PREVALENCE ASSESSMENT OF THE AMPHIBIAN CHYTRID FUNGUS *BATRACHOCHYTRIUM DENDROBATIDIS* ACROSS TWO HABITAT TYPES IN EAST TENNESSEE

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ABSTRACT

In light of the biodiversity crisis facing amphibian populations globally, studies investigating the pathogenic amphibian fungus *Batrachochytrium dendrobatidis* (*Bd*) are a foremost priority for biologists. Understanding effects of habitat variation on *Bd* prevalence is important for identifying populations that are most at risk and can help to inform management decisions. Using American Bullfrogs (*Lithobates catesbeianus*) and Green Frogs (*Lithobates clamitans*) as study organisms, this research sought to investigate how prevalence of *Bd* varies between natural wetlands and urban retention ponds in East Tennessee while also examining relevant habitat factors and morphometrics. A total of 373 frogs were sampled across six retention ponds and six wetlands distributed evenly between two basin level hydrologic unit codes. Of the frogs sampled, 11 tested positive for *Bd.* These data provide new insights into the status of *Bd* prevalence and distribution in Tennessee and provide information useful in future conservation and remediation efforts.

DEDICATION

This thesis is dedicated to my wife Sherri, whose unwavering support throughout this process made the whole thing possible.

ACKNOWLEDGEMENTS

There are a number of people I would like to acknowledge here as this research would not have been possible as a singular effort on my part. Firstly, I would like to acknowledge the members of Team Salamander, undergraduate and graduate students, as well as friends and family that spent late nights assisting me in the field. I would like to especially acknowledge my friend and colleague, Jonathan Carpenter for assisting me in the field on numerous occasions and functioning as a sounding board for discussion of current literature and streamlining field strategies. I would also like to especially acknowledge friend and lab-mate Nyssa Hunt for the spatial imagery in this document and in multiple poster presentations along the way.

Secondly, I would like to acknowledge Cameron Brocco for showing me the ropes in the genetics laboratory and doing the lion's share of the trouble shooting when things went awry.

Thirdly, I want to thank my committee members Dr. Ethan Carver, Dr. Jose Barbosa, and Dr. Bradley Reynolds for providing constant support, laboratory materials, and guidance. I would also like to thank Dr. Margaret Kovach for use of laboratory space, machine and instrument use, and for providing the occasional advice.

Lastly, but certainly not least, I want to thank my advisor Dr. Thomas Wilson for being not only a transformational mentor but also a friend. Without the guidance, support, and assistance of Dr. Wilson my academic career and professional growth would certainly not have progressed to where it is today.

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LIST OF ABBREVIATIONS

AUP, animal use protocol

Bd, *Batrachochytrium dendrobatidis*

DNA, deoxyribonucleic acid

dNTP, deoxynucleotide triphosphates

GIS, geographic information system

HUC, hydrologic unit code

HW, head width

km, kilometers

L., *Lithobates*

m, meters

mm, millimeters

PCR, polymerase chain reaction

POC, percent overstory canopy

POD, probability of detection

ranid, belonging to the family Ranidae

SE, standard error

TBE, tris/borate/EDTA

TBL, total body length

UTC, University of Tennessee at Chattanooga

LIST OF SYMBOLS

μ, micro

°, degree

λ, lambda

<, less than

≤, less than or equal to

≥, greater than or equal to

n, number

n, population

CHAPTER I

INTRODUCTION

Background

Batrachochytrium dendrobatidis (Bd) is a fungus that causes a disease known as chytridiomycosis that is responsible for widespread amphibian declines around the world and has been detected in 56 of 82 countries surveyed (Olson et al. 2013). *Bd* has been linked to extirpation and extinction events throughout the world and is a significant contributor to the current global biodiversity crisis (Olson et al. 2013). Not only is this pathogen responsible for the global decline of amphibian populations, it is also a significant component in the broader context of factors that are contributing to the sixth major mass extinction event facing this planet (Ceballos et al. 2015, Wake and Vredenburg 2008, Dodd Jr 2010). It is for this reason that studies investigating this disease are a critical piece of the conservation puzzle and why any new information on this topic should be viewed as valuable as it works to better inform future mitigation and management decisions.

The *Bd* lifecycle consists of two stages, the mobile flagellated zoospore stage and the sessile zoosporangium stage (Berger et al. 1998). The zoospore is free-living in the environment until it finds an amphibian host at which time it encysts in the epidermis and forms the zoosporangium. Penetration of the fungus into the amphibian cells is poorly understood but it is thought that this is achieved through proteolytic enzymes and the formation of a germ tube that functions to inject the nucleus into the amphibian cell (Berger et al. 1998, Berger et al. 2005,

Longcore, Pessier, and Nichols 1999, Van Rooij et al. 2012). Zoospores are produced by the zoosporangium and once mature are released into the environment or back onto the epidermis to reinfect the host (Berger et al. 2005).

Laboratory studies have demonstrated the effects of chytridiomycosis to include symptoms of sloughing of skin, lethargy, dilated pupils, reduced coordination, and parakeratotic hyperkeratosis (Berger et al. 1998, Carver, Bell, and Waldman 2010). Berger et al. (1998) was the first to identify *Bd* and suggests that mortality is ultimately caused by compromised respiration and osmoregulation.

Interspecific differential susceptibility to *Bd* has been documented where certain species have mortality rates as high as 100% while others, such as several species of true-frogs from the genus *Lithobates,* are able to persist with the disease and can function as reservoirs and vectors for spreading the pathogen (Blaustein et al. 2005, Gahl, Longcore, and Houlahan 2012, Ortiz-Santaliestra et al. 2013). It is largely expected that different species with similar physiologies, morphologies, life-history traits, and habitats would share similar rates of infection prevalence as demonstrated by Blaustein et al. (2005) and Gahl, Longcore, and Houlahan (2012), but Wilson et al. (2015) found that that *L. catesbeianus* and *L. clamitans* differed in infection prevalence at an isolated wetland in southeast Tennessee. Wilson et al. (2015) found that *L. clamitans* had higher rates of *Bd* prevalence compared to *L. catesbeianus*. These findings are interesting as *L. catesbeianus* and *L. clamitans* share many similarities that would suggest that infection rates might be similar between the two species. Additional studies are needed to determine if these differences exist on a broader scale and in other assemblages where *L. catesbeianus* and *L. clamitans* co-occur.

While interspecific variation in susceptibility to *Bd* is fairly well documented, intraspecific variation is not as well understood, especially in free-ranging populations (Bradley et al. 2015). The role of age class, sex, and various morphometrics on infection prevalence is an important area of research as it helps to elucidate which demographics are at the greatest risk of disease. Additionally, variation in certain habitat factors such as canopy cover as well as habitat type (i.e. natural or manmade) need to be further investigated as these factors may play a role in intraspecific infection prevalence variation. It should be noted that in studies such as Becker and Zamudio (2011), *Bd* has been detected at higher rates in remote populations that experience very little human interference than in disturbed habitats, thus raising questions about the role of habitat type on disease prevalence.

While habitat loss is often associated with species declines, studies have shown that amphibians are at greater risk of *Bd* infection in pristine habitats than disturbed habitats, with canopy cover having a significant positive relationship with disease risk (Becker et al. 2012, Becker and Zamudio 2011). One study found that pond margins with greater canopy cover resulted in cooler water, which is more favorable to *Bd* and ultimately results in greater infection prevalence (Becker et al. 2012). While studies such as Becker and Zamudio (2011) suggest that tropical populations in pristine habitats are more at risk for *Bd* infection than populations in disturbed habitats, additional studies are needed to evaluate whether this trend holds true for populations in ridge-and-valley ecoregions at temperate latitudes such as is found in East Tennessee. Saenz, Hall, and Kwiatkowski (2014) found significantly greater prevalence of *Bd* (62.9%) at forested sites than urbanized sites (19.5%) and suggests that urban sites may provide refuge from the disease.

To date, very little is known about the prevalence of *Bd* in anurans found in urban retention ponds as compared to conspecifics residing in nearby natural wetlands. Habitat generalists such as the American Bullfrog (*Lithobates catesbeianus*) and the Green Frog (*Lithobates clamitans*) are commonly found in urban retention ponds as well as natural wetlands in East Tennessee and so are ideal species for making comparisons between populations from the two habitat types. Usage of retention ponds by amphibians is well documented and may play an important role in maintaining populations in areas experiencing an increase in urbanization and a decrease in natural habitats (Hamer, Smith, and McDonnell 2012). Because retention ponds generally have less canopy cover than natural ponds or wetlands, they may also function in providing habitat with a reduced *Bd* infection risk. However, because both *L. catesbeianus* and *L. clamitans* are known to travel between bodies of water and are thought to be vectors of *Bd*, understanding possible differences in disease risk to populations of the two habitat types can yield information useful in informing habitat management decisions. *Bd* has been detected in several amphibian populations across the southeast including East Tennessee (Wilson et al. 2015) but additional studies are needed to fully characterize the extent of *Bd* spread in East Tennessee and the effect of habitat type on prevalence of infected individuals.

Objectives

This study seeks to answer the following questions: Is there a link between habitat conditions such as canopy cover and *Bd* infection prevalence? Does the seemingly paradoxical situation where disturbed habitats have lower *Bd* infection rates than pristine habitats compare to and hold true when evaluating *Bd* at wetlands and retention ponds in East Tennessee? Does occurrence of *Bd* vary between the two target species as noted by Wilson et al. (2015)? Does *Bd*

prevalence vary by certain morphometrics, sex, and age class and are the effects of these variables more pronounced in one of the two habitat types? Additionally, this study seeks to provide new information on the prevalence of *Bd* in ranid frog populations in East Tennessee and further characterize the distribution and prevalence of the disease within the state.

CHAPTER II

MATERIALS AND METHODS

Study Sites

Study sites were selected using geographic information systems (GIS) and are distributed across two distinct watershed basins in East Tennessee. These watershed basins are identified as unique hydrologic unit codes (HUCs). The East Tennessee counties in which sampling took take place across the three HUCs are as follows: Hamilton, Marion, Polk, Rhea, Roane, Loudon, Monroe, and Blount. The HUC scale was set at HUC 6, which is defined as the basin scale. This scale was selected because it allows for study sites within each HUC to be at least 10 km from one another, which is necessary for spatial and statistical independence (Wilson personal correspondence). Studies have indicated that watersheds may be an important predictor in modeling risk of *Bd* infection (Richardson, Govindarajulu, and Anholt 2014) and that the motile chytrid zoospores can be transported and remain infective for up to seven weeks in water (Johnson and Speare 2003). Within each of the basins, three retention ponds and three natural wetlands, all separated by a minimum of 10 km, were surveyed (Figure 1). The following sites are listed and described in the order they were surveyed and identified as either wetland or retention pond. A summary of the study sites can be found in Appendix C.

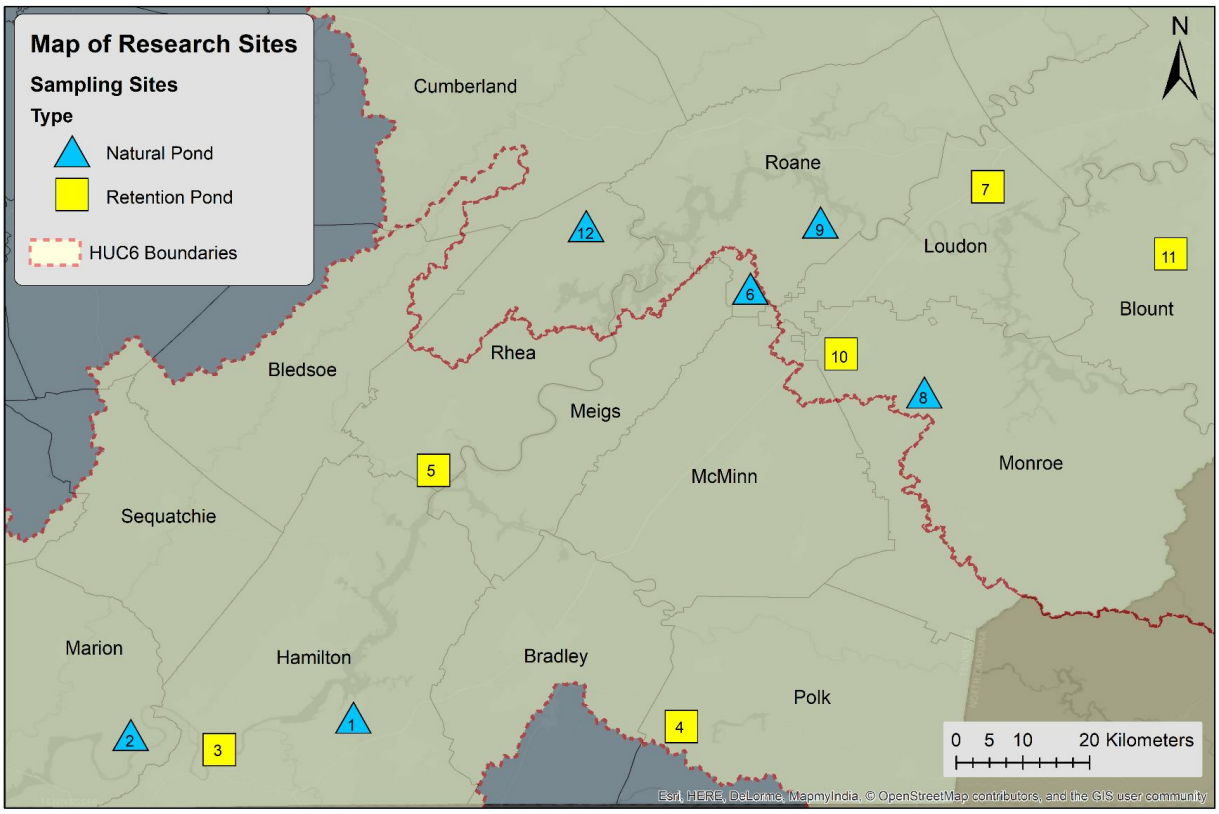


Figure 1 Map of study sites

Site 1 Wetland (LT6)

LT6 (Figure 2) is located in the southeast corner of Hamilton County at 35° 6'17.86"N, 85° 7'48.57"W. This site is owned by the University of Tennessee at Chattanooga and is the location of a long-term study examining amphibian communities. This site borders an industrial complex known as Enterprise South which houses a Volkswagen manufacturing plant and an Amazon distribution center as well as a nature park containing walking, driving, and mountain bike trails. The wetland is approximately five hundred and fifty meters in circumference with a site average canopy coverage of 97.87%. Sampling was conducted in 2016 on June 1-13. The frogs sampled at this site were twenty-five adult male *L. catesbeianus*, three adult female, *L. catesbeianus*, and two subadult *L. clamitans*.

Figure 2 Aerial image of Study Site 1 (LT6)

Site 2 Wetland (Davis Pond)

Davis Pond (Figure 3) is located in the southeast corner of Marion County at 35° 4'53.81"N, 85°25'48.65"W. This site is located near the center of Prentice Cooper Wildlife Management Area which is a part of Prentice Cooper State Forest. Prentice Cooper State Forest is 24,686 acres and is located roughly ten miles west of Chattanooga and is a regionally popular location for hunting, hiking, camping and all-terrain vehicle recreation (Tennessee Department of Agriculture n.d.). This site is only accessible via gravel road and is adjacent to a small campground. The wetland is approximately two hundred and seventy meters in circumference

with a site average canopy coverage of 91.00%. Sampling was conducted in 2016 on July 18, 19, and 26. The frogs sampled at this site were four adult male *L. catesbeianus,* sixteen adult male *L. clamitans,* four adult female *L. catesbeianus,* three adult female *L. clamitans,* and three subadult *L. clamitans.*

Figure 3 Aerial image of Study Site 2 (Davis Pond)

Site 3 Retention Pond (Renaissance Park Pond)

Renaissance Park Pond (Figure 4) is located in the southwest corner of Hamilton County and in north Chattanooga at 35° 3'41.37"N, 85°18'39.21"W. This pond is in a highly trafficked area known as Renaissance Park and is bordered on its south side by the Tennessee River and on its west side by industry, US Highway 27, and other miscellaneous impervious surfaces. This pond is bordered on its north and east sides by manicured park lawns, sidewalks, shopping centers, parking lots, apartment complexes, etcetera. This pond is approximately four hundred meters in circumference with a site average canopy coverage of 38.82%. Sampling was

conducted in 2017 on March 23, and April 3, 4, 6, and 10. The frogs sampled at this site were twenty adult male *L. catesbeianus,* and ten adult female *L. catesbeianus*.

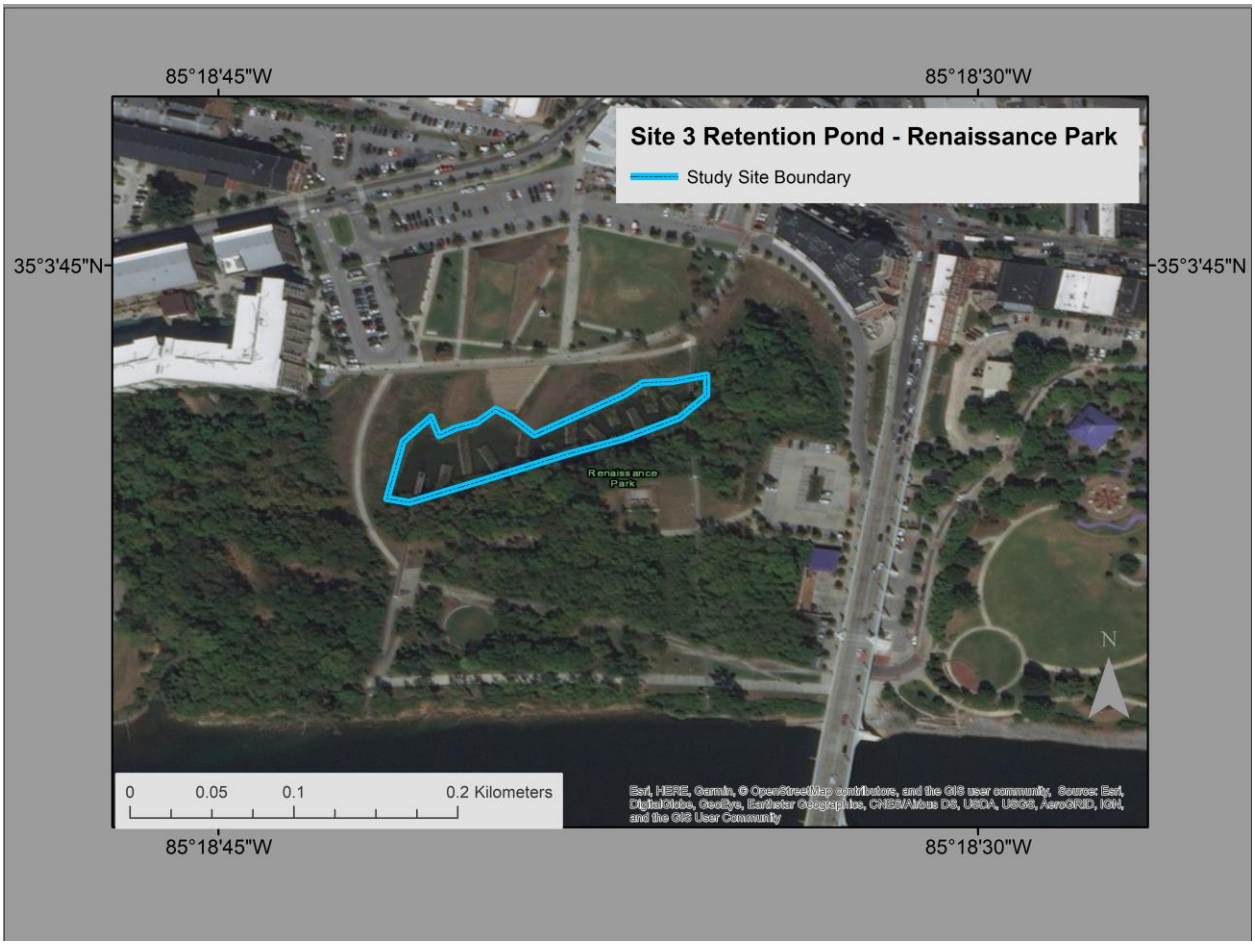


Figure 4 Aerial image of Study Site 3 (Renaissance Park)

Site 4 Retention Pond (Polk County Pond)

Polk County Pond (Figure 5) is in the southwest corner of Polk County on the private property of a housing community just inside the boundary of the Cherokee National Forest at 35° 5'32.73"N, 84°41'17.53"W. The neighborhood in which this pond is located is somewhat remote and gets very little vehicle traffic. This pond is bordered on its north and east sides by Mountain View Circle and a few houses. It is bordered on its south and west sides by forest and few scattered houses. This pond is approximately one hundred and seventy meters in circumference with a site average canopy coverage of 0.16%. Sampling was conducted in 2017 on May 2, 9, and 11, and September 18. The frogs sampled at this site were one adult male *L. catesbeianus,*

twelve adult male *L. clamitans*, three adult female *L. catesbeianus*, nine subadult *L. catesbeianus*, four subadult *L. clamitans*, and one adult male *L. sphenocephalus*.

Figure 5 Aerial image of Study Site 4 (Polk County Pond)

Site 5 Retention Pond (Golden Pond)

Golden Pond (Figure 6) is in the southeast corner of Rhea County on the private property of a small condominium community known as Cottages on Golden Pond at 35°26'16.09"N, 85° 1'19.61"W. This retention pond is bordered on its north side by what appears to be a natural pond and is separated by an impoundment and small gravel road. It is bordered on its west side by Tennessee State Route 60, on its south side by a manicured field and several small condominiums and on its east side by patches of forest and open fields. This pond is approximately three hundred and twenty meters in circumference with a site average canopy coverage of 16.67%. Sampling was conducted in 2017 on May 16, 17, and 20, and September 7.

The frogs sampled at this site were seven adult male *L. catesbeianus*, ten adult female *L. catesbeianus*, thirteen subadult *L. catesbeianus*, and one adult female *L. sphenocephalus*.

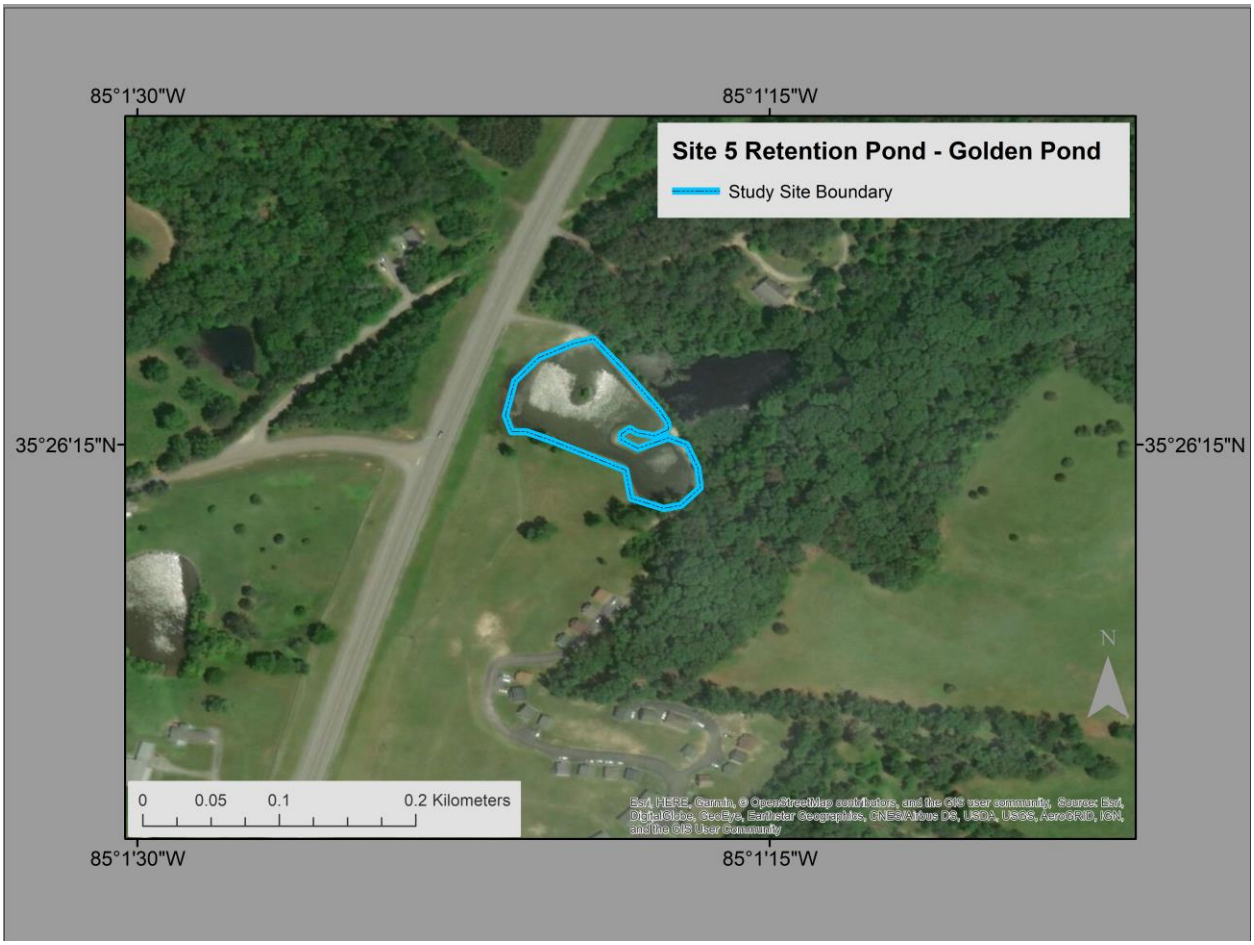


Figure 6 Aerial image of Study Site 5 (Golden Pond)

Site 6 Wetland (Roane County Wetland)

Roane County Wetland (Figure 7) is in the southeast corner of Roane County on private property at 35°40'58.85"N, 84°35'42.55"W. It is bordered essentially on all sides by forest with nearby open fields and a few distantly spaced houses. Hughes Hollow Road borders the south side. This wetland gets very little foot traffic other than the occasional duck hunter that the property owners allow onto the property during duck season. This wetland is approximately four hundred and thirty meters in circumference with a site average canopy coverage of 54.31%. Sampling was conducted in 2017 on May 30 and June 1 and 5. The frogs sampled at this site

were one adult male *L. catesbeianus*, thirteen adult male *L. clamitans*, nine adult female *L. catesbeianus*, six adult female *L. clamitans*, and one subadult *L. catesbeianus*.

Figure 7 Aerial image of Study Site 6 (Roane County Wetland)

Site 7 Retention Pond (Lenoir City Pond)

Lenoir City Pond (Figure 8) is located in the approximate center of Loudon County on the private property of a Farm Bureau Insurance Agency at 35°49'10.33"N, 84°16'31.01"W. This pond is bordered on its north side by the four lane Town Creek Parkway, on its south side by an equestrian facility called Blue Point Stables, on its west side by parking lots, buildings, and adjacent open fields, and on its east side by a small section of forest and a residential neighborhood. This pond has steep banks with two of its three edges comprised of grasses and sedges and the third side primarily containing thick Chinese privet and blackberry bushes. This

pond is approximately two hundred and ten meters in circumference with a site average canopy coverage of 25.86%. Sampling was conducted in 2017 on June 11, 15, and 17. The frogs sampled at this site were thirty subadult *L. catesbeianus* and one subadult *L. clamitans*.

Figure 8 Aerial image of Study Site 7 (Lenoir City Pond)

Site 8 Wetland (Echo Valley Farm Wetland)

Echo Valley Farm Wetland (Figure 9) is located in the northwest corner of Monroe County on a privately owned organic dairy farm called Echo Valley Farm at 35°32'34.33"N, 84°21'38.73"W. This wetland is surrounded on all sides by a dense buffer of woody vegetation and outside of that buffer are livestock pastures and scattered houses and barns. There is another small body of water separated by a gravel road on the south side of the wetland. This wetland is approximately four hundred meters in circumference with a site average canopy coverage of 85.94%. Sampling was conducted in 2017 on June 21 and 27 and July 5. The frogs sampled at

this site were six adult female *L. catesbeianus*, four adult female *L. clamitans*, and twenty subadult *L. catesbeianus*.

Figure 9 Aerial image of Study Site 8 (Echo Valley Farm Wetland)

Site 9 Wetland (Reed Wetland)

Reed Wetland (Figure 10) is located in east central Roane County on a large single residence privately owned tract of land at 35°46'19.88"N, 84°30'2.61"W. This wetland is bordered on its south and west sides by large sections of contiguous forest and on its north and east sides my manicured fields and one large house and outbuilding. This wetland is approximately two hundred and fifty meters in circumference with a site average canopy coverage of 82.08%. Sampling was conducted in 2017 on June 25 and 30 and July 10. The frogs sampled at this site were two adult male *L. catesbeianus*, six adult male *L. clamitans*, three adult

female *L. catesbeianus*, four adult female *L. clamitans*, thirteen subadult *L. catesbeianus*, two subadult *L. clamitans*, and one adult female *L. sphenocephalus*.

Figure 10 Aerial image of Study Site 9 (Reed Wetland)

Site 10 Retention Pond (Willow Creek)

This retention pond (Figure 11) is actually a pair of connected ponds that due to spatial proximity are treated as one site. This site is located in the northwest corner of Monroe County and is on the privately owned neighborhood of Willow Creek Housing Authority at 35°35'40.57"N, 84°28'22.91"W. This site is bordered on the north and west sides by Willowcreek Boulevard and the Willow Creek neighborhood. On the south and east side this site is bordered by manicured lawns and fields as well as restaurants, shops, parking lots and other miscellaneous impervious surfaces. The combined circumference of the two ponds is approximately six hundred and thirty meters with a site average canopy coverage of 5.39%.

Sampling was conducted in 2017 on July 15, 17, and 19. The frogs sampled at this site were one adult male *L. catesbeianus*, four adult male *L. clamitans*, five adult female *L. clamitans*, twenty-one subadult *L. clamitans*, and one subadult *L. sphenocephalus*.

Figure 11 Aerial image of Study Site 10 (Willow Creek)

Site 11 Retention Pond (Maryville Pond)

Maryville Pond (Figure 12) is located in the northwest corner of Blount County and is situated on the northwest side of an apartment complex called The Reserve at Maryville at 35°43'45.49"N, 84° 1'42.69"W. Maryville Pond is bordered to the south and east by parking lots and apartments and to the north and west by open fields, houses and a small patch of forest. The entire pond is encircled in a paved walking path and a manicured lawn reaching up to the edge of the water. This pond is approximately three hundred and ten meters in circumference but the majority of the frogs sampled at this site were captured on a one hundred and thirty-meter stretch of paved walking path on the northwest side of the pond. Site average canopy coverage for the

pond is 17.62% while the average canopy coverage for the path where the majority of the frogs were captured is 26.20%. Sampling was conducted in 2017 on July 25 and 27 and August 3 and 12. With the exception of one adult male *L. clamitans*, no other individuals from the two target species groups were able to be located and captured at this site. The frogs that were sampled at this site were three adult female *L. palustris*, twelve adult female *L. sphenocephalus*, thirteen subadult *L. palustris*, four subadult *L. sphenocephalus*, and one adult male *L. clamitans*.

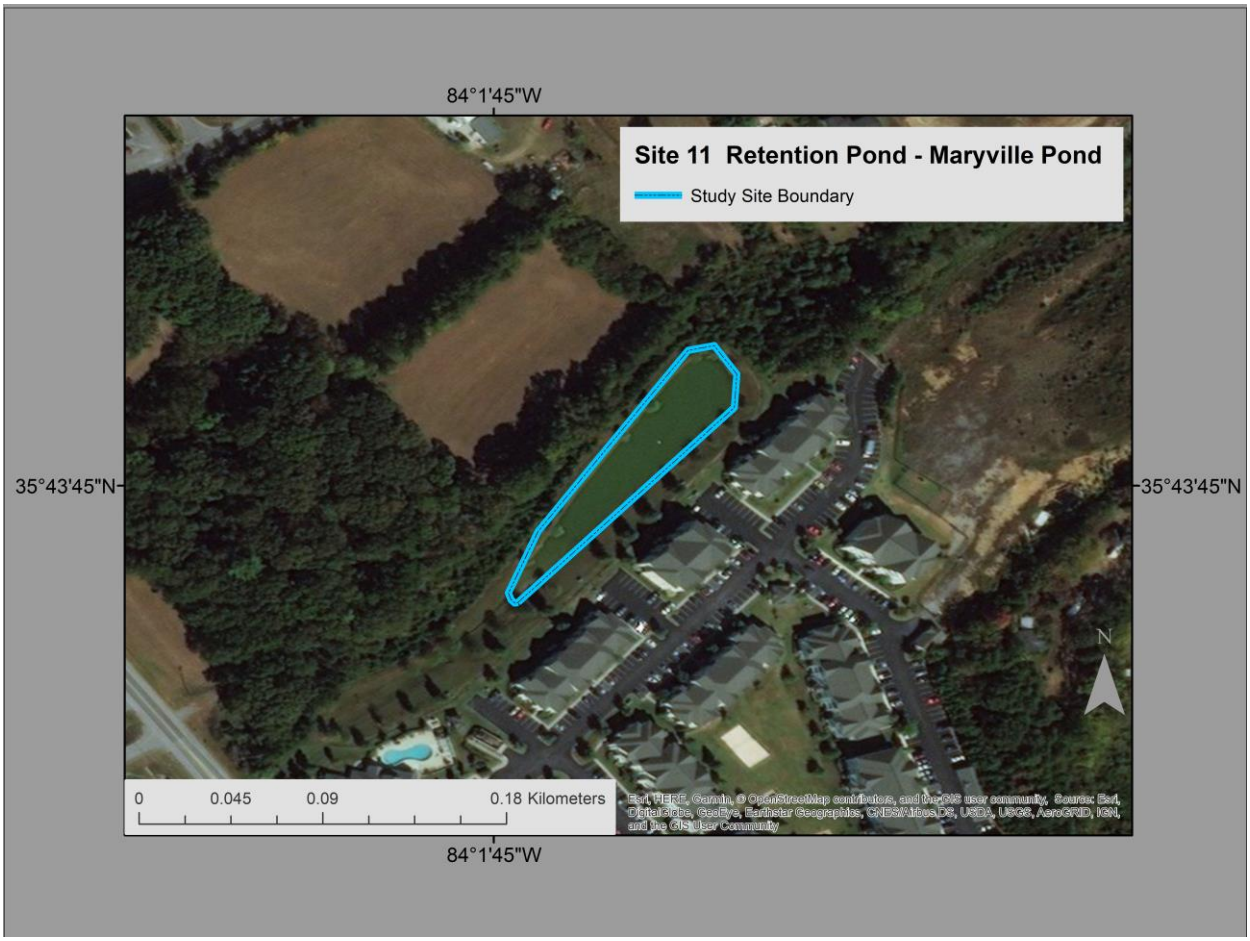


Figure 12 Aerial image of Study Site 11 (Maryville Pond)

Site 12 Wetland (Grandview Cottages)

Grandview Cottages wetland (Figure 13) is located in the northwest corner of Rhea County and is situated on a piece of privately owned property at 35°46'2.18"N, 84°48'59.59"W that has been run as an organic farm since the 1970s and more recently has been turned into a mountain vacation spot with small primitive cabins called Grandview Cottages. This wetland is open to guests of Grandview Cottages for fishing and picnicking, etcetera. The wetland is entirely encircled in forest and a buffer of approximately thirty meters separating the edge of the water from the nearest open field. This wetland is approximately one hundred and ninety meters in circumference and has a site average canopy coverage of 97.39%. Sampling was conducted in

2017 on August 29 and September 3 and 6. The frogs sampled at this site were one adult male *L. catesbeianus*, two adult male *L. clamitans*, five adult female *L. catesbeianus*, one adult female *L. clamitans*, eighteen subadult *L. catesbeianus*, three subadult *L. clamitans*, two adult female *L. sphenocephalus*, one adult female *L. palustris*, and one subadult *L. palustris*.

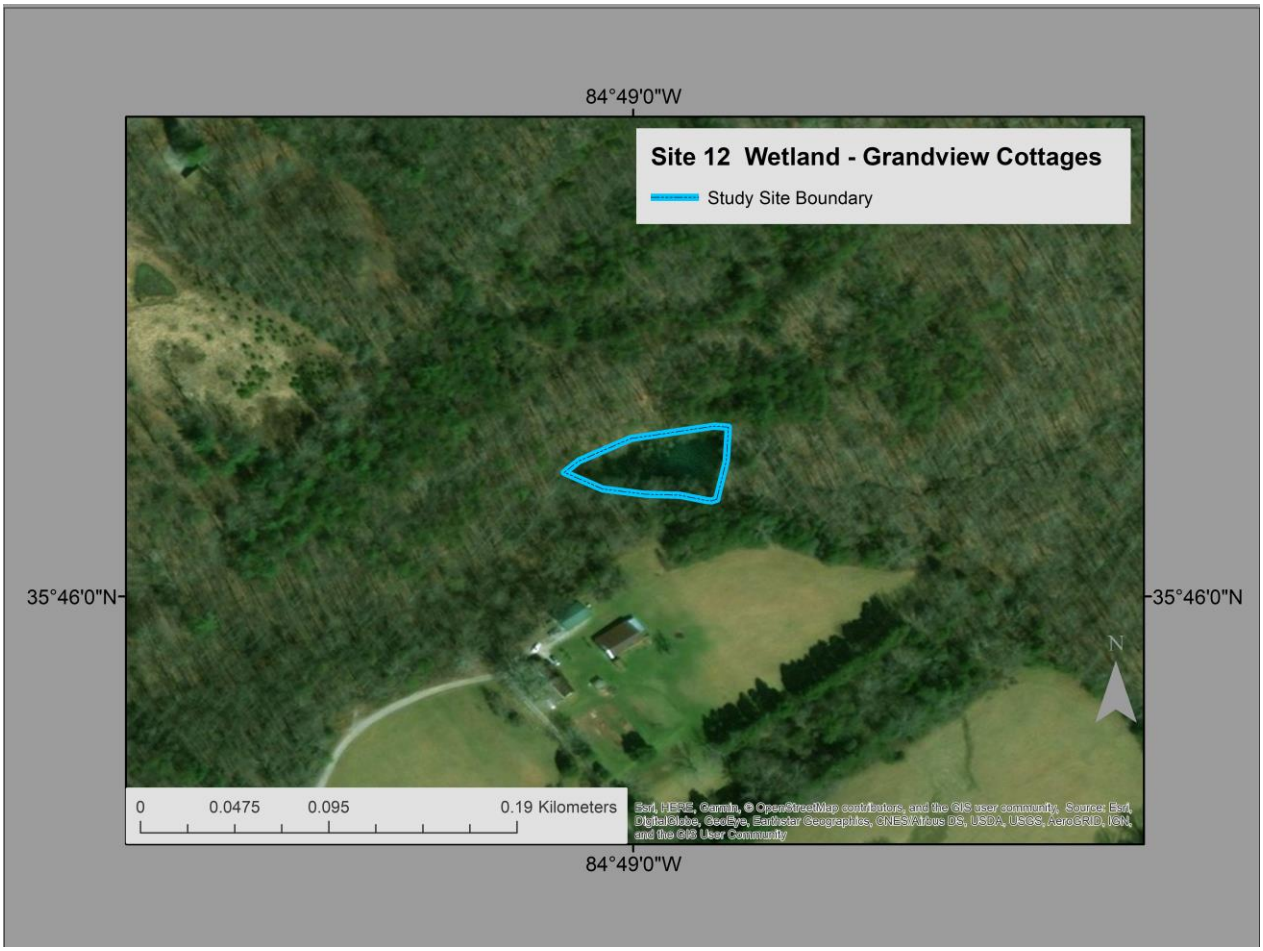


Figure 13 Aerial image of Study Site 12 (Grandview Cottages)

Study Organisms

L. catesbeianus and *L. clamitans* are both true-frogs of the genus *Lithobates* within the family *Ranidae* and occur sympatrically throughout their native ranges (Conant and Collins 1998). Both species have webbed hind feet and spend a great portion of their lives in or near water. Both species are sexually dimorphic as adults with males having a tympanum larger than the eye while the tympanum of females is equal to or smaller than the size of the eye (Conant and Collins 1998). Both *L. catesbeianus* and *L. clamitans* breed during the spring and summer months and tend to have restricted home ranges during that time with an average home range of 62 meters for *L. clamitans* and an average activity radius for *L. catesbeianus* of 2.6 meters

(Barbour 1971, Bury and Whelan 1985, Currie and Bellis 1969, Hamilton 1948, Mount 1975). This temporally restricted home range indicates that the majority of individuals tend to remain at one site and do not travel between ponds during breeding season (Willis, Moyle, and Baskett 1956).

Field Methods

To test for differences in chytrid prevalence between urban retention ponds and natural wetlands, six retention ponds and six wetlands evenly divided across two HUCs in East Tennessee were surveyed over a block of time in 2016 and 2017 when *L. catesbeianus* and *L. clamitans* breeding activity coincides with optimal conditions for *Bd*. Lannoo et al. (2011) found a strong temporal correlation between season and positive *Bd* samples, with spring and early summer yielding the greatest number of positive samples. The fact that peak chytrid season coincides with temporally restricted home ranges due to breeding activity make spring and early summer an ideal window of time to sample for *Bd*. Also, risk of pseudo replication is greatly reduced when samples are taken from localities that are separated by distances greater than 10 km. Canopy cover measurements were taken via spherical densitometer readings at 10-meter intervals along the entire edge of the water line and then values were averaged to derive an overall percent overstory canopy (POC) value for each site (Becker et al. 2012).

A goal of 30 frogs (15 *L. catesbeianus* and 15 *L. clamitans*) was set for each site (Kriger and Hero 2007). While Bullfrogs (*Lithobates catesbeianus*) and Green Frogs (*Lithobates clamitans*) were the focal species for this study, two other species of ranid frogs (*L. sphenocephalus* and *L. palustris*) were incidentally captured and processed at a few sites and at one site (Site 11 Retention Pond – Maryville Pond) were the only frogs captured aside from a single male *L. clamitans*. Due to ecological similarities to the focal species, these additional

species are included in some appropriate analyses where the only factor of interest is presence/absence of *Bd*. In this case all individuals sampled in this study can function as replicates, regardless of species.

The sex of all frogs captured was recorded. Each frog received two measures of body size, total body length (TBL) and head width (HW). To avoid sampling the same individuals across sampling events, each frog captured was batch marked via toe clipping the outer digit of the front right limb. Toe clips were placed in individual microcentrifuge tubes with 70% ethanol and stored in a -80°C freezer.

Each frog captured was placed in a one-time-use plastic sandwich bag from which it was swabbed with a sterile Dacron-tip applicator for a minimum of 45 seconds (Vredenburg and Briggs 2009, Wilson et al. 2015). The Dacron-tip applicator was then placed in a microcentrifuge tube with 70% ethanol. Samples were kept on ice until they could be placed in a -80°C freezer (Wilson et al. 2015). To reduce the likelihood of spreading *Bd* between sites, standard biosecurity protocol was followed as outlined by Phillott et al. (2010). Biosecurity protocol included measures such as changing gloves between handling each frog and sterilization of all equipment and footwear between sites.

Laboratory Methods

DNA Extraction

Each swab sample was dried in a speedvac (Labconco, Centrivap DNA Concentrator; Kansas City, Missouri, USA) prior to DNA extraction. DNA was extracted from samples using Qiagen DNeasy Blood & Tissue Kits under the Animal Tissue protocol (Qiagen, DNeasy Blood and Tissue Kit; Hilden, North Rhine-Westphalia, Germany). Qiagen DNeasy kits are preferred to other similar products due to their efficiency, relative affordability, and rapid nature (Kosch

and Summers 2013). Use of Qiagen kits standardized and simplified the DNA extraction process and reduced the likelihood of laboratory procedural errors (Kosch and Summers 2013, Shin et al. 2014).

PCR and Electrophoresis

Conventional polymerase chain reaction (PCR) was used to test for the presence of *Bd*. Each sample was run in triplicate. To attempt to minimize false negatives and conclusions that underrepresent reality, if a sample showed as a clear positive in one of the three runs it was considered positive regardless of the results of the other runs. This type of liberal approach has precedence in the literature and is preferential over more conservative methods that have potential to underrepresent true infection prevalence (Lannoo et al. 2011). Underrepresenting true infection prevalence carries significant implications for the populations in question.

Each PCR assay contained 13.7µL of DNA sample suspended in elution buffer, 4 µL of Promega 5x green reaction buffer, 1µL of Promega dNTP mixture, 0.5µL of Eurofins IST1 primer (5'-CCTTGATATAATATGTGCCATATGTC-3'), 0.5µL of Eurofins 5.8s primer (5'-AGCCAAGAGATCCGTTGTCAAA-3'), and 0.3µL of TAQ polymerase for a total volume of 20µL in each tube. Positive and negative controls contained all of the same reagents with the exception that negative controls contained 13.7µL of molecular grade water in place of DNA and the positive controls contained 13.2µL of molecular grade water and 0.5µL of chytrid plasmid.

PCR assays were run in a thermocycler (Px2 Thermal Cycler, SN: PX210785 Thermo Electron Corporation, Milford, MA, USA) under a modification of a program protocol written by Boyle et al. (2004) designed to maximize *Bd* DNA amplification. The thermocycler was set for one cycle of 2 minutes at 50°C followed by 10 minutes at 95°C then 50 cycles of 15 seconds at

95°C followed by 1 minute at 60°C. PCR products were held at 5°C in the thermocycler until they could be run on a 1.2% agarose gel with TBE, ethidium bromide, and a λ/ hindIII ladder.

Statistical Analysis

All statistical analysis was performed using SPSS (IBM Statistics 2017, version 25). For tests of normality on comparisons of interest I evaluated the results of Shapiro-Wilk's tests where the null hypothesis is that the data is normally distributed and is rejected when the p-value is less than 0.05 (Shapiro and Wilk 1965, Razali and Wah 2011). I also evaluated skewness and kurtosis by determining if z-values (calculated by dividing the skewness and kurtosis measures by their standard errors) fall between +/-1.96 (Cramer 1998, Cramer and Howitt 2004, Doane and Seward 2011). I also visually evaluated histograms, Q-Q plots, and box plots. Homogeneity of variance was performed using the Levene's test for normally distributed data and the non-parametric Levene's test for non-normally distributed data where a p-value greater than 0.05 indicates equality of variances (Nordstokke and Zumbo 2010, Nordstokke et al. 2011). Based on the results from tests for normality and homogeneity of variance, appropriate tests were then selected for further analyses of the data.

Pooled data across all sites meet the assumptions of the nonparametric Kruskal-Wallis test including similar shaped distributions across groups so this test was used to detect for the effect of habitat type (wetland or retention pond) on *Bd* prevalence (Kruskal and Wallis 1952, Zar 2010).

Because the data at the HUC level in both the southern and northern HUCs violate the Kruskal-Wallis and Mann-Whitney U assumption of similar shaped distribution across groups,

two-sample Kolmogorov-Smirnov tests were used to test if the distribution of positive samples was significantly different between the two habitat types within each HUC.

Because the data violated the chi-square assumption that no more than 20% of cells contain an expected value of less than 5 and because the contingency table was greater than 2 x 2, Fisher-Freeman-Halton tests were performed to evaluate the effect of species on *Bd* prevalence across all sites and within each HUC.

A one-way ANOVA was performed to compare canopy cover between wetlands and retention ponds (Fisher 1956, Lewontin 1974, Listopad et al. 2018). The canopy cover data met the assumptions for this test, making it an appropriate analysis for this portion of the dataset.

CHAPTER III

RESULTS

Of the 373 frogs sampled, eleven confidently tested positive for *Bd* (2.95% of all frogs sampled). The positive individuals are as follows: from LT6 wetland site, one female *L. catesbeianus* and four male *L. catesbeianus,* from Davis Pond wetland site, one female *L. catesbeianus,* from Roane County wetland site, one female *L. clamitans,* from the Lenoir City retention pond site, two subadult *L. catesbeianus,* from the Willow Creek retention pond site, one female *L. clamitans*, and from the Maryville retention pond site, one female *L. sphenocephalus* (Appendix D).

Across both HUCs, 50% of wetland sites (3 of 6) are positive for *Bd.* Likewise, 50% of retention pond sites (3 of 6) are positive for *Bd.* 3.65% (8 of 219) of *L. catesbeianus* sampled across all sites tested positive for *Bd* while 1.75% (2 of 114) *L. clamitans,* 5.00% (1 of 22) of *L. sphenocephalus,* and 0% (0 out of 18) of *L. palustris* sampled across all sites tested positive for *Bd*. In the southern HUC, all positive samples came from wetlands. In the northern HUC, all positive samples came from retention ponds.

The greatest number of positive samples at any one site was at the LT6 wetland site where 16.67% of frogs sampled tested *Bd* positive. This site also contained the highest percent canopy coverage of any site in this study with 97.87% coverage. Using the nonparametric Kruskal-Wallis test for pooled data from both HUCs, no significant difference was detected in number of positive samples between habitat types ($p = 0.930$). Additionally, when comparing distribution of positive samples between habitat types at the HUC level using two-sample

Kolmogorov-Smirnov tests, no significant difference was detected in the southern HUC ($p = 0.100$) or the northern HUC ($p = 0.100$).

The Fisher-Freeman-Halton test showed no significant difference in infection prevalence between species in the pooled data across all sites ($p = 0.583$). Likewise, no difference in infection prevalence was detected across species in the southern HUC ($p = 0.470$) or northern HUC ($p = 0.707$). The one-way ANOVA showed a significant difference in canopy cover between habitat types with wetlands containing significantly greater canopy cover than retention ponds ($F(1,10) = 57.77$, $p < 0.05$).

CHAPTER IV

DISCUSSION

The objectives of this study were to evaluate the effect of two different habitat types on prevalence of *Bd* as well as factors such as watershed basin, canopy cover, species, age class, sex, TBL, and HW. Because the number of positive samples was low (n = 11) no correlations with *Bd* prevalence and the above mentioned factors were possible. While certain numerical data appears to show trends in the dataset, no statistical significance could be established. An additional objective of this study was to determine if the difference in *Bd* prevalence between *L. clamitans* and *L. catesbeianus* as noted by Wilson et al. (2015) at a single site holds true across a larger geography in East Tennessee. This study found no significant difference in *Bd* prevalence between these two species.

Each site had a minimum sample size of 30 individuals. With this sample size, at least one infected individual can be detected in an infinitely large population with 95% confidence if prevalence of the disease is ≤0.10 (Cannon et al. 1982, Richards-Hrdlicka, Richardson, and Mohabir 2013). With this probability of detection (POD), it is reasonable to assume that the 6 sites with *Bd*-positive individuals, the prevalence of the disease at those sites is likely ≥0.10 as at least 1 positive individual was detected. Additionally, it is reasonable to assume that the sites with no *Bd*-positive individuals detected, the prevalence of infection is <0.10 if even present at all (Appendix E). Confidence in these results is further bolstered by the identical prevalence of infection detected in this study at LT6 of 0.17 (5 of 30) when compared to 0.17 (17 of 77) detected by Wilson et al. (2015) at the same site. Had this baseline prevalence rate been detected

at the other sites in this study, it is likely that correlations between infection prevalence and the stated metrics of interest could have been evaluated.

The low pooled prevalence of infection in this study (0.03) is not dissimilar to some other regional studies, however. Moffitt et al. (2015) detected very low prevalence of *Bd* in the Southern Appalachians across a variety of taxa. That study found an infection prevalence of 0.01 across 36 species of caudates and no *Bd* was detected in seven anuran species which included the ranid species *L. catesbeianus, L. clamitans,* and *L. sylvaticus.* Another study by Davidson and Chambers (2011) failed to detect *Bd* in either *L. catesbeianus* or *L. clamitans* in Wise County, Virginia. Additionally, the detection of *Bd* in this study at three of six wetlands and three of six retention ponds for a total of 50% of all study sites is not drastically different from the detection of *Bd* at 40% of sites of pond breeding amphibians (4 of 10) as found by Chatfield et al. (2009) in the Great Smokey Mountains of North Carolina and Tennessee.

Though the study organisms were salamanders instead of frogs and the habitat types were headwater streams instead of wetlands or retention ponds, a study by Hossack et al. (2010) failed to detect any *Bd* across four common species of salamanders in the Tennessee Southern Appalachians. Another study evaluating the prevalence of *Bd* in Fowlers Toads (*Anaxyrus fowleri*) in Memphis, Tennessee detected a prevalence rate of 0.07 (11 of 159) in adult individuals (Davis et al. 2012). The *Bd* prevalence in the pooled dataset from this study of 0.03 is comparable to prevalence rates ranging from 0.00 to 0.07 as detected in three UTC honors theses that evaluated *Bd* prevalence in stream dwelling salamanders in Middle and East Tennessee (Brocco 2017, Nabors 2017, Schrenker 2017). This further supports the notion that *Bd* prevalence in East Tennessee may not be as severe as in other parts of the world where the fungus occurs.

It is important to note that even though the low infection prevalence detected in this study is not without precedence in the literature, it is possible and maybe even probable that the data provided here is an underestimate of true disease prevalence. False negatives are a known issue in *Bd* research and this is incredibly problematic as it may lead to conclusions that *Bd* does not occur at a particular location and therefore that site is not managed properly going forward.

One potential issue leading to underestimates of infection prevalence is that skin swabs can fail to pick up *Bd* spores even on individuals known to be infected. Schock et al. (2010) found that swabs failed to detect *Bd* on several individuals that were confirmed via tissue sample (toe clips) to be infected. DiRenzo et al. (2018) demonstrated that disease detection using skin swabs is directly related to infection intensity and that prevalence underestimations of up to 71% are possible in populations persisting with low levels of infection. It is also possible to fail to detect *Bd* in a positive individual that has recently shed its skin containing the chytrid spores. A principal discussed by Hanley and Lippman-Hand (1983) called the "rule of three" asserts that if no individuals in a population test positive for the variable in question, there is 95% confidence that the chance of individuals testing positive for the variable is no more than three in *n*. This is not particularly helpful though as the rule allows for as much as thee positive individuals to be overlooked in a sample size of 30 which means a prevalence rate of up to 0.10 or 10% could potentially be missed entirely in a population where no animals test positive.

Despite the shortcomings of skin swabs as means of testing for the presence of *Bd*, this methodology remains common practice in *Bd* research for a number of reasons. Firstly, skin swabs are a non-destructive sampling methodology that allows the researcher to collect a sample *in situ* and release the animal unharmed following processing. Secondly, this methodology is far more time efficient than other approaches such as histopathology. Thirdly, this approach is

recommended by Hyatt et al. (2007) who compared various methodologies and concluded that skin swabs were preferential due to comparatively good DNA recovery, ease of use in the field, and reduction of contamination risk.

Despite being unable to evaluate potential correlations between *Bd* prevalence and the aforementioned factors of interest, the data presented here still provides valuable information that works to increase the collective understanding of *Bd* prevalence and distribution in East Tennessee, a stated objective of this study. With the exception of LT6, which had previously been established as a known location of *Bd*, five other sites have been determined to be *Bd* positive as a result of this study, thus expanding the known locations and distributions of the disease in East Tennessee.

While other studies had previously detected *Bd* in *L. catesbeianus, L. clamitans,* and *L. sphenocephalus* in the state of Tennessee, this study confirms that the presence of this disease in those three species is not limited to the sites where it was originally detected. Additionally, this study confirms the presence of *Bd* across life stages (2 subadults and 9 adults), sex (5 females, 4 males, 2 undetermined), TBL (38.3 – 103.6mm), and HW (13.4 – 36.4mm), suggesting that infection in ranid frogs is not limited to a particular species, life stage, sex, or size.

This study also set out to determine if there was a significant difference in *Bd* prevalence between wetlands and retention ponds. The fact that no difference was detected is noteworthy as it implies that both habitat types are in need of monitoring, management, and mitigation efforts. In this dataset, 50% of wetlands and 50% of retention ponds are positive for *Bd* which lends support to the hypothesis outlined by Duncan Pullen, Best, and Ware (2010) that urbanization is not correlated with *Bd* prevalence.

While no statistical significance could be established with regard to POC and *Bd* prevalence, it is worth noting that the site in this study (LT6) with the greatest infection prevalence (0.17, 5 of 30) was also the site with the greatest POC of all study sites (97.87%). Additionally, the retention pond site (Lenoir City Pond) with the greatest infection prevalence (0.06, 2 of 31) was also the site with the second highest POC of all retention ponds in this study at 25.86%. Also, the majority of *Bd*-positive samples in this study (7 of 11) came from wetlands, which on average have a significantly greater POC than retention ponds. Though not statistically significant, these results appear to parallel other studies that show a positive correlation between canopy cover and infection prevalence (Becker et al. 2012, Becker and Zamudio 2011, Beyer, Phillips, and Schooley 2015).

Originally, the study design included a third HUC containing three additional wetlands and three retention ponds. Due to time constraints and seasonality affecting the spring emergence of frogs, it was not possible to sample this third HUC during the sampling window. Had the third HUC been sampled, the sample size of this dataset would have been ≥553, which would have exceeded the necessary sample size for statistical power of 385 as determined by a power test that assumes a 95% confidence level, a 0.5 standard deviation, and a +/- 5% confidence interval. Since the modified study design resulted in a sample size of 373, the difference in disease prevalence between HUCs was not statistically evaluated, though a numerical difference was noted and further discussed below.

There is a small numerical difference in the number of positive samples detected in the southern HUC (7 of 11) compared to the northern HUC (4 of 11) which could possibly indicate a greater overall *Bd* prevalence in the southern portion of the study region than the northern portion. This possibility is supported by two other *Bd* studies out of the UTC herpetology

laboratory where no *Bd* was detected across three different sites in the northern HUC while *Bd* was detected in three samples across two different sites in the southern HUC (Nabors 2017, Schrenker 2017). Further research is needed to determine if the apparent trends mentioned above have statistical significance in a larger dataset.

Conservation Implications

Though this study appears to support previous studies demonstrating a relationship between canopy cover and infection prevalence, suggesting the removal of canopy as a means of mitigation is likely ill-advised as habitat loss and alteration is widely considered to be one of the greatest threats faced by amphibians globally (Hof et al. 2011), and so other forms of *in situ* mitigation and management should be investigated and pursued. Such mitigation could include treatment of frogs in the field using anti-fungal drugs such itraconazole as a means of slowing population declines (Hudson et al. 2016). Additionally, assurance breeding programs such as recommended in the Amphibian Conservation Action Plan are a useful measure for preventing extinctions of particularly imperiled species (Wren et al. 2015). Because *Bd* is able to persist in the environment without the presence of an amphibian host, *in situ* treatment of individuals in a *Bd* inoculated environment is only a temporary and half-way solution. Likewise, while assurance breeding programs can help to prevent the extinction of certain species, *Bd*-free environments are necessary for successful and sustainable reintroduction efforts. For these reasons, a combination of approaches is needed to fully address this issue. Though labor intensive, studies such as Bosch et al. (2015) have demonstrated that total elimination of *Bd* from both a population of amphibians and its associated environment is possible through a combination of *ex situ* anti-

fungal treatment of individuals and a liberal application of anti-fungal solution to the environment.

Knowing which geographic areas and specific amphibian populations face issues associated with the presence of *Bd* is important for informing future management decisions. The identification of previously unknown *Bd*-positive locations in Tennessee as a result of this study has provided new insight on where mitigation efforts, such as mentioned above, may be needed.

Directions for the Future

Building on the groundwork laid out in this study going forward, a potentially enlightening endeavor could be to sample a third HUC using the same methodology. This would function to increase the sample size of the pooled dataset and potentially allow for various correlational analyses. Additionally, it would provide another HUC replicate which could be used to evaluate differences in infection prevalence at the watershed basin level between the three HUCs. Alternatively, additional sampling divided evenly between the northern and southern HUC could be carried out to increase the existing sample size to the 385 samples needed for statistical power in comparing the northern and southern HUCs. This could work to definitively determine if infection prevalence is greater in the southern portion of the study region than the northern, as the current dataset seems to numerically suggest. This however is likely unnecessary as it is justifiable to simply combine this dataset with the three UTC honors thesis datasets for the purpose of evaluating overall *Bd* prevalence differences between the northern and southern HUCs.

Resampling all sites across different seasons could also shed light on the effect of seasonality on *Bd* prevalence. Some studies such as Raffel et al. (2010) demonstrate peak

prevalence during spring and fall months and a decrease in prevalence during the hottest parts of the summer. Though the majority of samples in this study were collected during the spring and early summer, it is possible that due to seasonality effects, the presence of *Bd* went undetected at sites visited during the hottest parts of the summer.

In light of the shortcomings of skin swabs in failing to detect accurate disease prevalence and combined with the fact that a tissue sample (toe tip) was taken from each animal processed in this study, as a follow up project, tissue samples will be analyzed for the presence of *Bd*. This will serve as either support for the results presented here or provide means of comparing methodologies in detecting infection.

Conclusions

Overall, these data appear to support previous research on positive relationships between canopy cover and infection prevalence. This study also seems to support the idea that infection prevalence may be greater in pristine habitats (wetlands) than in disturbed habitats (retention ponds). This study also determined that prevalence of *Bd* does not vary between the two target species. As previously mentioned, the low sample size of positive individuals made any sort of evaluation of relationships between species, morphometrics, sex, and age class with infection prevalence impossible and so further investigation in that vein is warranted. With regard to the objective of providing new information on the distribution and prevalence of this disease within Tennessee, this study meets that objective. The importance of this last point should not be understated as any new information on this disease is valuable in informing future research objectives.

The data presented here provides a framework for future investigation of *Bd* in Tennessee and the drivers of its prevalence. This study also functions to point a direction for where future research should focus in the aim of addressing factors that influence infection prevalence. The goal of chytrid research should be to sample for it everywhere that amphibians occur, across all amphibian taxa as well as other possible vectors such a fish, macroinvertebrates, reptiles, birds and even mammals. By intensive sampling across different habitat types, seasons, weather conditions, and taxa, a better understanding of how to tackle this threat to global biodiversity can be achieved. This study works towards that end as it helps to fill gaps in the body of knowledge concerning *Bd* in the southeastern United States and specifically East Tennessee, an amphibian biodiversity hotspot.

REFERENCES

Barbour, R.W. 1971. *Amphibians and Reptiles of Kentucky*. Lexington, Kentucky: University Press of Kentucky.

Becker, C. G., D. Rodriguez, A. V. Longo, A. L. Talaba, and K. R. Zamudio. 2012. "Disease Risk in Temperate Amphibian Populations Is Higher at Closed-Canopy Sites." *Plos One* 7 (10):7. doi: 10.1371/journal.pone.0048205.

Becker, C. G., and K. R. Zamudio. 2011. "Tropical amphibian populations experience higher disease risk in natural habitats." *Proceedings of the National Academy of Sciences of the United States of America* 108 (24):9893-9898. doi: 10.1073/pnas.1014497108.

Berger, L., A. D. Hyatt, R. Speare, and J. E. Longcore. 2005. "Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*." *Diseases of Aquatic Organisms* 68 (1):51-63. doi: 10.3354/dao068051.

Berger, L., R. Speare, P. Daszak, D. E. Green, A. A. Cunningham, C. L. Goggin, R. Slocombe, M. A. Ragan, A. D. Hyatt, K. R. McDonald, H. B. Hines, K. R. Lips, G. Marantelli, and H. Parkes. 1998. "Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America." *Proceedings of the National Academy of Sciences of the United States of America* 95 (15):9031-9036. doi: 10.1073/pnas.95.15.9031.

Beyer, S. E., C. A. Phillips, and R. L. Schooley. 2015. "Canopy cover and drought influence the landscape epidemiology of an amphibian chytrid fungus." *Ecosphere* 6 (5):18. doi: 10.1890/es14-00263.1.

Blaustein, A. R., J. M. Romansic, E. A. Scheessele, B. A. Han, A. P. Pessier, and J. E. Longcore. 2005. "Interspecific variation in susceptibility of frog tadpoles to the pathogenic fungus Batracbochytrium dendrobatidis." *Conservation Biology* 19 (5):1460-1468. doi: 10.1111/j.1523-1739.2005.00195.x.

Bosch, J., E. Sanchez-Tome, A. Fernandez-Loras, J. A. Oliver, M. C. Fisher, and T. W. J. Garner. 2015. "Successful elimination of a lethal wildlife infectious disease in nature." *Biology Letters* 11 (11):4. doi: 10.1098/rsbl.2015.0874.

Boyle, D. G., D. B. Boyle, V. Olsen, J. A. T. Morgan, and A. D. Hyatt. 2004. "Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay." *Diseases of Aquatic Organisms* 60 (2):141-148. doi: 10.3354/dao060141.

Bradley, P. W., S. S. Gervasi, J. Hua, R. D. Cothran, R. A. Relyea, D. H. Olson, and A. R. Blaustein. 2015. "Differences in sensitivity to the fungal pathogen *Batrachochytrium dendrobatidis* among amphibian populations." *Conservation Biology* 29 (5):1347-1356. doi: 10.1111/cobi.12566.

Brocco, C. 2017. "An analysis of prevalence of chytrid fungus in an amphibian assemblage in Middle Tennessee." Honors thesis, The University of Tennessee at Chattanooga.

Bury, R. B., and J. A. Whelan. 1985. Ecology and management of the bullfrog. In *Resource Publication*. Washington, DC.

Cannon, R. M., R. T. Roe, Health Australian Bureau of Animal, and Industry Department of Primary. 1982. *Livestock disease surveys: A field manual for veterinarians*. Canberra, Australia: Australian Government Pub. Service.

Carver, S., B. D. Bell, and B. Waldman. 2010. "Does Chytridiomycosis Disrupt Amphibian Skin Function?" *Copeia* 2010 (3):487-495. doi: 10.1643/CH-09-128.

Ceballos, G., P. R. Ehrlich, A. D. Barnosky, A. Garcia, R. M. Pringle, and T. M. Palmer. 2015. "Accelerated modern human-induced species losses: Entering the sixth mass extinction." *Science Advances* 1 (5):5. doi: 10.1126/sciadv.1400253.

Chatfield, M.W.H., B.B. Rothermel, C.S. Brooks, and J.B. Kay. 2009. "Detection of *Batrachochytrium dendrobatidis* in amphibians from the Great Smoky Mountains of North Carolina and Tennessee, USA." *Herpetological Review* 40:176-179.

Conant, R., and J.T Collins. 1998. *A field guide to reptiles & amphibians: Eastern and central North America*. edited by Joseph T. Collins. Boston: Houghton Mifflin.

Cramer, D. 1998. *Fundamental statistics for social research: Step-by-step calculations and computer techniques using SPSS for Windows*. New York: Routledge.

Cramer, D., and D. Howitt. 2004. *The SAGE dictionary of statistics: A practical resource for students in the social sciences*. London: Sage.

Currie, W., and E. D. Bellis. 1969. "Home Range and Movements of the Bullfrog, Rana catesbeiana Shaw, in an Ontario Pond." *Copeia* 1969 (4):688-692. doi: 10.2307/1441794.

Davidson, S. R. A., and D. L. Chambers. 2011. "Occurrence of *Batrachochytrium dendrobatidis* in amphibians of Wise County, Virginia, USA." *Herpetological Review* 42:214-215.

Davis, J. R., D. T. Eastlack, A. J. Kouba, and C. Vance. 2012. "*Batrachochytrium dendrobatidis* Detected in Fowler's Toad (Anaxyrus fowleri) Populations in Memphis, Tennessee, USA." *Herpetological Review* 43:81-83.

DiRenzo, G. V., E. H. C. Grant, A. V. Longo, C. Che-Castaldo, K. R. Zamudio, and K. R. Lips. 2018. "Imperfect pathogen detection from non-invasive skin swabs biases disease

inference." *Methods in Ecology and Evolution* 9 (2):380-389. doi: 10.1111/2041-210x.12868.

Doane, D. P., and L. E. Seward. 2011. "Measuring skewness: A forgotten statistic?" *Journal of Statistics Education* 19 (2):1-18. doi: 10.1080/10691898.2011.11889611.

Dodd Jr, C. 2010. *Amphibian Ecology and Conservation, A Handbook of Techniques*. 1st ed. New York, New York: Oxford University Press.

Duncan Pullen, K., A. M. Best, and J. L. Ware. 2010. "Amphibian pathogen *Batrachochytrium dendrobatidis* prevalence is correlated with season and not urbanization in central Virginia." *Diseases of Aquatic Organisms* 91 (1):9-16.

Fisher, R.A. 1956. "Statistical methods for research workers. By Sir Ronald A. Fisher. Edinburgh (Oliver and Boyd), 12th Ed., 1954. Pp. xv, 356; 12 Figs., 74 Tables. 16s." *Quarterly Journal of the Royal Meteorological Society* 82 (351):119-119. doi: 10.1002/qj.49708235130.

Gahl, M. K., J. E. Longcore, and J. E. Houlahan. 2012. "Varying Responses of Northeastern North American Amphibians to the Chytrid Pathogen Batrachochytrium dendrobatidis." *Conservation Biology* 26 (1):135-141. doi: 10.1111/j.1523-1739.2011.01801.x.

Hamer, A. J., P. J. Smith, and M. J. McDonnell. 2012. "The importance of habitat design and aquatic connectivity in amphibian use of urban stormwater retention ponds." *Urban Ecosystems* 15 (2):451-471. doi: 10.1007/s11252-011-0212-5.

Hamilton, W. J. 1948. "The Food and Feeding Behavior of the Green Frog, Rana clamitans Latreille, in New York State." *Copeia* 1948 (3):203-207. doi: 10.2307/1438455.

Hanley, J. A., and A. Lippman-Hand. 1983. "If nothing goes wrong, is everything all right? Interpreting zero numerators." *Journal of the American Medical Association* 249 (13):1743-1745.

Hof, C., M. B. Araujo, W. Jetz, and C. Rahbek. 2011. "Additive threats from pathogens, climate and land-use change for global amphibian diversity." *Nature* 480 (7378):516-U137. doi: 10.1038/nature10650.

Hossack, B. R., M. J. Adams, E. H. Campbell Grant, C. A. Pearl, J. B. Bettaso, W. J. Barichivich, W. H. Lowe, K. True, J. L. Ware, and P. S. Corn. 2010. "Low prevalence of chytrid fungus (Batrachochytrium dendrobatidis) in amphibians of U.S. headwater streams." *Journal of Herpetology* 44 (2):253-260. doi: 10.1670/09-058.1.

Hudson, M. A., R. P. Young, J. Lopez, L. Martin, C. Fenton, R. McCrea, R. A. Griffiths, S. L. Adams, G. Gray, G. Garcia, and A. A. Cunningham. 2016. "In-situ itraconazole treatment improves survival rate during an amphibian chytridiomycosis epidemic." *Biological Conservation* 195:37-45. doi: 10.1016/j.biocon.2015.12.041.

Hyatt, A. D., D. G. Boyle, V. Olsen, D. B. Boyle, L. Berger, D. Obendorf, A. Dalton, K. Kriger, M. Hero, H. Hines, R. Phillott, R. Campbell, G. Marantelli, F. Gleason, and A. Colling. 2007. "Diagnostic assays and sampling protocols for the detection of Batrachochytrium dendrobatidis." *Diseases of Aquatic Organisms* 73 (3):175-192. doi: 10.3354/dao073175.

Johnson, M. L., and R. Speare. 2003. "Survival of *Batrachochytrium dendrobatidis* in water: Quarantine and disease control implications." *Emerging Infectious Diseases* 9 (8):922-925.

Kosch, T. A., and K. Summers. 2013. "Techniques for minimizing the effects of PCR inhibitors in the chytridiomycosis assay." *Molecular Ecology Resources* 13 (2):230-236. doi: 10.1111/1755-0998.12041.

Kriger, K. M., and J. M. Hero. 2007. "Large-scale seasonal variation in the prevalence and severity of chytridiomycosis." *Journal of Zoology* 271 (3):352-359. doi: 10.1111/j.1469-7998.2006.00220.x.

Kruskal, W. H., and W. A. Wallis. 1952. "Use of ranks in one-criterion variance analysis." *Journal of the American Statistical Association* 47 (260):583-621. doi: 10.2307/2280779.

Lannoo, M. J., C. Petersen, R. E. Lovich, P. Nanjappa, C. Phillips, J. C. Mitchell, and I. Macallister. 2011. "Do frogs get their kicks on Route 66? Continental U.S. transect reveals spatial and temporal patterns of *Batrachochytrium dendrobatidis* infection." *PLOS ONE* 6 (7): e22211. doi: 10.1371/journal.pone.0022211.

Lewontin, R. C. 1974. "The analysis of variance and the analysis of causes." *American Journal of Human Genetics* 26:400-11.

Listopad, Cmcs, M. Kobel, A. Principe, P. Goncalves, and C. Branquinho. 2018. "The effect of grazing exclusion over time on structure, biodiversity, and regeneration of high nature value farmland ecosystems in Europe." *Science of the Total Environment* 610:926-936. doi: 10.1016/j.scitotenv.2017.08.018.

Longcore, J. E., A. P. Pessier, and D. K. Nichols. 1999. "*Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians." *Mycologia* 91 (2):219-227. doi: 10.2307/3761366.

Moffitt, D., L. A. Williams, A. Hastings, M. W. Pugh, M. M. Gangloff, and L. Siefferman. 2015. "Low prevalence of the amphibian pathogen *Batrachochytrium dendrobatidis* in the southern Appalachian mountains." *Herpetological Conservation and Biology* 10 (1):123-136.

Mount, R. H. 1975. *The reptiles and amphibians of Alabama*. Auburn, AL: Auburn University, Agricultural Experiment Station.

Nabors, M.A. 2017. "Prevalence of *Batrachochytrium dendrobatidis* (Bd) in watercourses situated in southeast Tennessee." Honors thesis, The University of Tennessee at Chattanooga.

Nordstokke, D. W., B. D. Zumbo, S. L. Cairns, and D. H. Saklofske. 2011. "The Operating characteristics of the nonparametric Levene test for equal variances with assessment and evaluation data." *Practical Assessment, Research & Evaluation* 16 (5):8.

Nordstokke, D., and B. Zumbo. 2010. "A New Nonparametric Levene Test for Equal Variances." *Psicológica* 31:401-430.

Olson, D. H., D. M. Aanensen, K. L. Ronnenberg, C. I. Powell, S. F. Walker, J. Bielby, T. W. J. Garner, G. Weaver, M. C. Fisher, and Grp Bd Mapping. 2013. "Mapping the global emergence of Batrachochytrium dendrobatidis, the amphibian chytrid fungus." *Plos One* 8 (2):13. doi: 10.1371/journal.pone.0056802.

Ortiz-Santaliestra, M. E., T. A. G. Rittenhouse, T. L. Cary, and W. H. Karasov. 2013. "Interspecific and postmetamorphic variation in susceptibility of three north american anurans to Batrachochytrium dendrobatidis." *Journal of Herpetology* 47 (2):286-292. doi: 10.1670/11-134.

Phillott, A. D., R. Speare, H. B. Hines, L. F. Skerratt, E. Meyer, K. R. McDonald, S. D. Cashins, D. Mendez, and L. Berger. 2010. "Minimising exposure of amphibians to pathogens during field studies." *Diseases of Aquatic Organisms* 92 (2-3):175-185. doi: 10.3354/dao02162.

Raffel, T. R., P. J. Michel, E. W. Sites, and J. R. Rohr. 2010. "What drives chytrid infections in newt populations? Associations with substrate, temperature, and shade." *EcoHealth* 7 (4):526-536. doi: 10.1007/s10393-010-0358-2.

Razali, N., and Y. Wah. 2011. "Power comparisons of Shapiro-Wilk, Kolmogorov-Smirnov, Lilliefors and Anderson-Darling tests." *Journal of Statistical Modeling and Analytics* 2 (1). doi: citeulike-article-id:12686206.

Richards-Hrdlicka, K. L., J. L. Richardson, and L. Mohabir. 2013. "First survey for the amphibian chytrid fungus Batrachochytrium dendrobatidis in Connecticut (USA) finds widespread prevalence." *Diseases of Aquatic Organisms* 102 (3):169-180. doi: 10.3354/dao02552.

Richardson, J. M. L., P. Govindarajulu, and B. R. Anholt. 2014. "Distribution of the disease pathogen *Batrachochytrium dendrobatidis* in non-epidemic amphibian communities of western Canada." *Ecography* 37 (9):883-893. doi: 10.1111/ecog.00360.

Saenz, D., T. L. Hall, and M. A. Kwiatkowski. 2014. "Effects of urbanization on the occurrence of Batrachochytrium dendrobatidis: Do urban environments provide refuge from the amphibian chytrid fungus?" *Urban Ecosystems* 18 (1):333-340. doi: 10.1007/s11252-014-0398-4.

Schock, D. M., G. R. Ruthig, J. P. Collins, S. J. Kutz, S. Carriere, R. J. Gau, A. M. Veitch, N. C. Larter, D. P. Tate, G. Guthrie, D. G. Allaire, and R. A. Popko. 2010. "Amphibian chytrid fungus and ranaviruses in the Northwest Territories, Canada." *Diseases of Aquatic Organisms* 92 (2-3):231-240. doi: 10.3354/dao02134.

Schrenker, E.A. 2017. "Measuring the presence of the amphibian pathogen *Batrachochytrium dendrobatidis* in East Tennessee." Honors thesis, The University of Tennessee at Chattanooga.

Shapiro, S. S., and M. B. Wilk. 1965. "An Analysis of Variance Test for Normality (Complete Samples)." *Biometrika* 52 (3/4):591-611. doi: 10.2307/2333709.

Shin, J., A. Bataille, T. A. Kosch, and B. Waldman. 2014. "Swabbing often fails to detect amphibian chytridiomycosis under conditions of low infection load." *Plos One* 9 (10):8. doi: 10.1371/journal.pone.0111091.

Tennessee Department of Agriculture. "Prentice Cooper State Forest." accessed March 5, 2018. https://www.tn.gov/agriculture/forests/state-forests/prentice-cooper.html.

Van Rooij, P., A. Martel, K. D'Herde, M. Brutyn, S. Croubels, R. Ducatelle, F. Haesebrouck, and F. Pasmans. 2012. "Germ tube mediated invasion of *Batrachochytrium dendrobatidis* in amphibian skin is host dependent." *Plos One* 7 (7):8. doi: 10.1371/journal.pone.0041481.

Vredenburg, V.T., and C. Briggs. 2009. "Chytrid swab protocol." accessed July, 11.

Wake, D. B., and V. T. Vredenburg. 2008. "Are we in the midst of the sixth mass extinction? A view from the world of amphibians." *Proceedings of the National Academy of Sciences of the United States of America* 105:11466-11473. doi: 10.1073/pnas.0801921105.

Willis, Y. L., D. L. Moyle, and T. S. Baskett. 1956. "Emergence, breeding, hibernation, movements and transformation of the bullfrog, Rana catesbeiana, in Missouri." *Copeia* 1956 (1):30-41. doi: 10.2307/1439241.

Wilson, T. P., J. Barbosa, E. Carver, B. Reynolds, D. Richards, Team Salamander, and T. M. Wilson. 2015. "*Batrachochytrium dendrobatidis* prevalence in two rand frogs on a former United States Department of Defense Installation in Southeastern Tennessee." *Herpetological Review* 46:37-41.

Wren, S., A. Angulo, H. Meredith, J. Kielgast, M. Dos Santos, and P. Bishop. 2015. *Amphibian Conservation Action Plan.* IUCN/SSC Amphibian Specialist Group. http://www.amphibians.org/publications/amphibian-conservation-action-plan/.

Zar, J. H. 2010. *Biostatistical Analysis*. 5th ed. Upper Saddle River, New Jersey: Prentice-Hall Pearson.

APPENDIX A

REPRESENTATIVE PHOTOGRAPHS OF STUDY SPECIES

American Bullfrog (*L. catesbeianus*)

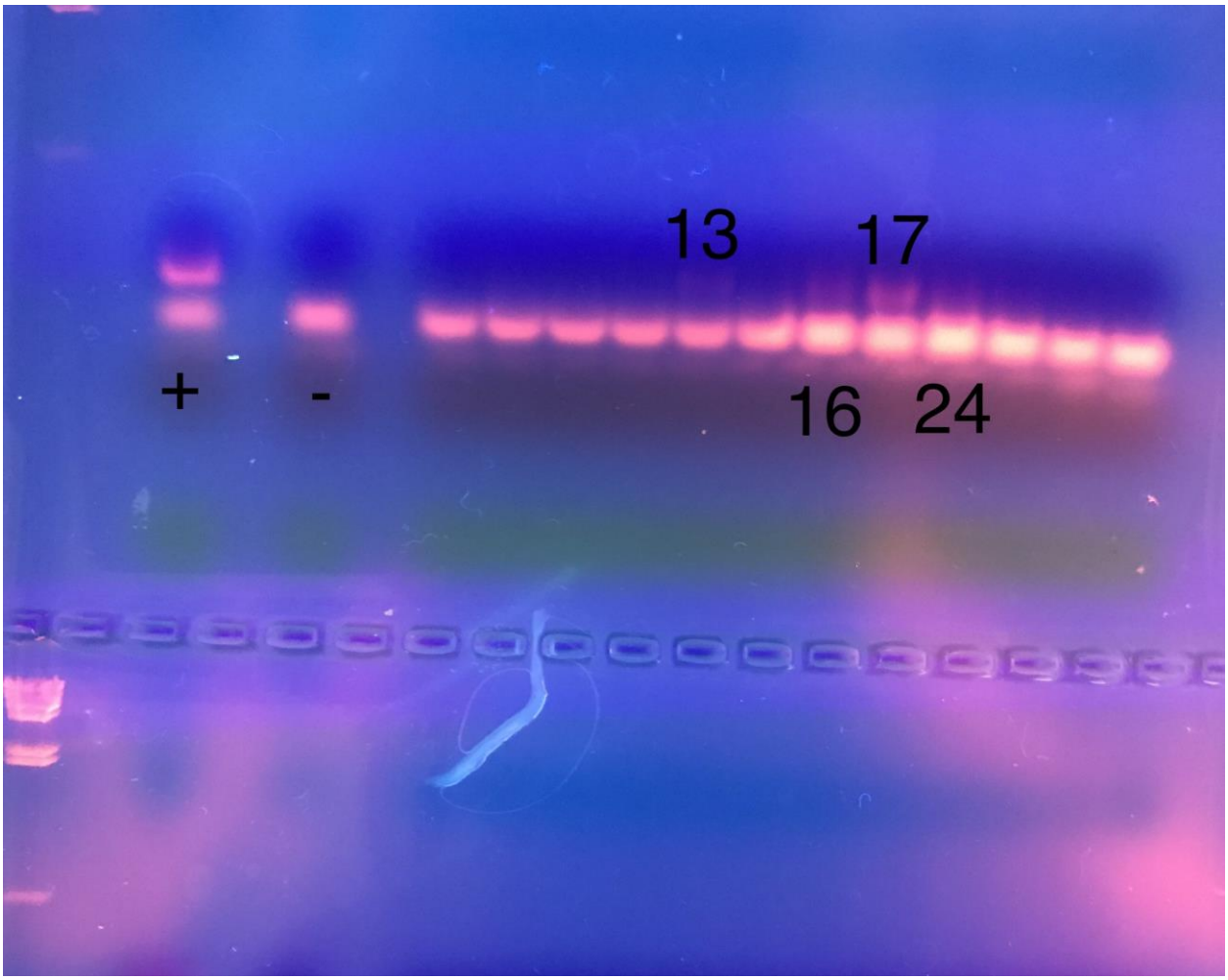
Green Frog (*L. clamitans*)

Southern Leopard Frog
(*L. sphenocephalus*)

Pickerel Frog (*L. palustris*)

APPENDIX B

EXAMPLE PHOTO OF AGAROSE GEL



Example agarose gel showing positive and negative controls marked with a + and –, respectively, and four positive samples with number 13 representing a weak positive

APPENDIX C

STUDY SITE SUMMARIES

Site #	Study Site	HUC	Lat-Long	Dates Sampled	# Sampled: *L. catesbeianus*, *L. clamitans*, *L. sphenocephalus*, *L. palustris*
1	LT6	Southern	35° 6'17.86"N 85° 7'48.57"W	6/1/2016 6/2/2016 6/3/2016 6/8/2016 6/11/2016 6/13/2016	30 (28, 2, 0, 0)
2	Davis Pond	Southern	35° 4'53.81"N 85°25'48.65"W	7/18/2016 7/19/2016 7/26/2016	30 (8, 22, 0, 0)
3	Renaissance Park	Southern	35° 3'41.37"N 85°18'39.21"W	3/23/2017 4/3/2017 4/4/2017 4/6/2017 4/10/2017	30 (30, 0, 0, 0)
4	Polk County Pond	Southern	35° 5'32.73"N 84°41'17.53"W	5/2/2017 5/9/2017 5/11/2017 9/18/2017	31 (13, 17, 1, 0)
5	Golden Pond	Southern	35°26'16.09"N 85° 1'19.61"W	5/16/2017 5/17/2017 5/20/2017 9/7/2017	31 (30, 0, 1, 0)
6	Roane County Wetland	Southern	35°40'58.85"N 84°35'42.55"W	5/30/2017 6/1/2017 6/5/2017	30 (11, 19, 0, 0)
7	Lenoir City Pond	Northern	35°49'10.33"N 84°16'31.01"W	6/11/2017 6/15/2017 6/17/2017	31 (30, 1, 0, 0)
8	Echo Valley Farm Wetland	Northern	35°32'34.33"N 84°21'38.73"W	6/21/2017 6/27/2017 7/5/2017	30 (26, 4, 0, 0)
9	Reed Wetland	Northern	35°46'19.88"N 84°30'2.61"W	6/25/2017 6/30/2017 7/10/2017	31 (18, 12, 1, 0)
10	Willow Creek	Northern	35°35'40.57"N 84°28'22.91"W	7/15/2017 7/17/2017 7/19/2017	32 (1, 30, 1, 0)
11	Maryville Pond	Northern	35°43'45.49"N 84° 1'42.69"W	7/25/2017 7/27/2017 8/3/2017 8/12/2017	33 (0, 1, 16, 16)
12	Grandview Cottages	Northern	35°46'2.18"N 84°48'59.59"W	8/29/2017 9/3/2017 9/6/2017	34 (24, 6, 2, 2)

APPENDIX D

PROFILE OF *BD*-POSITIVE INDIVIDUALS

Species	Site	Sex	Age Class	TBL (mm)	HW (mm)	% Canopy Cover
L. catesbeianus	LT6	female	adult	91.2	32.05	97.87
L. catesbeianus	LT6	male	adult	103.6	36.4	97.87
L. catesbeianus	LT6	male	adult	95.55	29.9	97.87
L. catesbeianus	LT6	male	adult	98.2	34.2	97.87
L. catesbeianus	LT6	male	adult	86.1	30.15	97.87
L. catesbeianus	Davis Pond	female	adult	66.85	24.8	91.00
L. clamitans	Roane County Wetland	female	adult	72.05	22.85	54.31
L. catesbeianus	Lenoir City Pond	undetermined	subadult	38.3	13.4	25.86
L. catesbeianus	Lenoir City Pond	undetermined	subadult	41.2	14.15	25.86
L. clamitans	Willow Creek	female	adult	71.55	22.35	5.39
L. sphenocephalus	Maryville Pond	female	adult	55.1	17.25	30.72

APPENDIX E

INFECTION PREVALENCE BY STUDY SITE

Name	Type	HUC	Approximate Circumference (meters)	% Canopy cover	*Bd* Prevalence (#positive/total)
LT6	Wetland	Southern	550	97.87	0.17 (5/30)
Davis Pond	Wetland	Southern	270	91.00	0.03 (1/30)
Renaissance Park	Retention Pond	Southern	400	38.82	0.00 (0/30)
Polk County Pond	Retention Pond	Southern	170	0.16	0.00 (0/31)
Golden Pond	Retention Pond	Southern	320	16.67	0.00 (0/31)
Roane County Wetland	Wetland	Southern	430	54.31	0.03 (1/30)
Lenoir City Pond	Retention Pond	Northern	210	25.86	0.06 (2/31)
Echo Valley Farm Wetland	Wetland	Northern	400	84.94	0.00 (0/30)
Reed Wetland	Wetland	Northern	250	82.08	0.00 (0/31)
Willow Creek	Retention Pond	Northern	630	5.39	0.03 (1/32)
Maryville Pond	Retention Pond	Northern	310	21.91	0.03 (1/33)
Grandview Cottages	Wetland	Northern	190	97.39	0.00 (0/34)

VITA

Paul-Erik Bakland was born in Loma Linda, CA, to parents Lars and Sandi Bakland. He is the second of four children with one older sister and a younger brother and younger sister. He attended Loma Linda Elementary in southern CA for kindergarten, was homeschooled for grades one through five and attended Glacier View Christian School in Ronan, MT for grades six through eight. Following graduation, he attended Upper Columbia Academy in Spangle, WA for his freshman year of high school and attended Georgia-Cumberland Academy in Calhoun, GA for his sophomore through senior years. Over a span of nine years and five majors, he attended Southern Adventist University in Collegedale, TN, Avondale College in Cooranbong, NSW, AU, Dalton State College in Dalton, GA, Chattanooga Technical Community College in Chattanooga, TN, and finally graduated with a Bachelor's degree in Biology with an Ecology concentration from The University of Tennessee at Chattanooga in 2014. Paul-Erik will graduate from UTC with a Master of Science degree in Environmental Science in August 2018.