The Prevalence of Chytridiomycosis in the Southern Appalachians

By

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ABSTRACT

Global amphibian population declines and extinctions have been well-documented over the past several decades. One of the most concerning biotic factors is the disease chytridiomycosis, which is caused by the fungal species *Batrachochytrium dendrobatidis* (*Bd*). Chytrid fungus has been documented across the United States with some areas having greater prevalence than others. The Southern Appalachian Mountains, particularly around Western North Carolina, are a diversity hotspot for salamanders including many endemic species. Historically *Bd* has been found only in relatively low densities in this area, however, there have been few surveys that focus on the prevalence of *Bd* in the region and the effects of chytridiomycosis on local amphibian populations. In 2014, 312 individuals were swabbed from 13 amphibian species at 19 study sites around Northwestern North Carolina. I used PCR-assays in triplicate and found that none of the samples had a confirmed presence of *Bd*. These data, along with past studies, suggest that *Bd* is very uncommon in this region of the Southern Appalachian Mountains. Interestingly, there was no evidence of *Bd* at one study site that has historically contained positive animals. The difference in detection could be attributed to multiple factors including sample size, seasonal variations, and subpar DNA samples.

KEYWORDS

DEDICATION

To my loving, supportive grandparents, "Big Shem" and Melissa DeVane:

I would not be where I am today without your guidance and enthusiasm.

Thank you for encouraging me to go outside and catch critters.

I love you both very much.

ACKNOWLEDGEMENTS

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INTRODUCTION

Amphibians around the world are experiencing rapid declines in population numbers. Researchers first became concerned with population declines around the time of the first World Congress of Herpetology in 1988 (Lips 2016). This was when scientists realized that a multitude of historically common amphibians had begun to disappear. These declines were documented all around the world in habitats that were often in very remote and protected areas (Blaustein et al. 1990). The IUCN published a Global Amphibian Assessment (GAA), which stated that approximately 43% of amphibian species out of >6000 described are in decline and 32% were considered threatened by extinction (Stuart et al. 2004). There are many factors that are thought to contribute to these population declines including traditional threats such as chemical pollution, habitat destruction, UV-B radiation, climate change and over exploitation. Novel threats include increased competition from invasive or introduced species and infectious disease (Beebee and Griffiths 2005). These novel threats are more difficult to assess and overcome because they are global issues not confined by political boundaries and their solutions require global collaborations (Lips 2016).

Batrachochytrium dendrobatidis (Phylum Chytridiomycota, Class Chytridiomycetes, Order Rhyzophydiales) is a zoosporic non-hyphal fungal species. *Bd* is a notable novel threat, affecting amphibians through the infectious disease chytridiomycosis. In the past few decades, this pathogen has been implicated in widespread population amphibian declines (Adams et al. 2010; Blaustein et al. 1990; Berger et al. 1998). Molecular studies of *Bd* have determined that the fungus has several genetically distinct lineages that vary in degree of virulence and phenotype. The lineage associated with population declines, mass mortality events, and extinctions is called the global pandemic lineage (GPL; Lips 2016).

The origins of chytridiomycosis have been greatly debated since it was first described in 1997. The first confirmed presence of virulent *Bd* in the wild was in South Africa in 1938 (Weldon et al. 2004). It is currently known to occur on all continents inhabited by amphibians. There are two main hypotheses for the origin of the amphibian chytrid epidemic. The "novel pathogen hypothesis" states that *Bd* was a clonal pathogen that was specific to one region and spread on a global scale causing new epidemics in naïve populations (Lips 2016; Rachowicz et al. 2005). The "endemic pathogen hypothesis" states that *Bd* has been present all along and went unnoticed by researchers until it increased in virulence or entered a new host species due to factors such as environmental change (Rachowicz et al. 2005).

Detrimental effects from chytridiomycosis have been documented in more than 700 species within three different orders of vertebrates (Caudata, Aunra, and Gymnophiona; Gower et al. 2013; Lips 2016). Histological examinations have determined that *Bd* spores are able to infiltrate the cells of the superficial epidermis, stratum granulosum, and stratum corneum of their host resulting in skin lesions. These skin lesions are caused by a large increase in the quantity of keratin in the stratum granulosum known as hyperkeratosis. Along with the lesions, hyperkeratosis also causes the superficial epidermis to thicken and allows for the colonization of bacteria in the sloughing skin (Berger et al. 2005). The distribution of *Bd* spores across the epithelial tissue of the host is never consistent and hyperkeratosis is usually only present where there are high densities of spores on the body. When spores are widespread and present in high numbers hyperkeratosis is also widespread leading to the inability for the host to perform gas exchange through cutaneous respiration. In severe cases this results in death by asphyxiation. Amphibian species also absorb water and essential electrolytes including sodium and potassium through their skin. Hyperkeratosis can result in

electrolyte imbalances, which can cause seizures and cardiac arrest (Voyles et al. 2009).

Another symptom chytridiomycosis expressed in amphibians is the loss of the righting reflex

(Searle et al. 2011). It is now known that the chytridiomycosis-amphibian system is very

complex and that the effects of chytridiomycosis on a host vary greatly depending on a

number of biotic and abiotic factors including host species, body size, antimicrobial peptides

present, temperature, moisture, habitat, and elevation (Woodhams et al. 2014; Harris et al.

2009; Lips et al. 2003).

Various studies have determined species-specific patterns of infection rate and

population declines in different amphibian communities (Lips et al. 2003; Olsen et al. 2013;

Kueneman et al. 2013). Lips et al. (2003) compared four sites with a confirmed presence of

Bd and discovered that the declining amphibian populations all shared similar elevation

ranges, aquatic habitats, and large body sizes. There have only been a small number of

studies that look at the species-specific response of *Bd* infection because the areas where

large epidemics have been documented generally also have low species richness (Vredenburg

et al. 2010).

Because there are species-specific effects and patterns of *Bd* infection, it is capable of

altering the composition of amphibian communities. This means different species could

either amplify or dilute the rate of infection within a given habitat and change the

community's response as a whole to the pathogen (Han et al. 2015; Lips 2016). While *Bd* can

affect approximately 10% (700) species, most if not all amphibians are capable of serving as

a reservoir, host, or vector for the disease (Lips 2016). The composition of species in tadpole

and adult communities could have various effects of the patterns of *Bd* transmission. In some

cases, the presence of a species could actually cause the rate of infection in a co-occurring

species to decrease (Becker et al. 2014; Han et al. 2015). This is a particularly important

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factor in areas with high amphibian biodiversity such as the tropics or the Southern Appalachian Mountains.

There over 600 caudate species worldwide and approximately one third of those are found in North America. The Southern Appalachian Mountains are known for their incredible salamander diversity including over 30 species with high endemism (Petranka 1998; Greene at al. 2013). Ecological niche studies (e.g. Ron 2005 or Xie et al. 2016) indicate that chytridiomycosis outbreak prevalence is most common in areas with high moisture levels, cool temperatures, and high elevations (Young et al. 2001). These optimal *Bd* conditions are present in the Southern Appalachians suggesting that *Bd* could flourish here. Even though *Bd* has been found throughout the United States, its intensity and prevalence are typically low in the Eastern US. Caruso and Lips (2013) hypothesized that Plethodontid salamanders in the Appalachian Mountains experienced dramatic population declines associated with *Bd*. These declines were first noted in the 1980s, but no cause had been determined. These same sites were resurveyed in 2009, which revealed that populations of multiple genera had declined in both occupancy and detection (Caruso and Lips 2013). *Bd* testing was performed on animals from these sites and it was found that a very low number had chytridiomycosis. Because the rates of mortality and disease outbreaks were so low, this may suggest that the genetic strain of *Bd* present in the Eastern United States has either been present for a very long time or that perhaps some of the species native to the area have characteristics that help prevent infection (Lips 2016).

Seasonality has also been an influencing factor of chytridiomycosis prevalence. Kriger and Hero (2006) performed a large-scale study to determine the season variations of chytrid prevalence and found that over a 21-month period in Australia, the presence of the disease peaked in the cooler months of early spring and dropped significantly during late

summer and early autumn. The study showed a significant negative relationship between disease prevalence and air temperature during the 30 days leading up to the sampling. Many salamander and frog species in the Southern Appalachians participate in breeding migrations during the spring months when the moisture levels are high and the temperatures are cool. During these migrations, large numbers of individuals can be found in concentrated areas, which may facilitate the transmission of the disease.

With the help of researchers at Appalachian State University, I conducted surveys to determine the presence of *Bd* in amphibian populations around Northwestern North Carolina in the Southern Appalachian Mountains. Throughout Watauga, Wilkes, Yancey, Ashe, and Allegheny Counties, 19 sites were surveyed and 312 individuals from 13 species of amphibians were swabbed. These samples were collected throughout the spring and summer months from aquatic, semi-aquatic, and terrestrial amphibians. I used nucleic acid extractions and polymerase chain reactions to confirm the presence of *Bd*.

MATERIALS AND METHODS

Permit Requirements and Ethics

All samples were collected under a wildlife collection permit issued by the NC Wildlife Commission (#16-SC01056) and an endangered species permit (#16-ES00460). Furthermore, all procedures were approved by the ASU Institutional Animal Care and Use Committee (IACUC).

Study Sites

Visual encounter surveys were conducted at 19 locations in Watauga, Ashe, Avery County, and Allegheny County (Fig. 1). The survey sites were chosen to encompass aquatic, semi-aquatic, and terrestrial species. Sites were also chosen to include areas where breeding

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migrations take place. Because the time of year has been shown to affect the prevalence of *Bd*, particularly in semi-aquatic species, the majority of the sampling was performed during the known peak months of prevalence, which starts in early spring and continues through the summer (Kriger and Hero 2006).

Sample Collection

Animals were typically captured by hand in small zip-lock and processed on site. This processing protocol was performed prior to swabbing each individual. For each individual, I recorded the species name, weight (g), total length (cm), snout-vent length (mm), and sex of each organism. After the information was recorded the individuals were swabbed by carefully rubbing a sterile cotton swab five times on each side including the neck, five times over the dorsum, five times over the ventral surface, and five times on the interior side of each leg. A clean bag was used for each animal during this process to prevent contamination. After taking the swab, each animal was released back where they were originally found. The swabs were placed in plastic vials containing 70% ethanol and then stored in a -80°C freezer until DNA extractions could be performed.

Study Species

The majority of the samples were taken during the spring breeding migrations because that time provided ideal conditions to collect large numbers of samples at one time. This study includes aquatic, semi-aquatic, and terrestrial species. The species that made up the majority of the sample size were *Ambystoma maculatum, Pseudocris crucifer,* and *Cryptobranchus alleganiensis.* However, because this study was not intended to survey any single species or even family of amphibian, all species captured were processed and swabbed for testing.

Laboratory Techniques

DNA extractions were performed using the MO BIO UltraClean® Tissue and Cells

DNA Isolation Kit (Catalog #12334-250). The swab samples were placed in bead tubes that

contained 0.7 mm garnet beads. Seven hundred µL of TD1, which is a proprietary aqueous

binding solution, was added to the bead tube to facilitate cell lysis. Twenty µL of Proteinase

K was also added to degrade the proteins and remove contamination. The tubes were then

placed in a horizontal vortex for 10 minutes to maximize homogenization. The samples were

centrifuged for 30 seconds at 6,200 rpm before transferring the mixture, including the bead,

into 1.5 mL Eppendorf tubes. This transfer was necessary because the bead tubes did not fit

in the heat block. The Eppendorf tubes were added to a heat block for 30 minutes at 60°C.

The samples were then centrifuged for 30 seconds at 6,200 rpm. The liquid was then

separated from the beads via micropipette and placed into a silica spin filter tube and

centrifuged again and the flow through was discarded. Four hundred µL of a solution

utilizing ethanol called TD2 was added to the spin filters before another round of

centrifugation. The flow through was discarded and the tubes were centrifuged again before

removing the spin filter and putting it in a new tube. Fifty µL of TD3, which is an elution

buffer of 10mM tris that adjusts the final pH of the DNA sample was added to the new spin

filter tube. The sample was centrifuged one last time for 30 seconds at 6,200 rpm. The spin

filter was discarded and the DNA in the bottom of the tube was stored in a -80°C freezer for

quantitative PCR. Polymerase Chain Reactions (PCR) were performed on each of the

samples in triplicate. All of the samples were added to a 1% agarose gel and electrophoresis

was performed. The samples in the gel were run alongside both positive and negative

controls to reduce the possibility of a false positive. The positive control was obtained from

swabbing animals in pet stores and had a confirmed presence of *Bd* spores. The gels were imaged under UV lighting in order to test for the presence of *Bd* in each of the samples.

RESULTS

A total of 312 animals representing 13 amphibian species (seven caudate and six anuran) at 19 sites in five different counties in Northwestern North Carolina were swabbed (Table 1). *Bd* was not found in the samples tested for any species or at any location. Sample sizes were large for several of the species including Spotted salamanders (*Ambystoma maculatum;* n= 137), Spring Peepers (*Pseudocris crucifer;* n= 50), and Eastern Hellbenders (*Cryptobranchus alleganiensis;* n= 60). Although some individuals encountered had leeches, various abrasions, and lacerations, none exhibited superficial signs of *Bd* infection such as irregular skin sloughing or a loss of righting reflex.

DISCUSSION

Bd was not found in any of the samples taken. This was surprising as the presence of *Bd* has been confirmed in the Southern Appalachians. There is the potential that laboratory malfunctions could provide inaccurate results, but I feel confident that the protocols were followed in a very meticulous manner. All of the samples that were tested were run and compared to a positive control that was clearly visible through imaging. These positive controls came from animals that were swabbed in the pet trade and confirmed that the protocols used were capable of detecting *Bd* even with very low densities of zoospores (see Moffitt et al. 2015). Previous studies have detected *Bd* in Northwestern North Carolina, but it has been found in very low densities in all cases (Moffit et al. 2015). The previous surveys along with the current study support the idea that *Bd* is in fact very low in prevalence in this

area. There is the possibility that the incidence of chytrid in the Southern Appalachians is sparse enough for the sampling to completely miss the detection of infected animals. Many of the study sites, particularly those in rivers, only provided opportunities to swab a few specimens from a single species and therefore I cannot confirm the complete absence of *Bd*.

The Meat Camp study site had a very diverse sample group that included 11 species some of which had large sample sizes. All of the sampling at the Meat Camp site was performed in late March and into mid April when the spring breeding migrations were taking place. Surveys have been performed at this exact study site in previous years during the breeding migrations (Moffit et al. 2015). Moffit's study included a sample of 50 Spotted Salamanders from the breeding migration populations at Meat Camp in 2013. Of the 50 specimens, only 5 tested positive for the presence of *Bd*.

Bd has also been documented in Eastern Hellbenders in Western North Carolina. In previous surveys Hellbenders from the Kanawha, French Broad-Holston, Upper Tennessee, and Lower Tennessee-Hiwassee River basins (Williams and Groves 2014). In their study, Williams and Groves (2014) found that of 165 wild and 15 captive animals, the prevalence if *Bd* was 27.9% and 26.7% respectively. This study did not report any significant relationships between *Bd* prevalence and water temperature or elevation. All of the Hellbender samples in my study came from these same drainages, which include major waterways such as the Little Tennessee, Hiawassee, Watauga, and New Rivers were negative. Although my study sites differed from that of Williams and Groves (2014), there has still been a confirmed presence of chytrid in the drainages where my sampling took place. The reasons for previous detection and no confirmation in this study maybe influenced by factors including land use, stream order, and habitat quality, which could play a role in limiting *Bd* exposure in this watershed (Pugh 2013).

There are a number of reasons that may contribute to my failure to detect chytrid in this study. The sample sizes used were not as large as some studies in the past. In Moffit et al. (2015), 668 swabs were taken from 603 animals of 43 different species and many of the animals were swabbed twice. Williams and Groves (2014) took samples of 165 wild Hellbenders from a total of 33 different streams in Western North Carolina. The streams that were sampled by Williams and Groves (2014) that were within the same drainages as our study, had lower densities of chytrid than their other sites. Areas that tend to have a low prevalence of *Bd* may require vast numbers of samples in order to confirm density. Our overall sample size of 312 animals may have been inadequate, especially because some species that were included in the study had fewer than 20 specimens captured.

There are a number of methods for confirming infection and laboratory error can occur in the detection of *Bd*. A review of literature (Kriger et al. 2006) shows that there are typically two commonly used methods to detect infection including swab-PCR assays and histological examinations of toe-clippings. The swab-PCR technique has only become popular in the last decade. It is non-invasive and has been shown to have a higher frequency of detection than histological examinations. My use of the swab-PCR assay techniques are still not without potential fault. The reliability of the swabbing techniques have not been fully evaluated in a field setting because wild animals are not typically exposed to high numbers of spores (10^3-10^7) compared to animals that are infected in a laboratory setting (Berger et al. 1998; Boyle et al. 2004; Kriger et al. 2006). This indicates that wild specimens are more likely to carry low densities of spores and light infections. In addition, the swabbing of wild animals typically results in dirt, plant matter, and microorganisms covering the swab. This combined with the mild temperatures and high levels of humidity that are typically

associated with field conditions could result in the degradation of fungal DNA, providing false-negatives in the lab (Kriger et al. 2006).

Amphibian species native to the Southern Appalachians have been sparsely surveyed for the presence of *Bd*. The areas that have been sampled are typically associated with high-quality and protected ecosystems (Moffit et al. 2015; Muletz et al. 2014). In order to get a better grasp on the effects *Bd* may have the native and endemic species of the Southern Appalachians, surveying must expand into new areas and include a wider range of amphibian species. Even though my study does not provide evidence for the presence of *Bd* in Northwestern North Carolina, it does indicate that it is extremely rare or may even fluctuate in detectability by season. Understanding the prevalence and density of *Bd* is important for conservation of amphibians across the globe. *Bd* prevalence could be very dynamic and must be monitored to ensure the population stability of amphibian species.

LITERATURE CITED

Adams M. J., Chelgren N. D., Reinitz D., Cole R. A., Rachowicz L. J., Galvan S., McCreary B., Pearl C.A., Bailey L.L., Bettaso J., Bull E. L., and Leu M. Using occupancy models to understand the distribution of an amphibian pathogen, *Batrachochytrium dendrobatidis*. *Ecological Applications* 20 (2010): 289-302.

Becker C.G., Rodriguez D., Toledo L.F., Longo A.V., Lambertini C., Correa D.T., Leite D.S., Haddad C.F.B., and Zamudio K.R. Partitioning the net effect of host diversity on an emerging amphibian pathogen. *Philosophical Transactions of the Royal Society B.* 281 (2015): 0141796.

Beebee T.J.C., and R.A. Griffiths. The amphibian decline crisis: a watershed for conservation biology? *Biological Conservation.* 125 (2005): 271–285.

Berger L., Speare R. Daszak P., Green D.E., Cunningham A.A., Goggin C.L., and Parkes H. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences.* 95 (1998): 9031-9036.

Berger L., Marantelli G., Skerratt L.F. and Speare R. Virulence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* varies with the strain. *Diseases of Aquatic Organisms.* 68 (2005): 47–50.

Blaustein A.R., Wake D.B. Declining amphibian populations: a global phenomenon? *Trends in Ecology and Evolution.* 7 (1990): 203-204.

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

Briggs C.J., Vredenburg V.T., Knapp R.A., and Rachowicz L.J. Investigating the population-
level effects of chytridiomycosis: an emerging infectious disease of amphibians.
Ecology. 86 (2005): 3149-3159.

Briggs C.J., Knapp R.A., and Vredenburg V.T. Enzootic and epizootic dynamics of the
chytrid fungal pathogen of amphibians. *Proceedings of the National Academy of
Sciences*. 107 (2010): 9695-9700.

Caruso N.M. and Lips K.R. Truly enigmatic declines in terrestrial salamander populations in
Great Smoky Mountains National Park. *Diversity and Distributions*. 19 (2013): 38-
48.

Gower D.J., Doherty-Bone T., Loader S.P. et al. *Batrachochytrium dendrobatidis* infection
and lethal chytridiomycosis in caecilian amphibians (Gymnophiona). *EcoHealth* 10
(2013): 173.

Green D.M., Weir L.A., Casper G.S., and Lannoo M.J. (2013) North American Amphibians:
Distribution and Diversity. University of California Press, Berkley, California.

Han B.A., Kerby J.L., Searle C.L., Storfer A., and Blaustein A.R. Host species composition influences infection severity among amphibians in the absence of spillover transmission. *Ecology and Evolution*. 5 (2015): 1432 -1439.

Harris R.N., Brucker R.M., Walke J.B., Becker M.H., Schwantes C.R., Flaherty D.C., et al. Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J*. 3.7 (2009): 818-824.

Kriger K. M. and Hero J.M. Survivorship in wild frogs infected with chytridiomycosis. *EcoHealth*. 3 (2006): 171-177.

Kueneman J.G., Parfrey L.W., Woodhams D.C., Archer H.M., Knight R., and McKenzie V.J. The amphibian skin-associated microbiome across species, space and life history stages. *Molecular Ecology.* 23 (2013): 1238-1250.

Lips K.R., Reeve J., and Witters L. Ecological factors predicting amphibian population declines in Central America*. Conservation Biology.* 17 (2003): 1078-1088.

Lips K.R. Overview of chytrid emergence and impacts on amphibians. *Philosophical Transactions of the Royal Society B.* 371(2016): 1709.

Moffit D., Williams L. A., Hastings A., Pugh M. W., Gangloff M. M., and Siefferman L. Low prevalence of the amphibian pathogen *Batrachochytrium dendrobatidis* in the

Southern Appalachian Mountains. *Herpetological Conservation and Biology*. 10.1 (2015): 123-136.

Muletz C., Caruso N.M., Fleischer R.C., McDiarmid R.W., and Lips K.R. Unexpected rarity of the pathogen *Batrachochytrium dendrobatidis* in Appalachian Plethodon salamanders: 1957–2011. *PLOS ONE*. 9 (2014): e103728.

Olson D.H., Aanensen D.M., Ronnenberg K.L., Powell C.I., Walker S.F., Bielby J., et al. Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. *PLOS ONE* 8.2 (2013): e56802.

Petranka J.W. Salamanders of the United States and Canada. (1998) Smithsonian Institution Press, Washington D.C., USA.

Pugh M.W. Effects of physicochemical parameters and land-use composition on the abundance and occurrence of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*). Unpublished Master's Thesis. (2013). Appalachian State University, Boone, NC.

Rachowicz L.J., Hero J.M., Alford R.A., Taylor J.W., Morgan J.A.T., Vredenburg V.T. et al. The novel and endemic pathogen hypotheses: competing explanations for the origin of emerging infectious diseases of wildlife. *Conservation Biology*. 19 (2005): 1441-1448.

Ron S.R. Predicting the distribution of the amphibian pathogen *Batrachochytrium dendrobatidis* in the world. *Biotropica*. 37 (2005): 209-221.

Searle C.L., Biga L.M., Spatafora J.W., and Blaustein A.R. A dilution effect in the emerging amphibian pathogen, *Batrachochytrium dendrobatidis*. *Proceedings of the National Academy of Sciences*. 108.39 (2011): 16322-16326.

Stuart S.N., Chanson J.S., Cox N.A., Young B.E., Rodrigues A.S., Fischman D.L., Waller R.L. Status and trends of amphibian declines and extinctions worldwide. *Science.* 306 (2004): 1783-1786.

Voyles J., Young S., Berger L., Campbell C., Voyles W.F., Dinudom A., Cook D., Webb R., Alford R.A., Skerratt L.F., and Speare R. Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science*. 326 (2009): 582-585.

Vredenburg V.T., Knapp R.A., Tunstall T.A., and Briggs C.J. 2010 Dynamics of an emerging disease drive large- scale amphibian population extinctions. *Proceedings of the National Academy of Sciences*. 107 (2010): 9689-9694.

Weldon C., Du Preez L. H., Hyatt A. D., Muller R., and Speare R. Origin of the amphibian chytrid fungus. *Emerging Infectious Diseases*. 10 (2004): 2100-2105.

Williams L.A and Groves J.D. Prevalence of the amphibian pathogen *Batrachochytrium dendrobatidis* in eastern hellbenders (*Cryptobranchus a. alleganiensis*) in western North Carolina, USA. *Herpetological Conservation and Biology*. 9 (2014): 454-467.

Woodhams, D.C., Brandt, H., Baumgartner, S., Kielgast, J., Küpfer, E., Tobler, U., et al. Interacting symbionts and immunity in the amphibian skin mucosome Predict disease risk and probiotic effectiveness. *PLOS ONE*. 9.4 (2014): e96375.

Xie G.Y., Olson D.H., and Blaustein A.R. Projecting the global distribution of the emerging amphibian fungal pathogen, *Batrachochytrium dendrobatidis*, based on IPCC climate futures. *PLOS ONE*. 11.8 (2016): e0160746.

Young B.E., Lips K.R., Reaser J.K., Ibáñez R., Salas A.W., Cedeño J.R., Coloma L.A., Ron S., Marca E.L.A., Meyer J.R., et al. Population declines and priorities for amphibian conservation in Latin America. *Conservation Biology*. 15 (2001): 1213-1223.

Table 1. Anuran and caudate species in Northwestern North Carolina that were swabbed for the presence of *Bd*. The number of samples at each site is in parentheses.
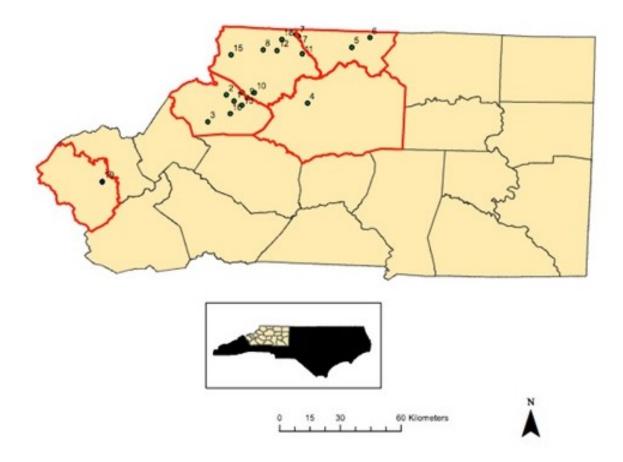
Species	Sample size	Study Site	Habitat Description
Ambystoma maculatum	137	Meat Camp Environmental Study Area	Wetlands
Pseudotriton montanus	11	Meat Camp Environmental Study Area	Wetlands
Pseudocris crucifer	50	Meat Camp Environmental Study Area	Wetlands
Notophthalmus viridescens	16	Meat Camp Environmental Study Area	Wetlands
Psuedotriton ruber	10	Meat Camp Environmental Study Area (7) Hopewell Church Rd (3)	Wetlands Damp leaf litter
Rana sp *	1	Meat Camp Environmental Study Area	Wetlands
Rana catesbeiana	4	Meat Camp Environmental Study Area	Wetlands
Anaxyrus americanus	8	Meat Camp Environmental Study Area	Wetlands
Desmognathus monticola	5	Meat Camp Environmental Study Area	Wetlands
Rana clamitans	2	Meat Camp Environmental Study Area	Wetlands
Rana palustris	1	Meat Camp Environmental Study Area	Wetlands
Necturus maculosus	1	New River State Park	Streams and rivers
Cryptobranchus alleganiensis	60	Shull's Mill (13) New River SP King's Creek (1) Little River US 21 (11) Little River US 18 (3) New River SP Pavilion (2) Big Horse Creek (1) SF New River at Todd Island (1) SF New River at Fleetwood Falls (3) Cranberry Creek (1) NF New River at Silas Creek (1) SF New River at Pine Run (3) SF New River at Big Hill (3) Big Laurel Creek (1) Old Greenway Dam (9) Grassy Creek Inlet at NF New River (1) Helton Creek at US16 (5) South Toe River (1)	Streams and rivers

Figure 1. *Bd* survey sites in Northwestern North Carolina. 1. Meat Camp Environmental Study Area, 2. Hopewell Church Rd, 3. Shull's Mill Rd, 4. New River State Park Kings Creek, 5. Little River US 21, 6. Little River US 18, 7. New River State Park Pavilion, 8. Big Horse Creek, 9. Todd Island at the South Fork of the New River, 10. Fleetwood Falls at the South Fork of the New River, 11. Cranberry Creek, 12. North Fork of the New River as Silas Creek Rd, 13. South Fork of the New River at Pine Run, 14. South Fork of the New River at Railroad Grade Rd, 15. Big Laurel Creek, 16. Old Greenway Damn, 17. Main Stem of the New River at Grassy Creek Inlet, 18. Helton Creek at US 16, 19. South Toe River at SR 1307. (Map Created by Olivia Thomas.)