096 |
| 9 | 40117611 | 27020324 | 53214957 | 1858.84 | 1251.98 | 2465.71 | 52.22 | 33.64 | 70.03 | 21583 |
| 10 | 66768658 | 44786609 | 88750758 | 2843.40 | 1907.27 | 3779.52 | 48.38 | 31.71 | 63.28 | 23483 |
| 11 | 39784517 | 25582826 | 53986307 | 1494.81 | 961.21 | 2028.42 | 43.18 | 28.51 | 56.87 | 26616 |
| 12 | 44422077 | 28556873 | 60287319 | 2006.23 | 1289.71 | 2722.76 | 56.70 | 36.97 | 75.63 | 22143 |
| 13 | 47404249 | 32573926 | 62234647 | 1968.77 | 1352.85 | 2584.71 | 53.64 | 35.30 | 71.03 | 24079 |
| 14 | 48867239 | 30876736 | 66857798 | 2264.47 | 1430.80 | 3098.14 | 57.05 | 36.48 | 76.62 | 21581 |
| 15 | 43152931 | 29891798 | 56414126 | 2968.08 | 2055.97 | 3880.19 | 73.48 | 46.32 | 100.30 | 14540 |
| 16 | 42827269 | 28315568 | 57339078 | 1884.09 | 1245.68 | 2522.50 | 40.40 | 26.66 | 53.05 | 27732 |
| Total | 648561070 | 424286686 | 872836656 | 29793.31 | 19538.08 | 40048.59 | 908.59 | 586.76 | 1212.92 | 352614 |
| Average | 40535066 | 26517917 | 54552291 | 1862.08 | 1221.13 | 2503.04 | 56.79 | 36.67 | 75.81 | 22038.38 |
| Median | 42990100 | 28436220 | 57394573 | 1967.47 | 1278.28 | 2625.11 | 54.50 | 35.56 | 73.64 | 22031 |

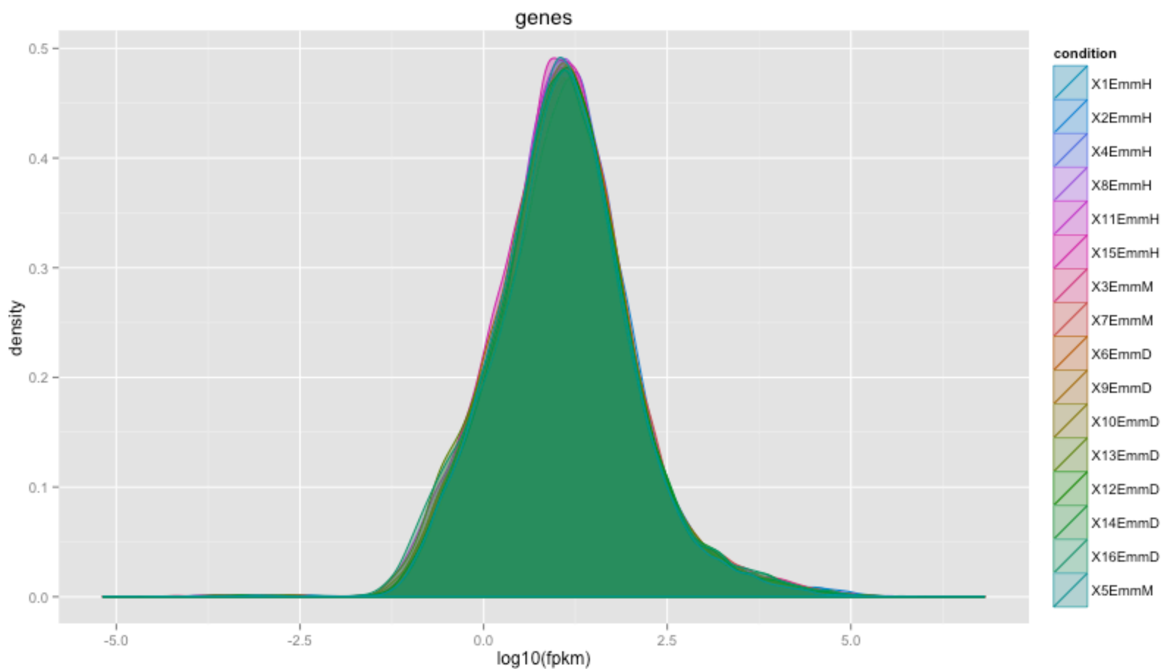


Figure 4.1. Gene Density (FPMK) plot for al 16 *Acropora palmata* RNA-Seq libraries. Gene density is consistent across libraries, making each library directly comparable to the next.

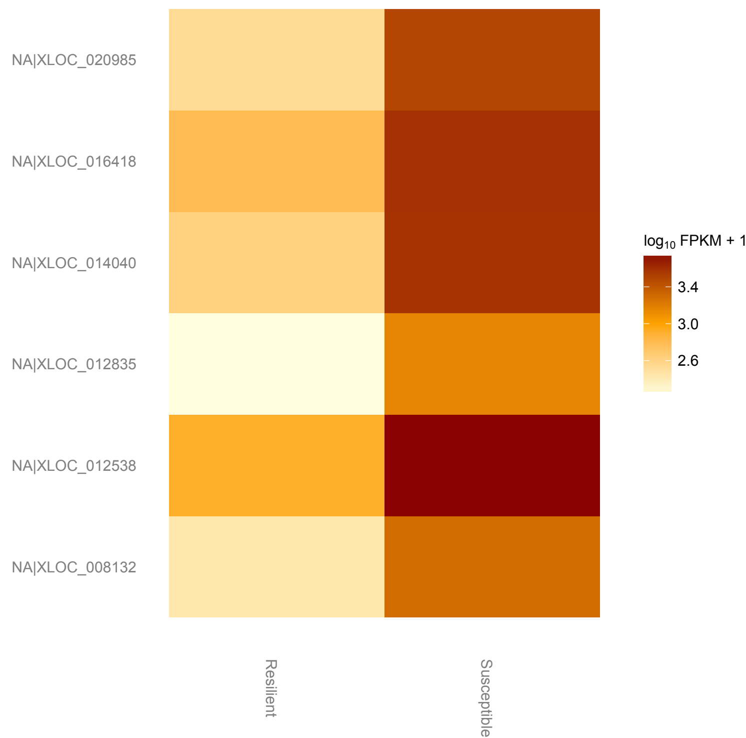


Figure 4.2: Heat map of differential expressed genes between resilient and susceptible *A. palmata* colonies.

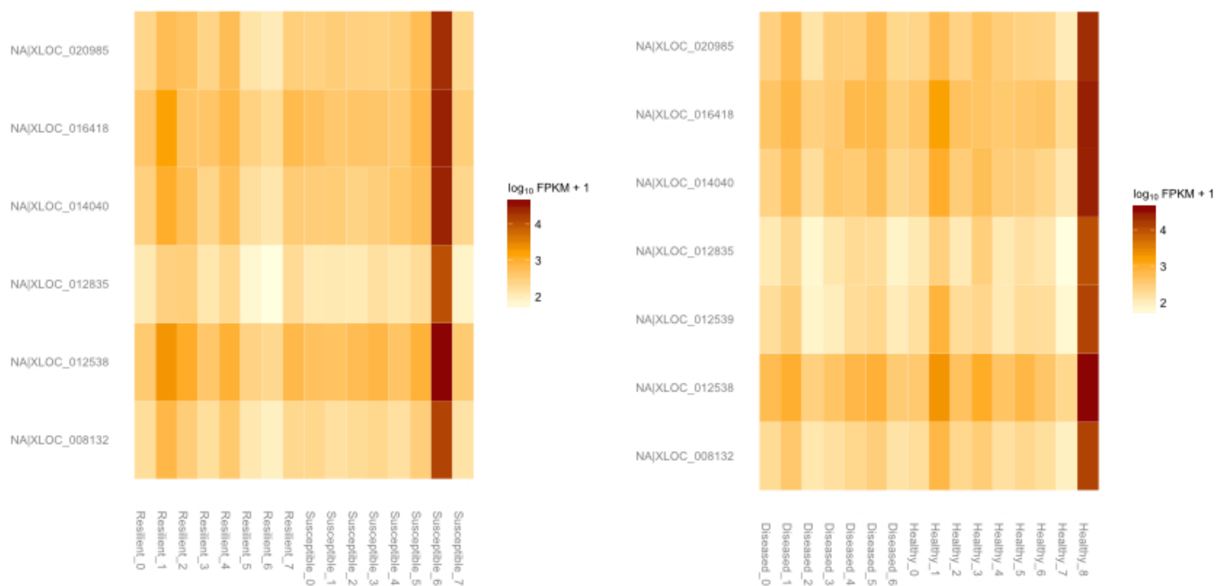


Figure 4.3. Gene expression heat maps for 16 USVI *A. palmata* RNA-seq libraries. Libraries are grouped into conditions: Healthy (no WSD), Diseased (WSD), Resilient (less occurrences of WSD), Susceptible (more occurrences of WSD). No significant differences in gene expression between libraries, with the exception of library 15.

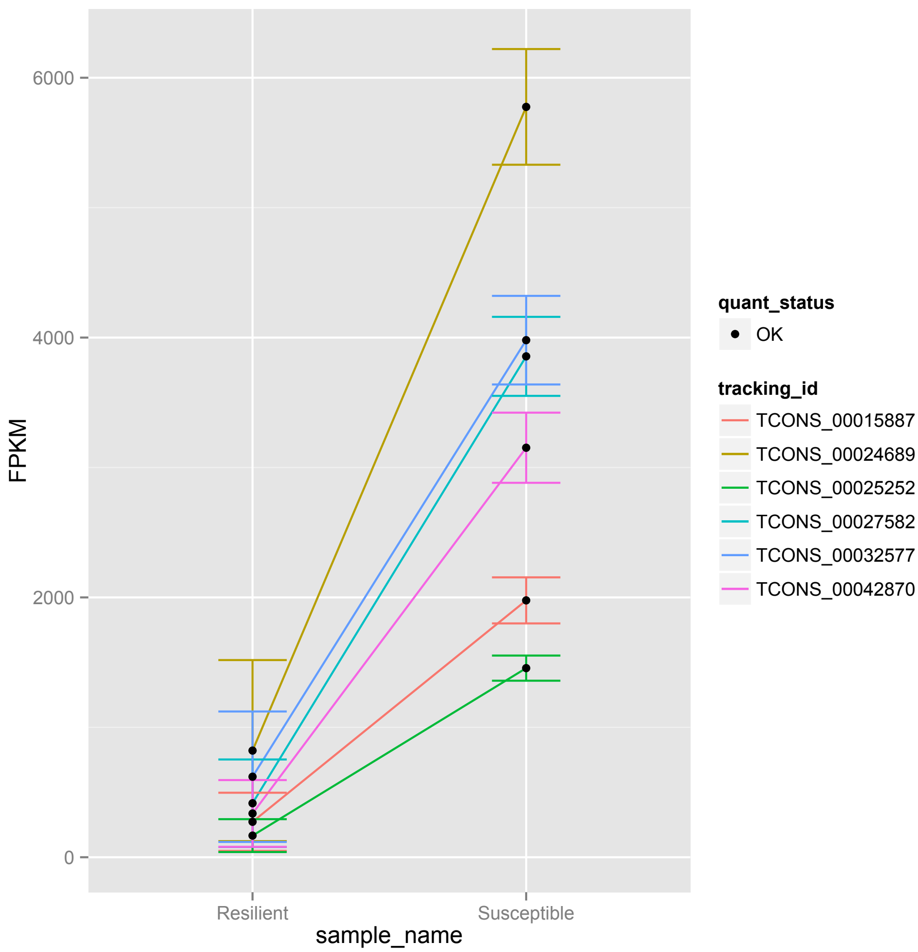


Figure 4.4: Differentially expressed splice variants between Resilient and Susceptible corals. Trend is driven by a single coral colony, colony 15.

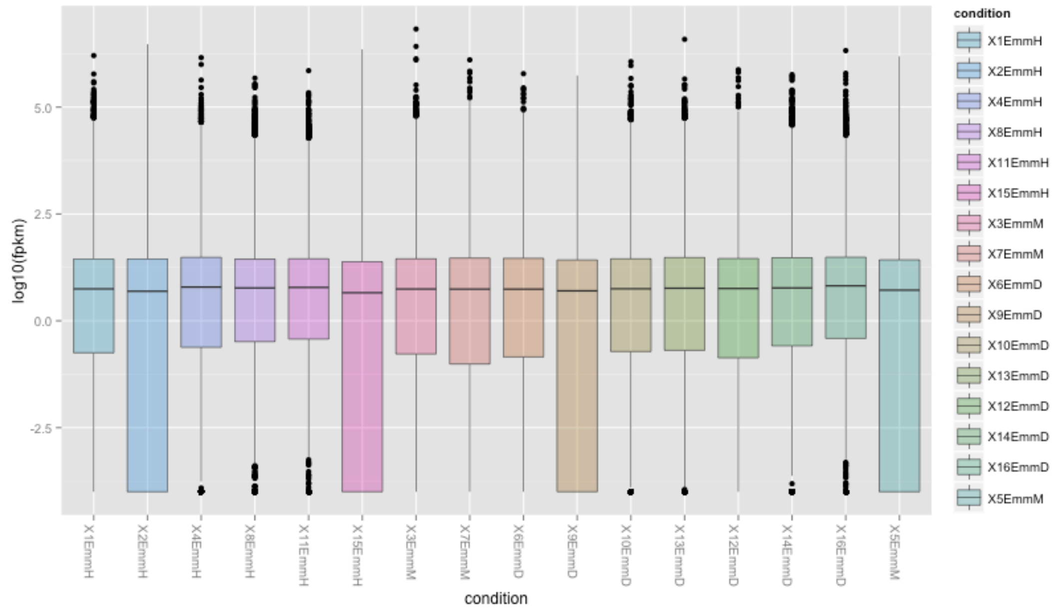


Figure 4.5. Expression level patterns across 16 *Acropora palmata* colonies. Transcript expression between libraries is consistent. There is some variation across libraries but no significant differences (P>0.05).

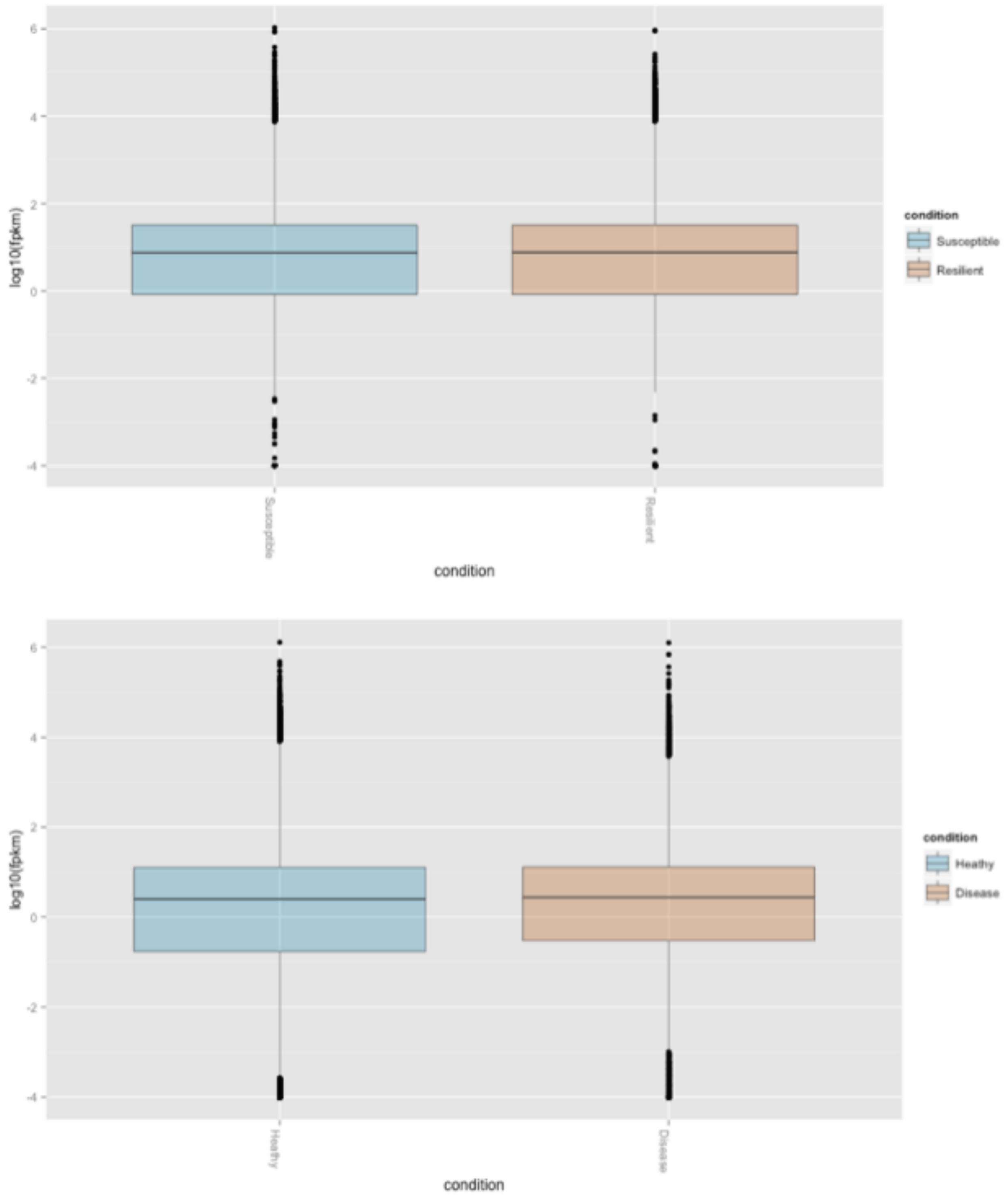


Figure 4.6. Expression level differences between “Resilient” and “Susceptible” and “Healthy” and “Diseased” *Acropora palmata* colonies. Gene expression between each condition is nearly identical.

# CHAPTER 5

## FUTURE DIRECTIONS

The concept of conservation biology is perfectly summed up by the father of conservation biology himself, Michael E. Soulé:

"With regard to breadth, conservation biology is as broad as biology itself. It focuses the knowledge and tools of all biological disciples, from molecular biology to population biology, on one issue – nature conservation.

- Michael E. Soulé and Bruce A. Wilcox

Conservation biology: an Evolutionary-Ecological Perspective, 1980

The goal of conservation biology is provide to tools and principles useful for the conservation of biodiversity in the face of human perturbation (Soulé 1985, Fares 2002). Because aquatic biodiversity is at a greater threat than terrestrial biodiversity (Sala et al. 2000), advancing genetic methods and furthering our knowledge of the basic life histories for understudied aquatic taxa is key. The goal for my dissertation was to learn many methods of conservation biology and, through applied research, make some contributions to the conservation of ecologically fundamental species. I believe I have achieved this goal. Because my dissertation focus was to prepare me for a career in conservation biology and not necessarily about filling in bits of missing information (though this was achieved as well), instead discussing the overall findings of

my dissertation, I would like to use this section to talk about the things about mussel mitochondria and reproduction that I am still curious about. There are aspects of my research that are incredibly interesting and I was only able to scratch the surface of the many questions I have. So, I would also like to discuss the things I would really like to continue working on in the future. With so much biodiversity in the Southeastern United States, and all the unknowns surrounding mussel life history and genetics, there really is a bottomless well of opportunity in freshwater mussel research.

FORF and its Potential Role in Reproductive Isolation:

When I was first investigating ways to identify glochidia encysted in fish tissue, there were many obstacles to overcome and, frankly, it took some time. What is poorly conveyed in publications are the things that don't work; things like getting markers like COI and ITS2 to cleanly amplify only mussel DNA when it is usually swamped by fish DNA. Things like how to get enough DNA out of glochidia that are at times smaller than half a millimeter in length, are mostly shell, and barely have tissue. What we do see are clean PCR bands, clear enzymatic cuts, and presumably an easy way to identify glochidia. Even with careful dissections attempting to remove as much host tissue as possible, I had limited success with published markers. Instead of amplifying genetic markers in mussels that are also easily amplified in fish, I needed a marker that is unique to mussels. Such a gene region would allow me to amplify glochidia DNA and not worry about host fish contamination (See Chapter 2: FORF for additional information). Thank the good graces of our PCR Gnome that Sophie Breton was interested in bivalve mitochondria and their peculiar means of mitochondrial inheritance, Double Uniparental Inheritance (DUI) (Zouros et al. 1994). Through her efforts, I was able to use Female-specific Open Reading

Frame (FORF) as my mussel specific barcode. In addition to being my favorite gene region, learning more about FORF only made me ask more questions.

Unlike most other animals, where all offspring inherited their mitochondrial genome from their mother, bivalves pass on both the father's and the mother's mitochondrial genome to their male offspring. We have known that male and female blue mussels (genus *Mytilus*) have separate and disparate mitochondrial genomes (Zouros et al. 1994), but until recently, we didn't know that there were actually whole gene regions of the mitochondria that were sex specific. Within the control region of the mitochondria, there are sex-specific open reading frames that are only found in their respective sex-specific mitochondrial genomes (Zouros, Oberhouser-Ball et al. 1994, Breton, Beaupré et al. 2009). Because female mitochondria are passed on to offspring per usual means - where every offspring receives their mitochondrial genome from their mother – the presence of a female mitochondria is nothing unusual. But because male mitochondria are only found in the gonads of male offspring, it is likely that the male-specific open reading frame of the mitochondria is a key component to sex determination (Breton et al. 2011). Additionally, it has been shown that female-specific open reading frame is expressed on the surface of eggs (Breton, Stewart et al. 2011). Little is known about the fine details of freshwater mussel reproduction, but from what we know about reproduction in marine bivalves, specifically oysters and mussels, proteins involved in recognition of conspecific sperm are also expressed on the surface of eggs. An additional interesting parallel between oyster reproduction and freshwater mussel reproduction is that in both, males release sperm in spherical structures called spermatozeugmata, and in both, the females take in the sperm while siphoning water (Foighil 1989, Breton, Stewart et al. 2011). The females will then move the spermatozeugmata to their

brooding structures to fertilize their eggs. With the potential for many different species releasing sperm at the same time, there must be some mechanism for freshwater mussels to prevent conspecific sperm from fertilizing their eggs – as most species are quite clearly genetically defined (Bockrath unpub data for ACF right?). Thus, with the localized expression of FORF and the clear parallels in bivalve reproduction, female-specific open reading frame may be involved in identifying conspecific sperm. Though it has not been investigated, it would be interesting to see if male-specific open reading frame is expressed on the surface of sperm. If so, I would venture to say that sex-specific open reading frames are absolutely involved in gametic recognition and may interact with known nuclear gametic recognition proteins, such as lysin and bindin (a ref here would be good too, Mytilus or urchin or something).

In addition to the reproductive parallels we can make between freshwater mussels and marine bivalves, another detail about FORF that makes me wonder if it is involved gametic recognition protein is the shear amount of nucleotide divergence in the face of maintained functionality. There is over 60% nucleotide divergence at FORF between some freshwater mussel species, yet FORF maintains its status as an open reading frame, thus a functional coding region. This in itself is kind of amazing. There has to be a reason why functionality is maintained in this region despite the huge nucleotide variation, and reproduction appears to be an obvious choice (given parallel findings in lysin and bindin, e.g. recent work by Mike Hart in seastars). Given more time and resources, this is one unanswered facet of my research I would like to explore.

Restoration of Anadromous Fish Migrations Role in the Restoration of Extirpated Mussels

Because FORF is specific to freshwater mussels, it is useful for the identification of glochidia recovered from infested fish. This method can be particularly useful when assessing how restoring fish migration patterns to improve their population numbers may also improve the population numbers of the mussels they use as hosts.

Dams have contributed to the decline of many freshwater mussel species, particularly those with glochidia that rely on migratory fish species as hosts (Watters 1996, Waller and Lasse 1997). Dams block migration of anadromous fish – those that live in the ocean as adults but migrate into rivers to spawn – (Vaughn and Taylor 1999) and as a result, populations of many migratory fish are not as abundant and as widespread as they once were (Ely et al 2008). Many mussel species that are suspected to be specialists on large-river migratory host fish have suffered declines above dams and some have even been extirpated (Kelner and Sietman 2000, Ely, Young et al. 2008, Haag and Warren Jr. 2010). In some cases, loss of fish hosts results in the creation of relic populations: populations of large, old individuals with little to no recruitment that persist until they die out of old age (Kelner and Sietman 2000).

In the Apalachicola-Chattahoochee-Flint River Drainage (ACF), impoundments, such as Jim Woodruff Lock and Dam (JWLD), have prevented anadromous fishes such as Alabama shad (*Alosa alabamae*) from making annual spawning migrations. Since 2005, however, "conservation locking" - where migrating fish are moved through a dam much like a ship - allowed *A. alabamae* to migrate upstream JWLD. By looking at the growth rings of their otoliths (bones in

the inner ear), it was inferred that 97% of the *A. alabamae* caught hatched upstream of JWLD and were returning to historical spawning grounds (Hinch, McMurray et al. 2012, Miller 2013).

Because Alabama shad are successfully moving upstream, we wanted to know what mussels were being carried along with them to the upper reaches of the Apalachicola-Chattahoochee-Flint (ACF) River Basin. After inspecting the gill arches from 30 of the Alabama shad, approximately 1/3 were infested with glochidia. Most shad only carried a few glochidia across all eight gill arches, but two were more heavily infested with over 20 glochidia across all gill arches. Using FORF, I identified the glochidia recovered from Alabama shad gills as *Elliptio crassidens* (K. Bockrath, in preparation). Though it is common in the ACF, *E. crassidens* is locally endangered and nearly extirpated in the upper reaches of the Mississippi, where their known fish host – Alabama shad and skipjack herring – are also extirpated. With these findings, we have additional support that opening large impoundments to allow anadromous fish to migrate upstream not only improves their numbers, but would allow for the repopulation of locally extirpated mussel species.

Because it takes a significant amount of time to inspect each set of gill arches, going through all 133 Alabama shad is a tall task. With more time, I would finish dissecting all remaining Alabama shad gills in order to determine how many of them are carrying glochidia, at what densities, and determine if the remaining recovered glochidia are Elliptio crassidens and the potential for multiple mussel species encysted simultaneously.

A dissertation is an experience that is like no other. It is one of the few times in a scientists career where they can explore their research interests, make mistakes, and really be scientifically creative. Through my experiences completing my dissertation, I have gained confidence as a researcher, learned to think outside the box, and to not be discouraged by failure. With every question that is examined in this dissertation, many more were generated, giving me with a jumpstart to potential research ventures and collaborations in the future.

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