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Measuring the presence of the amphibian pathogen Batrachochytrium dendrobatidis in East Tennessee

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IN EAST TENNESSEE

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ABSTRACT

I examined the presence and absence of the amphibian pathogen *Batrachochytrium dendrobatidis* in three survey areas in Eastern Tennessee. Amphibians were swabbed to assess for *Bd* and other ecological qualities like canopy cover, and air and water temperature are a few of the measurements evaluated. DNA was extracted from the swabs using a Qiagen DNeasy Blood and Tissue Kit and samples were PCRed in triplicate to confirm presence or absence. All 48 animals that were swabbed in the field were negative for *Bd*. These results parallel other findings that few *Bd* positive assays have been conducted in East Tennessee from juveniles. Bioassessments can be a powerful tool in expanding our knowledge of *Bd* in Tennessee and can be used to focus biological hazard protocols in respect to the conservation status of amphibians.

DEDICATION

To all female scientists; past, present, and future. Give ‘em hell.

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LIST OF ABBREVIATIONS

Bd, *Batrachochytrium dendrobatidis*

ICUN, International Union for Conservation of Nature

MLST, multilocus sequence typing

PCR, polymerase chain reaction

ssDNA, single strand DNA

LIST OF SYMBOLS

C, probability; DiGiacomo and Koepsell's equation (1986)

n, sample size

P, prevalence; DiGiacomo and Koepsell's equation (1986)

μ, 10^{-6}; micro

CHAPTER I

INTRODUCTION

Scientists argue that the natural world is either on the brink of the sixth greatest mass extinction or the event is already taking place (Wake and Vredenberg 2008). Amphibians — caecilians, frogs, and salamanders, are possibly the only major group threatened globally, and this threat will more than likely accelerate due to the environments that amphibians occur. The overwhelming majority of organisms in Class Amphibia require habitats located in tropics, subtropics, or temperate zones with ample access to fresh water (Wake and Vredenberg 2008). Amphibians are automatically placed into specific geographic ranges due to their innovative life histories, making the global threat more pressing. The subclass Lissamphibia contains caecilians (Order: Gymnophiona, ~175 species), frogs (Order: Anura, ~5600 species), and salamanders (Order Caudata, ~570 species). Frogs are the most abundant and speciose of the three, and thrive in the tropics, but can extend to the southern tips of Africa and South America and even as far north as the Artic Zone (Wake and Vredenberg 2008). Caecilians are also found in the tropics and typically not found elsewhere, but knowledge of caecilians is lacking and therefore incomplete (Thomas Wilson, personal communication, Sept. 2016). Salamanders, apart from the Plethodontidae family, are confined to subtropical and temperate areas of North America and Eurasia, including North Africa (Thomas Wilson, personal communication, Sept. 2016). Plethodontids are lungless and breathe entirely through cutaneous respiration, which has allowed them to radiate extensively into Central and South America. Organisms in this family still require moisture but can rely on leaf litter or soil more readily than other families; this

characteristic has enabled them to exploit and thrive in low competition areas (Thomas Wilson, personal communication, Sept. 2016). Amphibians' distribution parlays into the global biodiversity crisis. While amphibians themselves are not perpetrators of this decline, populations are decreasing and in some places completely disappearing, even from protected zones (Dodd 2010).

Today's extinction rate is estimated to be 100-1000 times greater than the historical norm. Anywhere from 10-50% of well-studied higher taxonomic groups have an increased risk of extinction, and over the last 30 years ~43% of all amphibian populations have declined drastically. This decline signifies that 32.5% of amphibians are globally threatened, and more than 34 species have gone extinct, although many more are thought to be extinct (Smith et al 2009). Current literature states that there are six major threats to amphibians: "habitat modification and destruction, commercial over-exploitation, introduced species, environmental contaminants, global climate change, and emerging infectious diseases, especially the chytrid fungus *Batrachochytrium dendrobatidis*" (Dodd 2010). Historically, scientists agree that habitat modification and destruction pose the greatest threat to amphibians. Recent analysis argues that infectious diseases can cause temporary, or sometimes permanent, declines in biological opulence which can greatly affect local populations (Smith et al 2009).

There is probable evidence that *Batrachochytrium dendrobatidis* (*Bd*) is responsible for the greatest disease-caused loss of biodiversity in documented history (Whittaker and Vredenberg 2011). *Bd* is an aquatic pathogenic fungus that causes chytridiomycosis, or hyperkeratosis of the superficial keratin-containing layers of skin. *Bd* has caused drastic declines or even extinction in at least 200 species of frogs, including populations in isolated and immaculate habitats (Whittaker and Vredenberg 2011). Recent studies have cited *Bd* as the proximate and ultimate cause of extinction in the Australian frog species *Taudactylus*

acutriostris (Smith et al 2009). Many of these declines occur over the course of one calendar year, meaning that *Bd* can affect communities, populations, and the overall environment rapidly. Current research states that there are over 350 amphibian species that are known to be infected by *Bd*, and infection has been recorded in numerous captive frog and salamander species and a single caecilian species (Whittaker and Vredenberg 2011). This pathogen occurs at all elevations that amphibians are found in the United States, and has been recorded on every known continent that amphibians occur (Lannoo et al. 2011).

Bd was originally thought of as an isolated disease responsible for die offs of Australian frogs, but in 1999 it was confirmed that chytridiomycosis is caused by the aquatic fungal pathogen *Bd*. Chytrid has been identified as early as 1938 by examining museum specimens of *Xenopus laevis*, an anuran species commonly used for developmental biology. This date coincides with the time when *X. laevis* was shipped worldwide for use in scientific research (Whittaker and Vredenberg 2011).

Diagnostic characteristics of *Bd* include its sequence of small subunit ribosomal DNA, its ultrastructural morphology of its zoospores, and that it occurs on an amphibian host. These differences provide evidence that *Bd* is unique compared to other known chytrids. Using multilocus sequence typing (MLST), genetic diversity of *Bd* has been examined across strands found in North America, Panama, Australia, and Africa. There are 5 variable nucleotide positions detected among 10 loci (5918 base-pairs; Berger et al 2005). Low variation suggests that *Bd* has recently emerged and the strains share a recent common ancestor (Whittaker and Vredenberg 2011).

Chytridiomycota, the phylum of *Bd*, is the most basal group of fungi and shares the most characteristics with the animal kingdom. An organism placed into this phylum has a motile zoospore with a single whiplash flagellum and reproduces asexually. Chytridiomycota consists

of a single class and five orders. Morphology of an organism's flagellar roots aids in identification down to Genus and is particularly useful in determining the order. Chytrids require a water film for zoospore dispersal, which introduces the possibility that zoospores can get trapped in water or soil (Division Chytridiomycota 2004).

The lifecycle of *Bd* is comprised of two stages: a thallus, or body, that is physically present in the amphibians' skin, and a free-living zoospore that is motile in an aquatic environment (Lannoo et al 2011). The thallus, or the growing organism, produces a sporangium, which is a sac like structure divided by walls to form zoospores (Division Chytridiomycota 2004). The growing thalli undergo numerous mitotic divisions and become multinucleate, in which the entire contents of the sporangium cleave into zoospores (Berger et al 2005). These newly cleaved zoospores exit through a discharge tube and their release typically signifies the death of the thallus (Division Chytridiomycota 2004). Sexual reproduction has yet to be observed in *Bd*. A single zoospore can form many sporangia and colonial development appears to be replicated in culture and in amphibian tissue. *Bd*'s lifecycle takes 4-5 days while being incubated at 22°C *in vitro*, and while these conditions have not been tested using amphibian skin they are assumed to be the same (Beyer et al 2005).

Sporangia characteristically infect the superficial epidermis, specifically the stratum granulosum and stratum corneum layers. As many as three sporangia have been found within one epidermal cell. Immature sporangia can be found deeper in viable cells, while mature or empty sporangia are more abundant in outer, keratinized layers of skin (Berger et al 2005). Typically, sporangia development has completed by the time it reaches the skin surface. Mature vessels are more likely to be found in outer layers because as zoospores are preparing to be released, the discharge tube projects towards the skin surface to enter the environment. Zoospores are released as the discharge tube creates a hole within the epidermal cell membrane,

and the keratinized membrane adheres closely to the surface of the tube. The tube is closed off from the environment by a plug that absorbs water and liquefies when the zoospores are ready to discharge. Following release, the zoospore is free-living and motile in an aquatic environment and as it encysts within a host, the flagellum is reabsorbed and a cell wall begins to form (Berger et al 2005).

Experimental testing has determined that *Bd*'s zoospore can swim approximately 2 cm before encysting (Piotrowski et al 2004). As it encysts into keratinizing epithelial cells, *Bd* causes the stratum corneum to thicken 2-5 times the normal amount (Longcore et al 1999). Within this layer of an infected organism, one will typically find large numbers of the spherical chytrid thalli. Chytrid thalli are the most numerous in the skin of the ventral abdomen, feet, and the hind limbs (Longcore et al 1999). Once in contact with a host, zoospores eventually lead to hyper keratinization that compromises the animal's ability for osmotic regulation. This leads to declines in their electrolyte blood levels, and the animal dies of cardiac arrest (Whittaker and Vredenberg 2011).

In anuran tadpoles, only the mouthparts of the animal are keratinized and therefore susceptible to *Bd* infection, which can cause defects and depigmentation (Whittaker and Vredenberg 2011). As the animal continues to develop the skin becomes increasingly keratinized and the fungal infection spreads. In juvenile and adult frogs, *Bd* encysts in skin cells on the ventral abdomen, digits, and pelvic drink patch (Whittaker and Vredenberg 2011). Similar to tadpoles, larval salamanders do not have numerous keratinized areas so the risk of being infected is lower for larval animals than for adults (Keitzer et al 2011). This holds true for both salamanders and frogs.

Bd has proven to be increasingly pathogenic and virulent in many frog species that have been infected experimentally. In some cases, lower levels of initial infection, e.g., 100

zoospores, can lead to death (Skerratt et al 2007). These findings suggest that even a small population of zoospores can lead to mortality in some species. Other frog species, such as *Lithobates catesbianus*, act as carriers for the pathogen because they can be infected but are not harmed (Whittaker and Vredenberg 2011). In a series of three experiments performed in 1998, *Bd* proved to be fatal to captive bred poison dart frogs. All frogs that were exposed to the disease developed a fatal skin disease while control animals did not. The most consistent clinical sign of exposed frogs was excessive skin shedding. Skin lesions typically affected the ventral surface of the legs and trunk where the skin was discolored and thickened. Further microscopic examination of shed skin confirmed the presence of chytrids (Nichols 2001).

A study found that 100% of field-collected, *Bd* positive *Batrachoseps attenuates* died when transferred into captivity (Weinstein 2009). Symptoms of the infected salamanders included excessive skin shedding and caudal autonomy. Dark brown spots were observed on the ventral surface of the animals, but disappeared as the skin was shed. Upon histological examination, the spots contained numerous chytrid sporangia. The uninfected *B. attenuates* specimens shed their skin in one cohesive piece, while their infected counterparts sloughed fragments of skin in large quantities. Concentrated spots of sporangia have also been observed in *Ambystoma tigrinum*, *Euproctus platycephalus*, and *Plethodon neomexicanus* (Weinstein 2009). Sporangia concentration suggests that zoospores encyst in the immediate area they were released (Piotrwoski et al 2004). In severely infected animals caudal autonomy was observed. These results suggest that tail loss may be a symptom of *Bd* and could help to identify infected organisms. In some species, tail loss occurs to avoid predation so this characteristic alone is not sufficient to identify an animal infected with *Bd* (Weinstein 2009).

Some salamander species have shown to resist, persist, or even clear the infection with a higher degree of success when compared to frogs (Whittaker and Vredenberg 2011). In the same

study that observed *Bd* symptoms in salamanders, *B. attenuates* that were inoculated with *Bd* and held in habitats that simulated summer estivation were able to clear the infection. *Bd* and its consequences materialize broadly across salamander species, some species are impacted more than others (Weinstein 2009).

Many factors contribute to *Bd*'s concerning nature. The pathogen is widely distributed in both Old and New worlds, spreads rapidly and is highly virulent, and can infect a wide range of host species (Dodd 2010). *Bd* can be spread from amphibian to amphibian through direct contact, such as mating or schooling of larvae. Human activities including outdoor recreation and amphibian trade have also been reported as reasons for concern (Smith et al 2009).

Bd perpetuates itself via a motile zoospore and in the laboratory 95% of zoospores stopped moving within the first 24 hours (Piotrowski 2004). Motility is an advantageous trait, but this data suggests that zoospores need to encyst quickly to continue their lifecycle. The zoospore can travel longer distances if carried through a water current, but this would cause the concentration of the pathogen to decrease and therefore decrease the likelihood of the zoospore encountering a host (Piotrowski 2004). Regarding amphibians, inhabiting areas with access to flowing water could lower their chances of infection.

Bd will be more likely to spread into uncontaminated habitats if those areas are ideal for the reproduction of the microorganism. *Bd* can grow and reproduce within the temperature ranges of 4-25°C, with the ideal temperature being anywhere between 17-25°C. The pathogen's wide range of temperatures suggest that it can survive in many types of environments. While *Bd* grows slowly at 4°C, this ability allows it to overwinter in its hosts, and as the temperature begins to increase, so does its reproductive abilities (Piotrowski 2004). *Bd* does not grow well above 25°C and areas with temperatures above this are unfavorable to outbreaks. Unless new strains develop different temperature ideals, *Bd* epidemics will be limited to cooler areas in the

tropics, such as Australia, and in temperate areas outbreaks will be limited to the warmer months. Infections at temperatures below 10°C or above 28°C may not be fatal to the host because the pathogen either does not growth or grows slowly (Piotrowski 2004). Another source states that temperatures above 25°C may actually aid the animal to clear the infection (Lannoo et al 2011).

High levels of canopy cover are another ideal environmental characteristic for the pathogen. Amphibians are motile for the majority of their lifecycle and are therefore able to determine their own microclimates, or local atmospheric conditions. Temperature, exposure, and elevation are a few qualities that amphibians can actively control. Canopy cover is a fundamental influence of microclimates in forested areas because large trees limit air movement and the amounts of solar radiation that reach the forest floor. Areas with larger percentages of canopy cover provide cool, moist conditions that are ideal for *Bd* growth and reproduction (Beyer et al 2015).

Chytridiomycosis has been linked to amphibian declines in North America. While *Bd* surveys have been conducted in the Southern Appalachian Mountains, few have been conducted in East Tennessee (Moffitt et al 2015). The Appalachians have broad salamander diversity and presumably many swabbed animals will be from the Plethodontidae family. Their physical adaptations and historical ranges suggest that these organisms are abundant in East Tennessee. Plethodontids occur in several body shapes, with some species being short and stocky, while others are elongate (Powell 2016). All species have four well-defined limbs. Plethodontids' physiological adaptations regarding environmental requirements overlaps broadly with microclimates of other small forest organisms (Welsh and Droege 2001). Microclimate overlaps suggest that plethodontids are indicator species, meaning that presence or absence of these species can reflect the fluctuations in the environment being monitored. Stressors, such as infectious diseases, can have adverse effects on entire ecosystems and threaten stability and

biodiversity (Welsh and Droege 2001). Biodiversity can be affected by disease-driven extinction on both local and global scales and *Bd* poses a serious threat to this viability (Smith et al 2009). Biological assays are needed to monitor and understand *Bd* and these surveys are particularly pertinent in areas of high salamander diversity, such as East Tennessee.

Objectives

The objectives of this study are to answer the following questions:

1. Is *Bd* present in Sullivan County, Tennessee?
2. Do ecological measurements, such as temperature and canopy cover, correlate with the presence of *Bd*?
3. Do impacted watersheds have a higher presence of *Bd*?

CHAPTER II

MATERIALS AND METHODS

Ethics Statement

This research was conducted under the scientific collection permit number 3082 issued by Tennessee Wildlife Resources Agency for the University of Tennessee at Chattanooga. UTC's Institutional Animal Care and Use Committee (AUP number 0408) approved the training methods which included both online CITI training and field training. No animals were harmed during this survey.

Study Sites

From August 7, 2016 —August 17, 2016 a total of 48 *Bd* swabs were taken from three different sites. Site one, referred to as Morrell Lane, is private property located in Bluff City, TN. Exact coordinates of the site are 36°28'55.23'' N and 82°07'16.40'' W. Morrell Lane is encompassed by Cherokee National Forest, sits at approximately 540 m in elevation, and has a stream that runs roughly 2.4 km through the sampled area. Permission for access was granted verbally from the landowner Brandy Schrenker on August 6, 2016. Due to the surrounding national forest, and its status as private property Morrell Lane is not exposed to the general public. These contributing factors imply that the chances for human related impacts are less frequent, and the observations of light to no foot traffic and few pieces of trash support this.

Morell Lane is considered unimpacted due to these characteristics, and it was not listed as an impacted waterbody by the state of Tennessee (Tennessee Department of Environment and Conservation 2016). Along with publications of impacted waterways, Tennessee also keeps records of exceptional waterways across the state. This sample site is not explicitly listed because it is not owned by the state, but many of the surrounding streams (Riddle Creek, Morrell Creek, etc) of the Cherokee National Forest are considered exceptional (Tennessee Department of Environment and Conservation 2017).

Steele Creek Park, located in Bristol, Tennessee, is the third largest municipal park in the state and covers more than 8.90 sq km (Friends of Steele Creek Nature Center and Park). The second and third sites are positioned on opposite sides of the park. While the park is large and aims to minimize human impacts, it is still nestled into the landscape of the city, meaning that its perimeter is surrounded by neighborhoods and roads. Permission was granted for both sites verbally from Jeremy Stout, a manager of Steele Creek Nature Center, on August 11, 2016. Steele Creek acts as a hub for outdoor recreation with the more popular attractions being hiking, biking, and running. Accessing both sites is relatively simple due to their public parking lots being adjacent to main roads.

Mill Creek (36°34'44.77'' N and 82°13'37.21'' W) is a flowing stream with banked edges that eventually empties into a small lake, and sits at approximately 496 m in elevation. This sampling site roughly follows the Quarry Cave Trail that measures approximately 0.644 km starting at the lake, and paved sidewalks are immediately next to the creek along its entirety. Mill Creek is listed as an impacted waterway (Tennessee Department of Environment and Conservation 2016). During sampling, there were everyday trash items such as wrappers and plastic bottles, but there were also items such as tires, old radiators, and large pieces of sheet

metal. Visual observations of pollution and its exposure to the public support Mill Creek's listing as an impacted waterway.

Rooster Front (36°33'35.66'' N and 82°07'16.40'' W) is a popular trail on the opposite side of the park from Mill Creek and operated as the third sampling site. Oftentimes there are children playing in the creek, people hiking to and from the dam, or families enjoying the playground equipment and tennis courts. The Lakeside Trail is 3.38 km long, and begins at Rooster Front Park and eventually connects to the lake and to Mill Creek. Rooster Front sits at approximately 471 m above sea level. The Rooster Front side of the park is divided by a large man made dam, and this divide guarantees the separation of salamander populations. Most plethodontids have small home ranges; meaning that it is highly improbable that an organism would be able to naturally transport itself from Mill Creek to Rooster Front (Welsh and Droege 2001). Many pieces of trash were incorporated into Rooster Front's ecosystem, but this site did not have as much macro pollution when compared to Mill Creek. Visual observations noted that Mill Creek had more foot traffic on the sidewalks than compared to Rooster Front, but the foot traffic occurring at Rooster Front involved stream disturbances. Rooster Front is also listed as an impacted waterbody (Tennessee Department of Environment and Conservation 2016).

Field Methods

Before sampling began at each site, all relevant materials, such as boots, buckets, etc, were disinfected with an approved disinfectant (Wilson et al 2015). This occurred before entering each new site and at the end of each sampling session. The use of a disinfectant is key to minimize the possibility of cross contaminating sites because the prevalence of *Bd* at this point

was unknown. A fifteen-minute waiting period was observed after the last application of disinfectant.

Because *Bd* is an amphibian disease the types of amphibians swabbed were not discriminated against, but no frogs were spotted during sampling. All swabs recorded were taken from salamanders. Organisms were found by carefully flipping rocks, searching leaf litter, and surveying rocky outcrops in creeks. Any disturbance created in the environment was carefully corrected before moving on to another area. Individuals were captured in un-used, individual plastic sandwich bags and remained there for processing and handling. While sampling, a pair of un-used Fisherbrand powder-free nitrile gloves were worn. Both gloves and plastic bags were kept in a separate trash bag after each amphibian encounter or use. Gloves were worn at all times during sampling and changed if any doubt of contamination was introduced. Ethanol was also used in the aseptic field protocol for cleansing the researcher's hand between glove changes. This, combined with the meticulous monitoring of equipment, decreases the probability of cross contaminating organisms and sample sites.

All field measurements and notes were recorded on a standardized Team Salamander data sheet. Measurements taken include morphometrics, densitometer readings, habitat information, and total dissolved solids readings. Following the random capture of a salamander, the air and water temperatures were recorded using a string thermometer. Animals are said to be captured randomly because there is not a systemic method to detect living, moving organisms. The sample sites represent habitats that amphibians would likely be found in and careful assessment lead to successful animal captures.

Total dissolved solids (TDS) water quality measurements were taken using an Eco Testr TDS Low by Eutech Instruments and Oakton Instruments (item number 35462-10). The measurement tip was properly submerged into the stream and rinsed in between each reading.

The TDS are the amounts of charged ions in the water including minerals, salts, or metals. This reading is an excellent indicator of stream health because the purity of water has a direct effect on the biotic and abiotic components of the stream ("What is TDS?" 2012).

Snout to vent length, tail length, and head width maximum were recorded to the nearest 0.05 mm using calipers. The mass of each salamander was recorded to the nearest 0.1 gram using a Cen-Tech 1000-gram digital scale (item number 60332). To minimize cross contamination, salamanders were weighed in their respective bags, with the average weight of the bag being 0.1 gram. Bag weight was noted and accounted for during analysis.

Organisms were swabbed with Fisherbrand sterile swabs with a Dacron polyester fiber tip (Cat. Number 14-959-90) and plastic handle for approximately 45 seconds. Swabbing was performed on the ventral surfaces and the legs/feet. The Dacron polyester fiber tip was then removed and stored in an orange 2mL micro centrifuge that contained approximately 1mL of 70% ethanol. Each tube was labeled similar to the following: EAS 08091601, meaning that this swab was August 9, 2016, and was the first sample of the session. Each micro centrifuge tube was labeled using a black pigma pen or pencil and a Tough-Tag.

Raw canopy coverage data was recorded using a spherical densitometer created by Robert E. Lemmon (Forest Densitometers, Spherical Densitometer, model-C). The instrument is held approximately 30 cm away from the operator's chest. I assumed four equidistant dots within each square of the mirrored grid, and counted the dots containing light or open canopy. At every location that an animal was swabbed, densitometer readings were taken for the four cardinal directions with the aid of a compass to determine the percentage of open canopy.

A square meter PVC pipe grid was used to describe the surrounding habitat. The grid consisted of 100 equally spaced squares and readings were taken at each capture site, and at randomly generated locations that were determined by a randomly selected angle and distance.

Using a random number application (RNG, available on iOS store) degrees were selected from the number range 0-360, and for meters, 0-30. After generating a random degree and a random meter away from the capture location, the grid was placed on the ground and used to describe the location encompassed within the 1 square meter. For example, descriptions were given as, "45 squares of slow moving water and 55 squares of creek edge", meaning that 45% of the location was water and 55% was bank. The maximum range was limited to 30 meters because this was the likely maximum distance a salamander would move and still be residing in similar habitat. The angle was identified using a compass.

Most salamanders were caught within the same area and it was not uncommon to find more than one salamander under the same rock. For these instances some measurements were identical, including the habitat and densitometer readings. Each animal was not kept in a plastic bag for longer than 15-20 minutes, and once released, the sampling site was shifted approximately 45 m upstream. Diagnostic characters of the animals were recorded with photo documentation to aid with identification along with range, habitat, phenology, and elevation.

At the conclusion of each sampling session, all gear was properly disinfected, and the samples were stored in their respective micro centrifuge tubes in a household refrigerator set at 4°C. All of the clothing worn was washed and dried before being used in another sampling session.

Lab Methods

All of the lab techniques were standardized across Team Salamander members to ensure consistency and replicability. Aseptic lab protocol was maintained throughout every lab process. Upon entering the lab, I would thoroughly wash my hands and immediately put gloves on. The

exact aseptic protocol varied depending on which lab technique was being performed, but the overall goal remained the same. For example, the aseptic protocol for drying samples differed from PCR because during the drying stages forceps need to be sterilized using ethanol and flames. PCR's protocol involves meticulously changing pipette tips. Without these protocols the possibility of cross contamination is introduced, and if this is a possibility none of the results are reliable.

At the conclusion of the sampling window, the micro centrifuge tubes containing ethanol and the swab were transported from Bristol to Chattanooga. The swabs were packed into a cooler and surrounded by ice, and after the three-and-a-half-hour drive, were placed back into a household refrigerator until they could be stored in a -80°C freezer on site at the University of Tennessee at Chattanooga, where they awaited processing.

The initial step of DNA extraction involves drying each sample. Because of the way the swab was stored, the DNA was captured within the Dacron polyester fiber tip and the ethanol. Each swab was aseptically scraped into its original micro centrifuge tube and dried using a Savant DNA120 SpeedVac Concentrator. Each swab contained approximately 1mL of ethanol so the drying times varied, but in general swabs took three to three and a half hour to dry. Drying time can vary based on the number of contaminants. The SpeedVac Concentrator spins and heats the samples for a set amount of time, and after drying, the ethanol evaporates leaving a pellet of DNA and other contaminants that were contained on the swab. This machine can hold 40 samples at one time but swabs were randomly selected to be dried as a preventative measure; in case of an unforeseeable malfunction, only a select number of swabs would be compromised, instead of the entire data set.

DNA extraction was performed using a Qiagen DNeasy Blood and Tissue Kit for 50 samples (catalog number: 69504). These kits are designed for rapid purification of DNA and are

appropriate for polymerase chain reaction (PCR). The principle of the procedure is essentially to lyse the target cells, selectively bind DNA to the spin column, remove contaminants by washing, and elute DNA to use it in further procedures. Neither phenol or chloroform extraction, or alcohol precipitation is required, and this enables one to extract multiple samples simultaneously (DNeasy Blood and Tissue Handbook 2006). Centrifugation aids in completely removing contaminants, divalent cations, and unwanted proteins. Results of the process yield purified DNA eluted in low-salt buffer, which is immediately available for use in PCR, and typically has a 260/280 ratio between 1.7 and 1.9. The DNeasy Blood and Tissue Kit can recover DNA fragments as small as 100 base pairs (DNeasy Blood and Tissue Handbook 2006). These kits were selected for their accuracy, the ease of performing multiple extractions, and because of the virtually immediate results.

Modifications were made to the purification of total DNA from animal tissues protocol to produce the highest yields of DNA. The original protocol can be found on page 28 of the DNeasy Blood and Tissue Handbook. After being dried, 180 microliters (μL) of Buffer ATL and 20 μL of Proteinase K were added to the pellet. Each sample was then vortexed to break apart the pellet and incubated for three hours at 56°C. Every hour the tubes were vortexed to fully break apart the dried pellet. Buffer ATL is used as a tissue lysis buffer (Buffer ATL 2017). Proteinase K is a broad-spectrum serine protease that digests contaminating proteins. This is particularly useful in this protocol because the enzyme is ideal for short digestion times and can remain stable over a range of temperatures and pHs. Proteinase K has a noticeable increase in activity at higher temperatures (Proteinase K Protocol 2016).

Following cell lysis, 200 μL of Buffer AL and 200 μL of ethanol are added. Samples are vortexed accordingly. Buffer AL contains the hydrochloride salt of guanidine, a strong organic base that is used in the lab as a protein denaturant (National Center for Biotechnology

Information). Ethanol effectively precipitates nucleic acids in the presence of a salt, which can then be collected by further centrifugation. After adding these two components, the liquid is transferred to a DNeasy mini spin column with a 2 mL collection tube and centrifuged for 30 seconds at full speed, or 13,200 RPMs. The sample is again transferred to a new 2 mL collection tube.

Buffers AW1 and AW2 act as wash buffers and are supplied as concentrates; ethanol was added at the initial opening of the DNeasy Kit (DNeasy Blood and Tissue Handbook 2006). Following transfer of the sample, 500 µL of Buffer AW1, which contains guanidine hydrochloride, is added and the tube is centrifuged for 30 seconds at full speed. The sample is transferred again to a new collection tube and 500 µL of Buffer AW2 is added, which acts as a second wash buffer (DNeasy Blood and Tissue Hanbook 2006). Following this addition, the tube is centrifuged for three minutes at full speed, and transferred to an orange 2 mL micro centrifuge tube, akin to the ones that were used in the field.

This collection tube will hold the primary elution buffer and purified DNA, and theoretically will contain the most DNA out of the two elution tubes. Buffer AE is added at 200 µL and the sample incubates at room temperature for one minute. It is then centrifuged for one minute, and this tube is completed and ready for use in other procedures. The mini spin column is placed in another orange micro centrifuge tube and 200 µL of Buffer AE is added. This tube is incubated and centrifuged, and the final solution contains the secondary elution buffer and purified DNA. Using Buffer AE provides optimal recovery and stability of eluted DNA (DNeasy Blood and Tissue Handbook 2006).

Following DNA extraction, each sample undergoes PCR for use on an agarose gel to be identified as either positive or negative for chytrid DNA. PCR is a relatively fast and inexpensive technique used to amplify specific segments of DNA. The sample is initially heated

so that DNA denatures and separates into single stranded DNA (ssDNA), and then *Taq* polymerase synthesizes two new strands of DNA, using the ssDNA as templates. Duplication of DNA proceeds in this format, and each of the subsequent strands can be used to create two new copies of DNA. PCR is cyclical and heating and cooling typically lasts 30 or 40 rounds, and creates millions to billions of new copies of DNA within a few hours. PCR is an automated reaction and is performed in a machine called a thermocycler (Polymerase Chain Reaction 2015). For this project, a Px2 Thermal Cycler by Thermo Electron Corporation was used. The cycles were set to one minute at 94°C once, then thirty cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, then five minutes of 72°C, and finally the samples were held at 4°C until they were removed from the machine. Thermocyclers can be programmed to ensure the same conditions for each round of PCR, and a program was set for these specific cycles in order to be consistent.

Similar to DNA extraction PCR requires a set protocol with specific reagents. For this project, 20 µL reactions were used and followed this formula:

DNA from sample:	13.7 µL
Reaction buffer:	4.0 µL
dNTP mix:	1.0 µL
5.8S primer:	.5 µL
ITS1 primer:	.5 µL
dNAP:	.3 µL
	20.0 µL total

The only variation was for the positive and negative chytrid controls, where the positive control had .5 µL of known chytrid plasmid DNA and 13.2 µL of DI water, while the negative

control had 13.7 μL of water. These controls were included with every PCR reaction in order to properly analyze the gel later on.

Initially the DNA was quantified using Nanodrop 2000 Spectrophotometer by Thermo Scientific, but after analyzing gels and consulting literature, it was decided that the DNA amount should be maximized (Mosen-Collar et al 2010). By maxing out the DNA amounts the chances of having a successful gel increased (See Table 1 of OD data).

The 5X Green GoTaq Reaction Buffer (by Promega) is necessary to create optimal conditions for *Taq* DNA polymerase (Polymerase Chain Reaction 2004). The buffer contains both blue and yellow dyes at a pH of 8.5, that separate during gel electrophoresis and aid in monitoring the migration process. On a 1% agarose gel, the yellow dye migrates faster than the primers (< 50bp), and the blue dye migrates at the same rate as 3-5kb DNA fragments. This allows for the process to be monitored visually during gel electrophoresis. Reaction buffer also helps the sample sink into the agarose well by increasing its density. The buffer contains 7.5 mM magnesium but when diluted to 1X concentration for the PCR reaction, this is present in a 1.5 mM concentration (GoTaq DNA Polymerase Protocol 2016).

The dNTP mix provides the essential building blocks for new strands of DNA. This mix, provided by Promega, is a premixed solution that contains the sodium salts of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate. Each of the dNTPs are concentrated at 10 mM in water (pH of 7.5) for a total of 40 mM (GoTaq DNA Polymerase Protocol 2016),

Typically, primers range from 15-30 base pairs and flank the DNA region of interest, allowing for the amplification of this specific region. The two primers used for chytrid amplification were as follows:

5.8SChytr: 5'-AGC CAA GAG ATC CGT TGT CAA A-3'

ITS1-3Chytr: 5'-CCT TGA TAT AAT ATG TGC CAT ATG TC-3'

MLST shows low variability of *Bd* DNA sequences across different strains. In eukaryotes, ribosomal DNA occurs in multicopy genes, and for fungi specifically, rDNA genes occur in 100-400 copies per haploid genome. The 5.8, 18, and 28S DNA are highly conserved regions and are separated by internal transcribed spacers (ITS-1 and ITS-2), and these are known to evolve quickly (Boyle et al 2004). This explains why the 5.8SChytr primer sequence matches the literature exactly and why the ITS1-3Chytr has a six-nucleotide difference.

GoTaq DNA Polymerase, also referred to as dNAP, is an enzyme derived from bacteria that allows the PCR reaction to continue. This enzyme is a nonrecombinant, modified form of *Taq* DNA polymerase and lacks the proofreading 5' → 3' exonuclease activity (GoTaq DNA Polymerase Protocol 2016). The .3 μL of dNAP was consistently loaded last into the PCR tubes because upon its addition the chemical reactions began. All the PCR tubes were then vortexed, spun down, and then added to the thermocycler.

Electrophoresis is a widely-used technique used to fractionate proteins that depends on the ability of charged molecules to migrate when placed into a magnetic field. During this portion of the lab work, a 0.8% agarose gel was utilized. By combining 20 mL of 10X Tris-borate-EDTA (TBE) buffer, 180 mL of water, and ~1.6 g of SeaKem Genetic Technology Grade agarose, a 200-mL agarose gel was created. TBE buffer is the most commonly used buffer for PAGE and aids in separation of nucleic acids. TBE is stable, has a higher buffering capacity, and obtains higher resolution of smaller DNA fragments (TBE Buffer (5X) 2017). This mixture is then heated in a microwave to allow the agarose to go into solution, cooled, and poured into a gel box apparatus. As the gel mixture is hardening in the gel box, 6 μL of ethidium bromide (EtBr) is added and evenly distributed to stain the DNA for later visualization (TBE

Buffer (Tris-borate-EDTA) (10X) 2017). Combs with a specific number of lanes, either 20 or 12, are suspended within the hardening liquid to create the wells that will hold the PCRed samples.

Once the gel is fully prepared, 6X DNA loading dye is added to the freshly PCRed samples. This dye prepares DNA samples and markers for use on an agarose gel. The samples are then vortexed, spun down, and pipetted into separate lanes in the gel. The first lane was reserved for 10 μL of Lambda DNA/HindIII ladder in order to properly compare the size of the resulting bands. Specifically, this ladder has a range of eight fragments of base-pairs, including 6557 and 4361 base-pairs; *Bd* DNA has 5918 base-pairs ("Lambda DNA/HindIII Marker" 2017). The ladder is utilized to locate approximately where *Bd* DNA would be located on the gel, which would fall between the specific fragments of the ladder. Then the positive control, negative control, and subsequent samples were loaded. TBE buffer, diluted to 1X, was poured over the top of the gel and 3 μL of EtBr was added to each side of the gel box to ensure proper conductivity. Since DNA is negatively charged, the gel box was set up to run from negative to positive and typically ran for 80 minutes. Following the completed run, the gel was moved to a UV light box and examined for results. All samples were run in triplicate to ensure accuracy (Boyle et al 2004).

Statistical Analysis

Samples meeting the statistical burden of proof were analyzed. Following the statistical analysis by Wilson et al 2015, the probabilities of detection (POD) were calculated for each species swabbed. The POD values were determined by using DiGiacomo and Koepsell's 1986 equation:

$$C = 1 - (p)^n$$

Where C is the probability of detecting at least one positive *Bd* sample, *n* is the sample size for each species, and *p* is the plausible prevalence of the disease expressed as a decimal between 0-1. Using Wilson et al 2015 and Keitzer et al 2011 as precedence, the plausible prevalence was set at 1, 5, 10, and 30%.

Hanley and Lippman-Hand's 1983 "rule of three" can provide support for statistical confidence. This rule states that if none of *n* salamanders test positive for *Bd*, I can be 95% confident that the chance of the event is at most 3 in *n*. The rule of three can be rewritten to determine an approximate sample size needed to detect a particular prevalence of disease:

$$n = \frac{3}{p}$$

Where *n* is the approximate sample size and *p* is the prevalence of the disease (Wobeser 2005).

CHAPTER III

RESULTS

All 48 animals that were swabbed in the field were conclusively negative for *Bd*. There were no positive swabs, so no statistical correlation can be drawn between *Bd* and environmental characteristics (See Table 3 for Habitat Measurements). Every animal swabbed was a member of the Plethodontidae family; these salamanders were the most speciose and were commonly found within the study areas (See Table 4 for Locations of Swabbed Amphibians). Only two genera were sampled, *Desmognathus* and *Eurycea*, for a total of six species. The most abundant species was *Desmognathus ochrophaeus*, which was captured 31 times, followed by *Desmognathus monticola* that was swabbed 11 times, *Eurycea cirrigera* was captured 3 times, and *Desmognathus fuscus*, *Desmognathus orestes*, and *Eurycea guttolineata* were swabbed once. Because there were no positive samples, the probabilities of detection were calculated, and there is 79.61% confidence that *Bd* would be detected at a prevalence rate of at least 5% for *Desmognathus ochrophaeus* (see Table 2 for POD data). Using the rule of three, there was 95% confidence that the prevalence of *Bd* was no greater than 3/48, or 6.25% within the community surveyed. A representative range of 43 – 60 animals would need to be swabbed to report 95% confidence at a prevalence rate of 6 – 7%, and with 48 animals, this study is within the range.

CHAPTER IV

DISCUSSION

Analytical techniques and standard collection protocols were used to address questions regarding the presence of *Bd* in Sullivan County, Tennessee. Zero animals were positive for *Bd* so correlations between presence and ecological factors in this area cannot be quantified.

Confidence in one's answer is challenged when all of the animals swabbed are negative. The prevalence of *Bd* was expected to be less than 6.25% within the community, and the rule of three functions under the assumption that the tests were 100% accurate and the sample is truly representative of the population. Realistically both assumptions are unlikely because of the introduction of human bias and the small sample size from each site (Wobeser 2005). This is not stating that there is a possibility that a percentage of the animals physically swabbed are false negatives, but instead provides evidence that there is no more than 6.25% prevalence of *Bd* in the areas surveyed.

Even with the mathematical evidence that at least one swab would be positive, none of them were. There are many explanations as to why this survey generated zero positive results. The first reason could be that the species sampled are resistant in *Bd* infection. Resistance is thought to occur due to antimicrobial peptides secreted by some amphibian species (Harris et al 2006). Population level variations such as life history, behavior, and skin shedding rate can impact *Bd* resistance (Harris et al 2006). It is highly unlikely that the animals swabbed in this study were resistant to infection because *Bd* in plethodontids has been reported (Keitzer et al

2011). A 2009 study based in North Carolina identified two *Bd* positive plethodontid salamanders in the field and experimentally infected two other plethodons, including *D. orestes* (Chinnadurai et al 2009). A similar study in the Appalachian Highlands identified a wild *D. monticola* as positive for *Bd* (Vazquez et al 2009). These studies surveyed roughly the same area and biological community, therefore it is highly unlikely that the animals I swabbed were resistant to *Bd*.

A second explanation could be that *Bd* was present in the areas surveyed, but it was too low to be detected. Out of the *Bd* surveys that have been conducted in the Southern Appalachian Mountains, few amphibians have tested positive for Bd, despite evidence suggesting that *Bd* would thrive in the areas that were sampled. *Bd* is known to occur at every elevation that amphibians are found, which would include the elevation range of 471 – 540 m of the sites surveyed. *Bd*'s life history states that the microorganism can survive in water as a free-living zoospore and water was a large part of the abiotic makeup of each site (Lannoo et al 2011). *Bd* is also known to reproduce in temperatures of 4°C to 25°C, with the ideal range between 17 - 25°C (Piotrowski et al 2004). The air temperatures of my sample sites range from 21 – 26.5°C. While the range does go above *Bd*'s ideal temperature range, this alone is not enough to discourage the microorganism. *Bd* also prefers areas of increased canopy cover (Beyer et al 2015), and the average canopy cover across the three sites is 92.7% closed canopy. These characteristics provide support that the sample sites appear to sustain *Bd*'s life history, and it is not simply that I was surveying uninhabitable areas. According to the *Bd* mapper, there are five recorded instances of the pathogen in the Great Smoky Mountain National Park (*Bd*-Maps.net). Recent analysis assumes that the prevalence of *Bd* in the national park is low, approximately 1-4%, but exact measures of prevalence could not be reliably stated due to the low sample size

(Chatfield et al 2009). This study suggests that the disease might be present, and if so, *Bd* is present in low prevalence. The Great Smoky Mountains National Park is close to the areas surveyed in this study, meaning that it would not be improbable that the prevalence of *Bd* was too low to be detected. Additional support for this argument stems from a seven-year (1999-2006) study in which over 1200 amphibians were swabbed across 30 sites, including the Great Smoky Mountains National Park and UT Arboretum (Rothermel et al 2008). *Notophthalmus viridescens* was the only species *Bd* positive and no amphibians swabbed from Tennessee were positive (Rothermel et al 2008). Comparing results from Rothermel et al 2008 to Chatfield et al 2009, one study did not detect any positive samples in the Great Smoky Mountains National Park while the other estimated a very low prevalence. Either *Bd* was not present in the park during the first study, or *Bd* was not present in high enough amounts to be detected. A survey conducted in 2010 sampled 452 tailed frogs (two species of *Ascaphus*) and 304 stream salamanders (Dicamptodontidae and Plethodontidae) found in U.S. headwaters. Three streams were sampled in the southern Appalachian Mountains and species swabbed include *D. monticola* and *D. ochrophaeus*. Out of the entire survey, only 7 specimens tested positive for *Bd*, and none of the animals sampled in this area were positive. This study had a large sample size and the prevalence was only 0.93% across the different sites (Hossack et al 2010). *Bd* was surveyed in Western North Carolina and Northeastern Tennessee for a period of five years, and it was determined that the pathogen was extremely uncommon (1.00% prevalence) in Caudata and was not detected in Anurans. A total of 668 swabs were taken during the sampling window, but only six salamanders tested positive for *Bd*. None of the positive species were from Tennessee (Moffitt et al 2015). The culmination of these studies, which range from 1999-2015, provide support for the fact that *Bd* may be present but too low to detect in Sullivan County.

Another reason that no positives were detected during this survey could be that the majority of animals were larval or juvenile. Adult salamanders were not intentionally avoided during sampling, it just so happened that many animals were in an earlier life stage. *Bd* encysts into keratinizing squamous epithelial cells, and larval salamanders do not have many keratinized portions of their bodies (Lannoo et al 2011). If an animal does not have keratinized tissues, *Bd* is limited because there are fewer appropriate cells in which the pathogen can continue its lifecycle. Juvenile salamanders are older and more developed than larval, but have not been alive as long as fully metamorphosed adults. This lowers the probability that the animal has been exposed because the juvenile has not had the same amount of time to become exposed compared to an adult.

For the fourth explanation, *Bd*'s life history is again examined. Experimentally, it has been determined that over 95% of motile zoospores stop moving with 24 hours and swim less than 2 cm before encysting (Piotrowski et al 2004). Each one of the three sites had moving bodies of water traveling through them, and every animal was captured directly from the water, the stream's edge, or within the riparian habitat. If *Bd* was present it is probable that the zoospore was unable to encyst due to the moving water because of how far it is able to move before it encysts.

Negative samples could indicate that *Bd* was not present at the time of sampling. While possible, this scenario is unlikely due to the pathogen's widespread occurrence in the United States (Wilson et al 2015) and supporting evidence from literature. Most likely there are no positive samples in this study because *Bd* was present but undetected during sampling. Canopy cover and water and air temperatures of the three survey areas suggest that the locations were suitable for *Bd*, but even across multiple studies, very few salamanders in East Tennessee have

tested positive. The findings of this study are relatively consistent with the results of published literature, suggesting that further research is needed to adequately evaluate *Bd*'s impacts on the Southern Appalachian Mountains region.

CHAPTER V

CONSERVATION IMPLICATIONS AND DIRECTIONS FOR THE FUTURE

Declines in biodiversity can affect local and global ecosystems that humans and wildlife depend on. Our overall stability as a biosphere depends on species extinction and we must strive to fully understand its drivers (Smith et al 2009). Creating and implementing sustainable solutions to counteract amphibian extinctions and declines is one of the greatest conversation challenges of our time (Wren et al 2015). Amphibian declines are due to numerous contributing factors, including infectious diseases.

Many amphibian conservation efforts focus on *Bd* due to its nearly global distribution and devastating effects (Woodhams et al 2011). While *Bd* does not affect all populations in the same manner, there is substantial evidence that the pathogen can lead to species extinctions. For example, *A. tigrinum* and *X. laevis* can tolerate infection and do not show clinical symptoms of chytridiomycosis, while the species *C. panamensis* is experiencing drastic population declines due to infection (Pessier 2008; Woodhams et al 2011). Conservation of amphibians regarding chytridiomycosis is focused on preventing the spread of the disease, establishing off site assurance colonies, and on site treatment strategies. Not all solutions are appropriate for every location due to differences in habitats and life histories. Treating infected organisms and environments, decreasing host density, and selective reintroduction are some of the management

programs currently in use. Complete elimination of *Bd* microorganism populations is typically not a viable option due to the complicated logistics and high possibility of reintroduction (Woodhams et al 2011).

Amphibian conservation has constraints that need to be understood and navigated in order to be effective. Immediate priorities have been identified by the Infectious Diseases Working Group of the Amphibian Survival Alliance and Amphibian Specialist Group, with the most urgent being to close critical gaps in basic knowledge (Langhammer and Harris N.d.). Research should be focused on developing a deeper understanding of global diversity and distribution of amphibian infectious diseases, identifying disease associated declines, preventing pathogen spread, and gathering baseline population data for amphibians (Langhammer and Harris N.d.). Ideally the scientific community would monitor all amphibians across all habitats, but this is neither realistic or cost effective. Therefore, our efforts should be focused on species that are able to represent a broad majority, such as plethodontid salamanders (Welsh and Droege 2001). *Bd* directly influences disease-driven extinction and by understanding its effect on plethodontids, we can better serve the representative whole.

Extraordinary amphibian diversity is a prominent feature of the Southern Appalachian Mountains, and special recognition must be given to the endemic and speciose salamander populations (Dodd N.d.; Moffitt et al 2015). Using plethodontids as study specimens in a diverse area of the world can help to close the data gaps for *Bd*. This study assembles baseline knowledge of a plethodontid salamander community in East Tennessee and contributes to the growing research of a devastating amphibian disease. Applying these results to amphibian conservation in Tennessee can help focus biological hazard protocols and community awareness. Conservation is rooted in knowledge, and further studies on the presence of *Bd* are needed to

deepen the scientific community's understanding of this disease and how it affects this region. To develop a more definitive perception of *Bd* and its impacts on this biologically diverse area, this study should be improved upon and repeated numerous times. Future studies would benefit from larger sample sizes taken over longer sampling windows, and by incorporating a wider range of taxa.

Bd's emergence, although damaging, provides the scientific community an opportunity to examine disease dynamics in complex ecological systems (Woodhams et al 2011). Sustainable amphibian conservation incorporates multifaceted concepts that can be difficult to navigate, but its end goals are achievable. Ideally the skill sets developed from these challenges will translate to other pivotal aspects of science and humanity, enabling effective conservation and technological advancements.

Table 1: OD Measurements						
Sample	260	280	260/280	ng/µL	Avg ng/µL	ABS
1	0	-0.001	-0.263	0.249	0.2985	0.2985
1	0	-0.001	-0.259	0.348		
2	0	-0.002	-0.128	0.252	-0.353	0.353
2	-0.001	-0.002	0.514	-0.958		
3	0.001	-0.001	-1.211	1.164	1.065	1.065
3	0.001	-0.001	-0.905	0.966		
4	0.001	-0.001	-2.273	1.273	0.866	0.866
4	0	-0.002	-0.243	0.459		
5	0.002	0	-39	1.964	1.209	1.209
5	0	-0.002	-0.29	0.454		
6	0.001	-0.001	-1.727	0.952	1.3	1.3
6	0.002	0	-6.6	1.648		
7	0.001	-0.001	-1.588	1.354	1.9025	1.9025
7	0.002	0	49	2.451		
8	0.001	0.001	2.417	1.444	1.2445	1.2445
8	0.001	0	5.25	1.045		
9	0.001	-0.001	-1.357	0.957	1.008	1.008
9	0.001	0.001	2.1	1.059		
10	0.002	0.001	3.417	2.075	0.097	0.097
10	-0.002	-0.002	1.028	-1.881		
11	0.002	0.001	3.071	2.189	0.61	0.61
11	-0.001	-0.001	0.792	-0.969		
12	0.003	0.002	2.031	3.273	2.2165	2.2165
12	0.001	0	-11.5	1.16		
13	0.002	0.001	3.1	1.553	1.101	1.101
13	0.001	0	-3.25	0.649		
14	0	0	6.48518E+13	0.451	0.701	0.701
14	0.001	0	3.167	0.951		
15	0.004	0.001	2.867	4.283	3.186	3.186
15	0.002	0	4.2	2.089		
16	0.001	-0.001	-1.429	1.007	1.008	1.008
16	0.001	0	????	1.009		
17	0.003	0.001	5.6	2.835	2.0275	2.0275
17	0.001	0	-4	1.22		
18	0.002	0	10	2.037	2.2425	2.2425
18	0.002	0	????	2.448		
19	0.004	0.002	2.471	4.23	4.283	4.283
19	0.004	0.002	2.687	4.336		
20	0.001	0	14	1.403	0.8515	0.8515
20	0	0	-0.75	0.3		
21	0.004	0.001	3.8	3.812	4.3075	4.3075
21	0.005	0.002	3.2	4.803		
22	0.002	0	7.4	1.843	1.8415	1.8415
22	0.002	0	37	1.84		
23	0.004	0.001	3.32	4.18	3.8305	3.8305
23	0.003	0.001	2.379	3.481		
24	0.001	-0.001	-1.182	0.658	0.202	0.202
24	0	-0.001	0.2	-0.254		
25	0	-0.001	-0.176	0.153	-0.153	0.153
25	0	-0.002	0.231	-0.459		
26	0.001	-0.001	-1.364	0.755	0.554	0.554
26	0	-0.001	-0.333	0.353		
27	0	-0.001	-0.538	0.351	0.55	0.55
27	0.001	-0.001	-1.364	0.749		
28	0.001	-0.001	-1.308	0.853	0.5015	0.5015
28	0	0	-3	0.15		
29	0.001	-0.001	-1.813	1.444	0.9955	0.9955
29	0.001	-0.001	-0.367	0.547		
30	0.002	-0.001	-4.9	2.468	2.7725	2.7725
30	0.003	0.001	3.389	3.077		
31	0.003	0	8.375	3.391	3.704	3.704
31	0.004	0	9.875	4.017		
32	0.002	0	-23.5	2.393	0.61	0.61
32	-0.001	-0.002	0.523	-1.173		
33	0.002	0	-4.875	1.964	1.562	1.562
33	0.001	-0.001	-1.278	1.16		
34	0.003	0	32.5	3.256	2.352	2.352
34	0.001	0	-4.833	1.448		
35	0.001	-0.001	-0.864	0.953	0.8515	0.8515
35	0.001	-0.001	-0.625	0.75		
36	0.001	0	-2.444	1.096	0.5975	0.5975
36	0	-0.001	-0.08	0.099		
37	0.001	-0.001	-1.043	1.209	0.453	0.453
37	0	-0.002	0.14	-0.303		
38	0	-0.002	0.242	-0.405	0.916	0.916
38	0.002	-0.002	-1.419	2.237		
39	0	-0.001	0.174	-0.204	-0.357	0.357
39	-0.001	-0.002	0.256	-0.51		
40	0	-0.002	0.242	-0.403	-0.857	0.857
40	-0.001	-0.002	0.578	-1.311		
41	-0.001	-0.001	0.444	-0.601	-0.7	0.7
41	-0.001	-0.001	0.552	-0.799		
42	0	-0.001	0.286	-0.301	0.0995	0.0995
42	0.001	-0.001	-0.667	0.5		
43	0	-0.001	0.714	-0.498	-0.9455	0.9455
43	-0.001	-0.001	1	-1.393		
44	-0.002	-0.002	1.048	-2.216	-2.4195	2.4195
44	-0.003	-0.002	1.238	-2.623		
45	-0.002	-0.002	1.111	-2.025	-2.0295	2.0295
45	-0.002	-0.002	1.111	-2.034		
46	-0.002	-0.002	1.2	-1.833	-2.0895	2.0895
46	-0.002	-0.002	1	-2.346		
47	-0.001	-0.001	0.429	-0.604	-0.201	0.201
47	0	-0.001	-0.2	0.202		
48	-0.002	-0.001	1.308	-1.703	-1.85	1.85
48	-0.002	-0.001	1.333	-1.997		

*blacked out boxes were used for computing in Excel

Table 2: POD Results of *Bd* sampling across six salamander species.					
Species	Date Sampled	Prevalence		POD	
D. fuscus	8/7/16	0/1		p=.01	0.01
				p=.05	0.05
				p=.10	0.1
				p=.30	0.3
D. monticola	8/7/16 to 8/15/16	0/11		p=.01	0.1047
				p=.05	0.4312
				p=.10	0.6862
				p=.30	0.9802
D. ochrophaeus	8/7/16 to 8/17/16	0/31		p=.01	0.2677
				p=.05	0.7961
				p=.10	0.9618
				p=.30	0.9999
D. orestes	8/10/16	0/1		p=.01	0.01
				p=.05	0.05
				p=.10	0.1
				p=.30	0.3
E. cirrigera	8/13/16 to 8/17/16	0/3		p=.01	0.0297
				p=.05	0.1426
				p=.10	0.271
				p=.30	0.675
E. guttolineata	8/11/16	0/1		p=.01	0.01
				p=.05	0.05
				p=.10	0.1
				p=.30	0.3

Table 3: Mean, Median, Mode, and Range for Habitat Measurements for All Sample Sites												
	Air Temperature (C)				Water Temperture (C)				% Closed Canopy			
Site	Mean	Median	Mode	Range	Mean	Median	Mode	Range	Mean	Median	Mode	Range
Mill Creek	24.96	25.5	24	2	21.96	22	21.1	4	92.74	91.16	88.82	11.96
Morrell Lane	22.37	22	23.8	28	20.25	20	20	1	96.41	96.23	94.02	6.24
Rooster Front	26.18	26	26	4	23.5	23.5	23.5	4.5	87.46	85.18	77.9	20.54

Table 4: Locations of Swabbed Amphibians						
ID #	Location	Coordinates	Date	Time	*Genus species*	Age
1	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/7/16	11:00-15:00	*D. monticola*	adult
2	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/7/16	11:00-15:00	*D. monticola*	adult
3	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/7/16	11:00-15:00	*D. ochrophaeus*	adult
4	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/7/16	11:00-15:00	*D. ochrophaeus*	adult
5	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/7/16	11:00-15:00	*D. ochrophaeus*	juvenile
6	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/7/16	11:00-15:00	*D. fuscus*	adult
7	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/7/16	11:00-15:00	*D. monticola*	juvenile
8	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/7/16	11:00-15:00	*D. ochrophaeus*	adult
9	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/9/16	17:30-19:30	*D. monticola*	adult
10	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/9/16	17:30-19:30	*D. monticola*	adult
11	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/9/16	17:30-19:30	*D. monticola*	adult
12	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/9/16	17:30-19:30	*D. ochrophaeus*	adult
13	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/9/16	17:30-19:30	*D. ochrophaeus*	juvenile
14	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/9/16	17:30-19:30	*D. monticola*	juvenile
15	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/9/16	17:30-19:30	*D. monticola*	adult
16	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/10/17	15:00-17:00	*D. ochrophaeus*	adult
17	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/10/17	15:00-17:00	*D. ochrophaeus*	adult
18	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/10/17	15:00-17:00	*D. orestes*	adult
19	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/10/17	15:00-17:00	*D. ochrophaeus*	larval
20	Morrell Lane	36°28’55.23’’ N and 82°07’16.40’’ W	8/10/17	15:00-17:00	*D. monticola*	adult
21	Mill Creek	36°34’44.77’’ N and 82°13’37.21’’ W	8/11/16	09:00-13:00	*E. guttolineata*	adult
22	Mill Creek	36°34’44.77’’ N and 82°13’37.21’’ W	8/11/16	09:00-13:00	*D. ochrophaeus*	larval
23	Mill Creek	36°34’44.77’’ N and 82°13’37.21’’ W	8/11/16	09:00-13:00	*D. ochrophaeus*	larval
24	Mill Creek	36°34’44.77’’ N and 82°13’37.21’’ W	8/11/16	09:00-13:00	*D. ochrophaeus*	larval
25	Mill Creek	36°34’44.77’’ N and 82°13’37.21’’ W	8/11/16	09:00-13:00	*D. ochrophaeus*	larval
26	Mill Creek	36°34’44.77’’ N and 82°13’37.21’’ W	8/11/16	16:00-18:00	*D. ochrophaeus*	larval
27	Mill Creek	36°34’44.77’’ N and 82°13’37.21’’ W	8/11/16	16:00-18:00	*D. ochrophaeus*	larval
28	Mill Creek	36°34’44.77’’ N and 82°13’37.21’’ W	8/11/16	16:00-18:00	*D. ochrophaeus*	larval
29	Mill Creek	36°34’44.77’’ N and 82°13’37.21’’ W	8/11/16	16:00-18:00	*D. ochrophaeus*	larval
30	Mill Creek	36°34’44.77’’ N and 82°13’37.21’’ W	8/11/16	16:00-18:00	*D. ochrophaeus*	juvenile
31	Rooster Front	36°33’35.66’’ N and 82°07’16.40’’ W	8/13/16	11:00-17:00	*E. cirrigera*	larval
32	Rooster Front	36°33’35.66’’ N and 82°07’16.40’’ W	8/13/16	11:00-17:00	*D. ochrophaeus*	larval
33	Rooster Front	36°33’35.66’’ N and 82°07’16.40’’ W	8/13/16	11:00-17:00	*D. ochrophaeus*	larval
34	Rooster Front	36°33’35.66’’ N and 82°07’16.40’’ W	8/13/16	11:00-17:00	*D. ochrophaeus*	larval
35	Rooster Front	36°33’35.66’’ N and 82°07’16.40’’ W	8/13/16	11:00-17:00	*D. ochrophaeus*	larval
36	Rooster Front	36°33’35.66’’ N and 82°07’16.40’’ W	8/13/16	11:00-17:00	*D. monticola*	larval
37	Rooster Front	36°33’35.66’’ N and 82°07’16.40’’ W	8/13/16	11:00-17:00	*D. ochrophaeus*	larval
38	Mill Creek	36°34’44.77’’ N and 82°13’37.21’’ W	8/15/16	11:00-13:00	*D. ochrophaeus*	larval
39	Rooster Front	36°33’35.66’’ N and 82°07’16.40’’ W	8/15/16	11:00-13:00	*D. ochrophaeus*	larval
40	Rooster Front	36°33’35.66’’ N and 82°07’16.40’’ W	8/15/16	11:00-13:00	*D. ochrophaeus*	larval
41	Rooster Front	36°33’35.66’’ N and 82°07’16.40’’ W	8/15/16	11:00-13:00	*D. monticola*	larval
42	Rooster Front	36°33’35.66’’ N and 82°07’16.40’’ W	8/16/16	11:00-11:30	*D. ochrophaeus*	larval
43	Rooster Front	36°33’35.66’’ N and 82°07’16.40’’ W	8/16/16	11:00-11:30	*D. ochrophaeus*	larval
44	Rooster Front	36°33’35.66’’ N and 82°07’16.40’’ W	8/17/17	13:00-16:00	*D. ochrophaeus*	larval
45	Rooster Front	36°33’35.66’’ N and 82°07’16.40’’ W	8/17/17	13:00-16:00	*E. cirrigera*	adult
46	Mill Creek	36°34’44.77’’ N and 82°13’37.21’’ W	8/17/17	13:00-16:00	*E. cirrigera*	larval
47	Mill Creek	36°34’44.77’’ N and 82°13’37.21’’ W	8/17/17	13:00-16:00	*D. ochrophaeus*	larval
48	Mill Creek	36°34’44.77’’ N and 82°13’37.21’’ W	8/17/17	13:00-16:00	*D. ochrophaeus*	larval

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VITA

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