DETERMINATION OF CHYTRIDIOMYCOSIS IN AMPHIBIANS AT ARNOLD AIR FORCE BASE

by

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ABSTRACT

Batrachochytrium dendrobatidis (Bd) is a fungal pathogen that infects the skin of amphibians and is implicated in global amphibian declines. This study determined Bd presence and prevalence in middle Tennessee by analyzing skin swabs of Eastern Newts (*Notophthalmus viridescens*), Cope's Gray Tree Frogs (*Hyla chrysoscelis*), and Barking Tree Frogs (*Hyla gratiosa*), at seven wetlands at Arnold Air Force Base, Coffee and Franklin counties, Tennessee. Bd is widespread in the area, and amphibians seem relatively tolerant of the fungus. Bd prevalence and Bd loads were much higher for Eastern Newts (82.7% prevalence; 26090.8 ± 6934.8 mean ITS1 copies) than for Cope's Gray Tree Frogs (7.1% prevalence; 53.7 ± 32.6 mean ITS1 copies) and Barking Tree Frogs (7.9% prevalence; 103.6 ± 98.3 mean ITS1 copies), and prevalence and loads decreased during summer as air temperatures rose. Future studies should further examine the interactive effects of amphibian life histories and environmental factors on Bd resistance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I: INTRODUCTION

Discovery of Chytridiomycosis

Chytridiomycosis, a lethal fungal disease that afflicts amphibians, is caused by the aquatic fungal pathogen *Batrachochytrium dendrobatidis* (Bd) (reviewed in Skerratt et al. 2007). Since the identification of chytridiomycosis in the late 1990s, the devastating effects of Bd on amphibian populations has been recorded on nearly every continent (except Antarctica; reviewed in Fisher et al. 2009). Bd has been detected on more than 520 of the 1,252 species of amphibians tested (Global Bd Mapping Project 2017), and it has contributed to the decline of about 500 species and the extinction of approximately 90 species (Scheele et al. 2019; Stuart et al. 2004; reviewed in Skerratt et al. 2007), including the Sharp-Snouted Day Frog (*Taudactylus acutirostris*) (Schloegel et al. 2006) and species of the Neotropical toad genus *Atelopus* (La Marca et al. 2005). The Bd panzootic may have originated in East Asia, according to O'Hanlon et al. (2018), who provided genetic evidence of high Bd biodiversity in the region, as well as the discovery of an ancestral population of Bd in the Korean peninsula.

During 2013, a new species of *Batrachochytrium* was described (Martel et al. 2013). This species, *Batrachochytrium salamandrivorans* (Bsal), is thought to be a driving force behind declines of salamander populations in Europe, including the Fire Salamander (*Salamandra salamandra*; Martel et al. 2013). The life cycle of Bsal is similar to Bd (Van Rooij et al. 2015), although some differences in pathogenesis exist

(Martel et al. 2013), and Bsal is more likely to cause disease in urodeles, although the range of susceptible species is unknown (i.e., it has not been found in either anurans or gymnophionans as yet; Martel et al. 2014). Although laboratory studies have demonstrated that several species of salamanders inhabiting North America are susceptible to Bsal, the pathogen has not been detected on the continent (Martel et al. 2014). Nonetheless, researchers continue to test for the pathogen in wild populations in hopes of averting outbreaks in North America (Bales et al. 2015).

Batrachochytrium Life Cycle and Pathogenesis

The life cycle of Bd has been studied in culture (Longcore et al. 1999; Berger et al. 2005a; Van Rooij et al. 2015) and on amphibian skin (Berger et al. 2005a). A motile, flagellated Bd zoospore comes in contact with the target substrate and encysts to form a germling, which develops into a zoosporangium that undergoes mitosis to produce new zoospores (Berger et al. 2005a). When mature, these new zoospores exit the zoosporangium via a discharge tube and are released into the environment (Van Rooij et al. 2015), where they can either reinfect the same host or migrate to infect new hosts (Berger et al. 2005a).

Bd can spread rapidly within amphibian populations with little regard to host density, which makes it a particularly dangerous pathogen (Woodhams and Alford 2005). The exact pathogenesis of chytridiomycosis has been difficult to determine because many organ systems shut down before host death occurs (Voyles et al. 2009). Although many details of Bd pathogenesis are still unknown, Bd infection has been shown to affect the

epidermis, triggering hyperkeratosis of the stratum corneum and stratum granulosum, hyperplasia of the stratum spinosum, and, occasionally, skin ulcerations (Berger et al. 2005b). These modifications to the epithelium impair electrolyte transport and osmoregulation, which can result in cardiac arrest (Voyles et al. 2009; Carver et al. 2010).

Disease Ecology of Chytridiomycosis

The disease ecology of chytridiomycosis is also being investigated (Johnson et al. 2003; Berger et al. 2004; Piotrowski et al. 2004; Kriger and Hero 2007; Kilpatrick et al. 2010; Pullen et al. 2010). Since the discovery of the disease more than 20 years ago, many studies have examined how environmental factors, including temperature and moisture levels, can affect the spread of Bd. Temperature has been found to be a strong influence on Bd growth and proliferation, and the fungus thrives in relatively cool temperatures, with high Bd prevalence at temperatures between 17° and 25° C, both in field (Berger et al. 2004; Pullen et al. 2010) and laboratory (Piotrowski et al. 2004) settings. High levels of Bd prevalence have been reported at temperatures as low as 12° C (Kriger and Hero 2007). The fungus ceases growth at 28° C (Piotrowski et al. 2004) and experiences 100% mortality at 32° C (Johnson et al. 2003). Desiccation has also been effective in killing Bd zoospores, with three hours of desiccation causing 100% mortality of the fungus (Johnson et al. 2003).

The evidence from some environmental studies points to climate change as a potential driver for future amphibian declines from Bd infection. Raffel et al. (2015)

examined the impact of temperature, temperature variability, and moisture on Bd abundance in infected juvenile Eastern Newts (*Notophthalmus viridescens*). Under wet conditions, a change in temperature at the time of Bd exposure resulted in higher Bd levels in these newts compared to those already acclimated to the end temperature at the time of exposure (Raffel et al. 2015). Thus, rapidly fluctuating temperatures, which are characteristic of climate change, may exacerbate the spread of Bd in some amphibian populations (Pounds et al. 2006; Raffel et al. 2015).

Chytridiomycosis in the United States

Since the beginning of the 21st century, more than 2,000 Bd-positive samples have been confirmed in the United States (Global Bd Mapping Project 2017), including many in the southeastern U.S. (Rothermel et al. 2008). Twelve cases have been confirmed in Tennessee, including two in Shelby County and 10 in the Great Smoky Mountains National Park (Global Bd Mapping Project 2017). Most of these cases have been of postmetamorphic individuals, including Southern Leopard Frogs (*Rana sphenocephala*), Pickerel Frogs (*Rana palustris*), and Eastern Newts (*Notophthalmus viridescens*). However, Bd-positive tadpoles of Wood Frogs (*Rana sylvatica*) and bufonid species have also been reported (Global Bd Mapping Project 2017). So far, no data has been publicly released on amphibians tested in middle Tennessee (Global Bd Mapping Project 2017).

The primary objective of my project was to determine prevalence of Bd occurrence (proportion of individuals that tested positive for Bd) and quantify and compare Bd pathogen burden within and among two species of frogs, Barking Tree Frog

(*Hyla gratiosa*) and Cope's Gray Tree Frog (*Hyla chrysoscelis*), and one species of salamander, the Eastern Newt (*Notophthalmus viridescens*), at seven pond sites at Arnold Air Force Base (AAFB), Coffee County and Franklin County, Tennessee (Figures 1 and 2; Table 1).

CHAPTER II: MATERIALS AND METHODS

Project Overview

My thesis research was conducted as part of a larger collaborative project involving research laboratories from several universities [Vanderbilt University Medical Center and University of Pittsburg as part of a Strategic Environmental Research and Development Program (SERDP) project funded by the Department of Defense]. As such, field sampling was performed often in collaboration with other researchers gathering data for the collective project. Amphibians used for my study were collected from 6 March 2017 to 8 August 2018.

Study Species

In Tennessee, Cope's Gray Tree Frogs typically breed from about mid-April to mid-August (Ritke et al. 1990). Males often call while perched in vegetation above water. Females select males based on some aspect of the call; males mount females that approach or contact them, and the amplexed pair then enter water, where they seek appropriate sites to deposit eggs (Ritke et al. 1990). After oviposition, females and males leave water. Eggs hatch into aquatic larvae (tadpoles), which then undergo

metamorphosis into arboreal juveniles. Cope's Gray Tree Frogs are arboreal throughout much of their life, entering water only during the breeding season. They likely hibernate under leaf litter of the forest floor during winter in a similar manner as a closely related species, *Hyla versicolor* (Schmid 1982).

The breeding season for Barking Tree Frogs extends from June through August, during which time males call while floating on the surface of the water (Murphy 2003). Females select males based on their call before amplexing in the water (Murphy 1994). After amplexus, submerged females lay eggs individually at the bottom of a body of water (Livezey and Wright 1947). Aquatic larvae (tadpoles) hatch from eggs and then undergo metamorphosis into arboreal juveniles (Travis 1980). Although regarded as arboreal after metamorphosis, adult Barking Tree Frogs are more fossorial than other species in the genus, and they typically overwinter in underground burrows, returning to water only to breed (Neill 1952, Wright and Wright 1949).

As with many newts in the family Salamandridae, Eastern Newts are aquatic for much of their life (Bishop 1943). In Tennessee, the breeding season extends from winter through early spring (Petranka 1998). Eastern Newts have an elaborate courtship that culminates in males amplexing with females before depositing a spermatophore (Petranka 1998). The male then coaxes the female to position the vent of her cloaca above the spermatophore and pick up the sperm (Petranka 1998). A female deposits each egg of her clutch individually, wrapping each egg in underwater vegetation or leaf litter (Morin 1983). Eggs hatch into aquatic larvae, which can then undergo metamorphosis into terrestrial juveniles (efts). Efts represent a wandering or dispersal stage. In middle

Tennessee, individuals reportedly remain in the eft stage for two to seven years (mean 3.5 years; Parker 2015) before undergoing a second metamorphosis and maturing into adults. Adults have a suite of structural, behavioral, and physiological adaptations associated with an aquatic life. Adults remain aquatic throughout life, but will seek refuge in small burrows or under logs, leaf litter, rocks, or other cover objects, in seasonally drying wetlands or during drought conditions (Brimley 1921; Hurlbert 1969). Also, in some populations, Eastern Newts apparently skip the eft stage and develop directly into adults from the larval stage (Noble 1929).

Study Site

Arnold Air Force Base spans 39,000 acres in an ecosystem known as the Barrens. This ecosystem, unique to the Eastern Highland Rim physiographic province, is relatively flat with low-lying areas that are prone to flooding. Soils in the Barrens contain a fragipan (Deselm 1994b), which is a layer of sediment that is relatively impermeable to water. Shallow bodies of water situated above this layer can be permanent or semi-permanent; other wetlands that have depths penetrating the fragipan can quickly and completely drain during the dry season as the water table drops (Gattinger 1887). As a result of this process, seasonal wetlands are scattered within the Barrens, and support a wide diversity of amphibians (more than 25 species; Miller et al. 2005). Although a prominent ecosystem of middle Tennessee historically, much of the Barrens has been logged, drained, or otherwise modified for agricultural use or urban development (Deselm 1994a). Arnold Air Force Base constitutes the largest public land area of the

remaining Barrens ecosystem (Pyne 2000), providing sites that can each be sampled for a diversity of amphibian species.

Bingham and Winford (1998) identified, mapped, and provided designations for several hundred wetlands on AAFB. Five wetlands, which included sites in the northeastern and southwestern ends of AAFB, were chosen as sites to sample amphibians at the outset of the collaborative project. These wetlands included relatively large, natural bodies of water, e.g. W514 (Westall Pond) and W538 (Sinking Pond) that dry seasonally, and relatively smaller artificial wetlands that are permanent or semi-permanent, e.g., W260 (Newt Pond), W518 (Pine Pond), and W25 (Deer Pond) (Table 1, Figure 2). I added two additional ponds to my component of the study to increase sample size and site diversity (Table 1, Figure 2). The first of these is W113 (Miller Pond), a relatively small pond that was artificially deepened several decades ago to provide year-round water availability to the deer population (B.T. Miller, pers. comm). As a consequence of this deepening, Miller Pond is a semi-permanent to permanent body of water that holds water throughout the year during most years, but is subject to drying in particularly dry years. This pond was selected because of the large Eastern Newt population known to occur there (B.T. Miller, pers. comm.). The second additional site is Borrow Pit, a moderately-sized wetland located in the northeastern end of AAFB that was unintentionally constructed during soil harvesting activities during the late 1990s or early 2000s. Since its construction, this seasonal pond is known to support a large breeding population of Barking Tree Frogs (B.T. Miller, pers. comm.). This wetland has no official designation number, as it was constructed relatively recently, after wetlands were

characterized and described by Bingham and Winford (1998). Henceforth in this document, I refer to all sites by common name.

Field Surveys

We performed both daytime and nighttime searches to collect amphibians. Before most searches, we recorded a brief description of the weather, placed a Kestrel weather meter (Kestrel Instruments, Boothwyn, Pennsylvania, USA) in a centrally located tree to record data on air temperature and humidity levels, and used a HOBO recorder (ONSET, Cape Cod, Massachusetts, USA) to measure average water temperature at the time of the survey. We used weather history reports from a weather station near AAFB (Weather Underground 2018) to calculate average air temperatures for 30 days prior to each sampling date, which is an appropriate timeframe for the climate to exert an effect on Bd prevalence (Kriger and Hero 2007). Most Barking Tree Frogs and Cope's Gray Tree Frogs sampled were collected at night during their breeding seasons; Eastern Newts sampled were typically captured using nets during daytime surveys. We searched for amphibians during nighttime surveys by slowly walking along pond margins, using headlamps to aid in our search for amphibians in vegetation, pond margins and surfaces, and on dirt roads and forest floor near the pond surface. We used an Etekcity infrared thermometer (Etekcity Corporation, Anaheim, California, USA) to determine the external body temperature of most individuals encountered, and of their immediate substrate, before capturing them either by hand or by net. At time of capture, we recorded a brief description of the microhabitat in which the amphibian was found. Categories of

microhabitat included water, leaf litter, soil, and vegetation less than and greater than one meter in height. We noted whether the substrate was wet or dry, and in some cases, additional descriptors were added (under log, under rock, etc.). To reduce the risk of cross contamination, we used clean latex gloves to capture each amphibian, which we then placed in a clean plastic bag to transport it to our processing station. Also, we rinsed nets with pond water between captures.

Sample Collection

We followed the protocol developed by Brem et al. (2007) to process the amphibians, and we used fresh latex gloves while processing each individual. Although resampling of the same individual is unlikely to occur given the high densities of amphibians at each site, we recorded the weight (to the nearest 0.1 g) and snout-vent length (SVL, to the nearest mm) for each captured animal, as well as tail length for each salamander and each larval frog (tadpole) for identification purposes. To sample each amphibian for chytrid fungus, we used sterile, cotton-tipped applicators to swab the forelimbs, hindlimbs, dorsum, ventrum, and lateral sides five times each. Cope's Gray Tree Frog and Barking Tree Frog tadpoles were sampled by gently swabbing the keratinized mouthparts, which are the only sites on tadpoles that Bd has been shown to infect (Marantelli et al. 2004). After collection of each sample, we placed the applicator tips in sterile, individually labeled vials (Brem et al. 2007), which we stored on ice in a cooler. After processing, we released all animals near the point of capture, and, to prevent the potential spread of Bd, we washed boots and nets in a 10% bleach solution before

moving to another sampling site. We stored swabs in a freezer at -20° C, usually within 3 hours after collection.

Sample Analysis

Because the number of genomic copies per Bd zoospore can vary widely among strains (Longo et al. 2013), we report the number of copies of the highly conserved Internal Transcribed Spacer 1 (ITS1) region as an analog for Bd load. I used Taqman Quantitative PCR (qPCR) to estimate the number of amplified ITS1 copies found on each sample swab. I extracted DNA from sample swabs and from Bd-negative and -positive (1000 zoospore-count) control swabs using a Qiagen DNeasy Blood and Tissue Kit. I used pre-mixed stock solutions of Pisces Molecular plasmid standards with concentrations ranging between 2.1×10^0 and 2.1×10^6 ITS1 copies per μL for my dilution standards. I made a qPCR master mix by combining 2X SensiFAST buffer, 36 μM forward primer ITS1 Chytr 3, 36 μM reverse primer 5.8S Chytr, 12.5 μM Taqman MGB probe, and molecular grade water (Boyle et al. 2004). A subset of the total number of samples was also analyzed for Bsal.

I prepared reactions of 25 μL in each well of a 96-well plate, including one set each of my Bd DNA dilution standards, no template controls (NTC), and positive and negative controls. I mixed reactions using 20 μL master mix and 5 μL extracted DNA solution (or molecular grade water for NTC reactions). I set up all reactions in triplicate (Boyle et al. 2004) and analyzed them using a Stratagene Mx3000 qPCR machine. The

PCR cycle was set for a hot start of 10 minutes at 95° C, followed by 40 cycles alternating between 20 seconds at 95° C and 60 seconds at 60° C.

The qPCR machine measured the change in fluorescence (Δ Rn) of each sample replicate. A critical Δ Rn threshold was calculated automatically at the midpoint of the log Δ Rn distribution. Results from the qPCR analysis were given as Cycle Threshold (Ct) values, which represented the PCR cycle at which fluorescence from each sample replicate crossed the critical Δ Rn threshold (Boyle et al. 2004). Samples crossing this critical threshold were labeled as Bd-positive, and then the number of ITS1 copies per sample replicate was calculated using the plasmid standard curve. If two to three replicates indicated positive results, I averaged the results of the positive replicates (Table 2). If only one replicate of a sample indicated DNA replication, I repeated the reaction with the sample in triplicate on a new plate.

Statistical Analysis

Data was analyzed using R Statistical Software (R Development Core Team 2018). I performed a Fisher's Exact Test on Bd prevalence data, as well as a zero-inflated, negative binomial linear regression analysis (function "zeroinfl" in package "pscl") on Bd load data (Raffel et al. 2010). This model was used in place of a simple linear regression model due to the large number of Bd-negative sample results and the high variance associated with the Bd load data (Fletcher et al. 2005). I used a Vuong Closeness Test to confirm that the zero-inflated model was a better fit for the data than a standard negative binomial model.

CHAPTER III: RESULTS

We captured, swabbed, and tested samples from 255 Cope's Gray Tree Frogs, 76 Barking Tree Frogs, and 98 Eastern Newts across all sites from March 2017 to August 2018. Skin swabs from amphibians found at five of the seven sample sites harbored detectable levels of Bd (Table 3), although no amphibians sampled exhibited obvious external symptoms of infection (Voyles 2009). Bd loads on swabs taken from individuals testing positive varied widely, from a low of 27 ITS1 copies to a high of 467221 ITS1 copies (mean = 6010; median = 0). All sampled tadpoles (n=6) were negative for Bd, and no Bsal was detected on any of the analyzed samples.

Approximately 24.5% of the three sampled amphibian species tested positive for Bd, although Bd prevalence and individual Bd loads were much higher for Eastern Newts than for either Cope's Gray Tree Frogs or Barking Tree Frogs ($p < 0.0001$; Table 3; Figures 3 and 4). Cope's Gray Tree Frogs and Barking Tree Frogs had 7.1% and 7.9% Bd prevalence, respectively; whereas, Eastern Newts had a Bd prevalence of 82.7% (Table 3; Figure 3). Also, the average Bd load of Eastern Newts was 26,091 ITS1 copies, which was between two and three orders of magnitude higher than that of Cope's Gray Tree Frogs (54 ITS1 copies) and Barking Tree Frogs (104 ITS1 copies) (Table 3; Figure 4).

Season was a significant predictor of Bd prevalence and Bd loads in Eastern Newts, with summer having much lower Bd prevalence ($p < 0.0001$) and Bd loads ($p < 0.001$) than all other seasons (Figures 5 and 6). Spring, fall, and winter showed no statistically significant differences in Bd prevalence or loads ($p > 0.05$; Figures 5 and 6). No seasonal differences were seen in Cope's Gray Tree Frogs or Barking Tree Frogs ($p >$

0.05), potentially because few tree frog samples were collected outside of summertime mating seasons. Air temperature at the time of collection ranged from a low of 3.0° C in the winter to a high of 29.2° C in the summer – crossing the 28° C threshold for Bd to cease growth (Piotrowski et al. 2004). The mean air temperature for 30 days prior to the collection of each sample was inversely related to the Bd load of each sample (Figure 7). In other words, the higher the average temperature 30 days prior to sampling, the lower the Bd load. The maximum water temperature recorded at the time of collection was 29° C; substrate and animal temperature recordings were similar, with each reaching a high of 28° C.

The zero-inflated, negative binomial regression model was the best fit model for our data ($p < 0.001$), using species (coefficient = 2.778, $p < 0.001$) and mean 30-day air temperature (coefficient = -0.078, $p = 0.020$) as predictors. Animal body temperature, substrate temperature, water temperature, air temperature at the time of collection, and mean 30-day air temperature were all collinearly related ($p < 0.001$). We selected mean 30-day air temperature as a predictor for the model because (a) it accounted for the most variance in Bd loads, and (b) it was the measurement most likely to exert a climatic effect on Bd loads based on prior literature (Kriger and Hero 2007). No other independent variable significantly improved the model after controlling for the effects of species and mean 30-day air temperature. A Vuong Closeness Test confirmed that the zero-inflated model was a better fit for the data when compared to a standard negative binomial model ($p < 0.001$).

CHAPTER IV: DISCUSSION

At least three species of amphibians inhabiting wetlands in the Barrens of middle Tennessee have Bd on their skin, and none tested positive for Bsal. Amphibians from five of the seven surveyed sites possessed detectable levels of Bd. Possibly, the two "Bd-negative" sites (Pine Pond and Deer Pond) also contained individuals harboring Bd. Eastern Newts had much higher Bd prevalence than Cope's Gray Tree Frogs or Barking Tree Frogs; only one Eastern Newt was sampled at Deer Pond, and none were sampled at Pine Pond. Furthermore, Bd could have been spread between ponds by hunters and wildlife, and both sites were located less than two kilometers away from sites testing positive for Bd.

All amphibians testing positive for Bd appeared physically healthy, and no amphibians of any species observed during field sampling exhibited symptoms characteristic of chytridiomycosis. This, along with a lack of reported Bd-implicated amphibian die-offs in the area, suggests that amphibians in middle Tennessee are tolerant to Bd, as they appear to be throughout much of the American Southeast (Rothermel et al. 2008). Nonetheless, monitoring of Bd prevalence in middle Tennessee is prudent because coinfection by other diseases, such as ranavirus (Warne et al. 2016), and changes in moisture levels (Raffel et al. 2015), temperature (Pounds et al. 2006), and temperature variability (Raffel et al. 2015) may increase pathogenicity of the fungus.

The significantly higher Bd prevalence in Eastern Newts (82.7%) compared to Cope's Gray Tree Frogs (7.1%) and Barking Tree Frogs (7.9%) is not surprising. Other studies have also found high Bd prevalence among Eastern Newts throughout their native

range (Ouellet et al. 2005; Rothermel et al. 2008; Raffel et al. 2010) and low Bd prevalence among all sampled species of genus *Hyla* in North America (Frías-Alvarez 2008; Rothermel et al. 2008; Brannelly et al. 2012). Differences in Bd loads between study species may be associated with interspecific differences in the skin microbiome (Woodhams et al. 2007). The amphibian skin microbiome includes symbiotic microorganisms and secreted antimicrobial peptides (AMPs), which can limit Bd growth rate (Rollins-Smith et al. 2002; Woodhams et al. 2007). High Bd prevalence among Eastern Newts may also be associated with their reliance on an aquatic habitat throughout adulthood. Adult Eastern Newts are generally aquatic throughout the year, which potentially increases their susceptibility to Bd, at least in comparison to species with terrestrial or arboreal adults. Adult Cope's Gray Tree Frogs and Barking Tree Frogs enter water only to breed during summer, when water temperatures at our sites occasionally exceeded 28 °C – the temperature at which Bd growth has been shown to stop completely (Piotrowski et al. 2004). This may partly explain the low incidence of Bd among the two species of tree frogs studied in the Barrens of middle Tennessee.

This study provides further evidence of the low Bd prevalence of tree frogs (family Hylidae) in eastern North America. Species belonging to other anuran families, including true frogs (family Ranidae) and toads (family Bufonidae), have shown higher average Bd prevalence in the eastern United States and Canada (Ouellet et al. 2005; Pearl et al. 2007; Rothermel et al. 2008). Bd-implicated mortality events have occurred in North American anurans, although evidence of these events has been limited to the western United States. For example, declines have been observed in the toad species *Bufo*

boreas in the western United States (Pearl et al. 2007); however, no such declines have been reported in toad species found in the eastern United States (Ouellet et al. 2005; Rothermel et al. 2008) despite species, including *Bufo americanus*, testing positive for Bd (Longcore et al. 2007). Similarly, ranid species such as *Rana chiricahuensis* and *Rana yavapaiensis* have experienced population declines in the western United States as a result of chytridiomycosis (Bradley et al. 2002). Ranid species in the eastern United States and Canada have high Bd prevalence (Ouellet et al. 2005; Longcore et al. 2007), though most, especially *Rana catesbeiana*, appear to be very tolerant of high Bd loads (Daszak et al. 2004). Ranid species possess significantly higher Bd loads than hylids and bufonids in eastern North America (Longcore et al. 2007; Rothermel et al. 2008), possibly because ranids, like newts, have a stronger reliance on aquatic habitats throughout adulthood than hylids or bufonids.

Sex, microhabitat type, relative humidity, animal weight, snout-vent length (SVL), and tail length had no effect on Bd prevalence or Bd load in this study after controlling for species and air temperature. Similar field studies have also found that sex (Longcore et al. 2007), microhabitat (Richards-Zawacki 2009), relative humidity (Richards-Zawacki 2009), and body condition (as a function of animal weight and SVL; Woodhams and Alford 2005; Murray et al. 2009; Richards-Zawacki 2009) are poor predictors of Bd prevalence.

Because of rapid spread and high mortality rates during the past two decades, chytridiomycosis is considered one of the most destructive panzootics to threaten a class of vertebrates (Scheele 2019; Gascon 2007). As more knowledge about the disease is

acquired, better strategies can be made to mitigate its effects. One method of preventing further spread of the disease is to enact legislation to increase biosecurity by further regulating the amphibian pet trade, ensuring that transported animals are not infected with Bd or Bsal (Lötters et al. 2011). Biosecurity measures seemed to be successful for preventing the introduction of Bd onto the island of Madagascar, which until recently had zero confirmed cases of Bd infection (Weldon et al. 2008; Vredenburg et al. 2012). Tragically, the fungus has since been introduced and is now widespread in species of frogs from all native families that inhabit the island (Bletz et al. 2015). The effects that the pathogen will have on Madagascar's native amphibians are yet to be studied (Lips 2016).

Research into targeted bioaugmentation treatments using probiotics and antifungal compounds has shown some limited potential for eliminating chytrid infection in wild amphibian populations (Becker et al. 2011; Bletz et al. 2013; Bosch et al. 2015; Rebollar et al. 2016). One successful application by Bosch et al. (2015) effectively eradicated Bd from four of five infected populations of Mallorcan Midwife Toads (*Alytes muletensis*) by treating tadpoles with antifungals and by using antifungal compounds for bioaugmentation of the infected ponds. The four successful sites have remained Bd-free for two years since the treatment (Bosch et al. 2015).

Evidence of Bd presence has now been provided for amphibians from all three geographic regions of Tennessee. Amphibians examined during this study, as well as many amphibian species throughout the American Southeast, appear to be tolerant to Bd; however, the lasting effects of the introduction of Bd to the region are yet to be seen.

Tennessee hosts a great diversity of amphibian species (Niemiller and Reynolds 2011), and a widespread and potentially deadly pathogen such as Bd must be actively monitored in the state. It is paramount to continue research on chytridiomycosis to protect the susceptible animals and to preserve the biodiversity that has been sustained by amphibians.

Table 1. Common names, official designations (Bingham and Winford 1998), and GPS coordinates of sampling sites at Arnold Air Force Base, Franklin and Coffee counties, Tennessee, USA.

Site #	**Site name (wetland designation number)**	**Latitude (°)**	**Longitude (°)**
1	Newt Pond (W260)	35.350720	-86.139930
2	Pine Pond (W518)	35.343295	-86.129539
3	Westall Pond (W514)	35.410780	-86.079660
4	Deer Pond (W25)	35.425170	-86.074520
5	Sinking Pond (W538)	35.409820	-86.069880
6	Miller Pond (W113)	35.404612	-86.071006
7	Borrow Pit (no official designation)	35.430823	-86.093313

Table 2. Example of qPCR results from one sample analyzed in triplicate. ITS1 copies for this sample would be calculated by averaging the results for Wells D4 and D6.

Well	**Sample #**	**Threshold (Δ Rn)**	**Ct**	**Quantity (ITS1 copies)**
D4	12	0.0411	34.48	596.4
D5	12	0.0411	No Ct	No Ct
D6	12	0.0411	38.07	58.4

Table 3. Mean Bd loads (± SE) and Bd prevalence (number of individuals testing positive / number of individuals sampled) per species for each site sampled.

	Bd Load per Species mean ± SE (prevalence)		
	Cope's Gray Tree Frog	**Barking Tree Frog**	**Eastern Newt**
Newt Pond	4.8 ± 3.5 (2/50)	196.0 ± 191.6 (3/39)	26837.6 ± 8727.2 (56/67)
Pine Pond	0 (0/46)	-	-
Westall Pond	177.5 ± 134.7 (4/61)	-	-
Deer Pond	0 (0/52)	0 (0/11)	0 (0/1)
Sinking Pond	1.2 ± 1.2 (1/22)	0 (0/1)	0 (0/3)
Miller Pond	-	-	28102.8 ± 12937.3 (25/27)
Borrow Pit	107.9 ± 36.2 (11/24)	9.2 ± 5.4 (3/25)	-
Collective means ± SE (prevalence)	53.7 ± 32.6 (18/255)	103.6 ± 98.3 (6/76)	26090.8 ± 6934.8 (81/98)

Figure 1. Location of Arnold Air Force Base in middle Tennessee, USA.

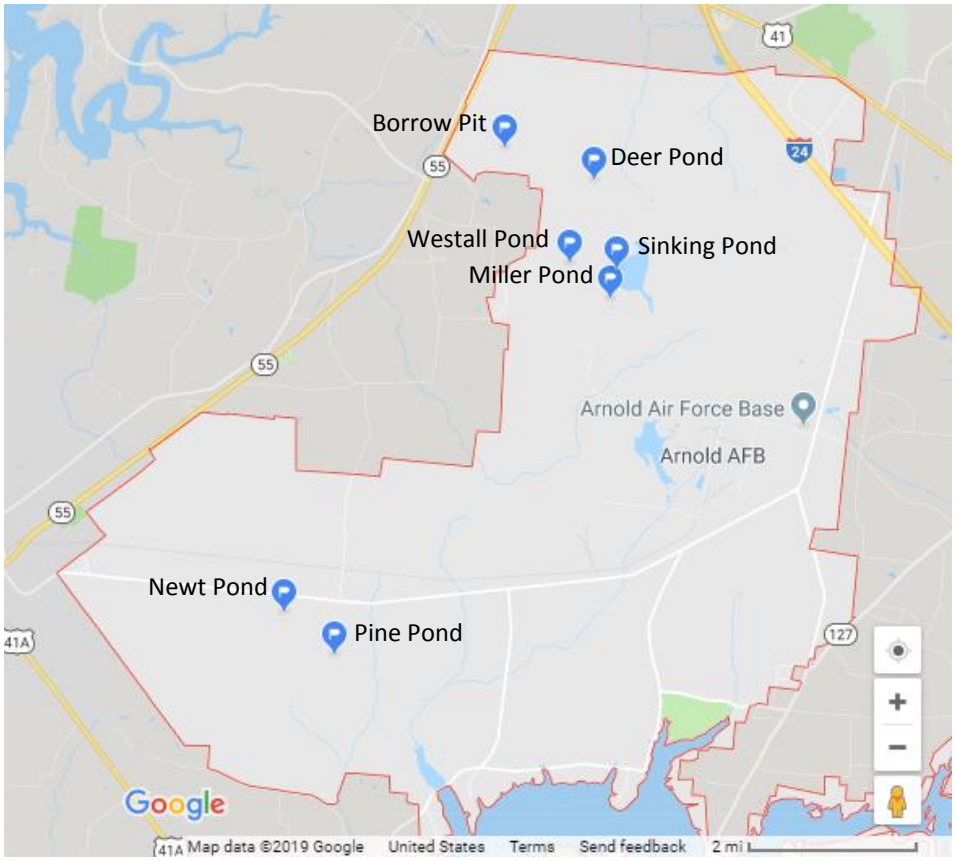

Figure 2. Boundary of Arnold Air Force Base, Franklin and Coffee counties, Tennessee, USA. Sampling sites are marked by blue flags.

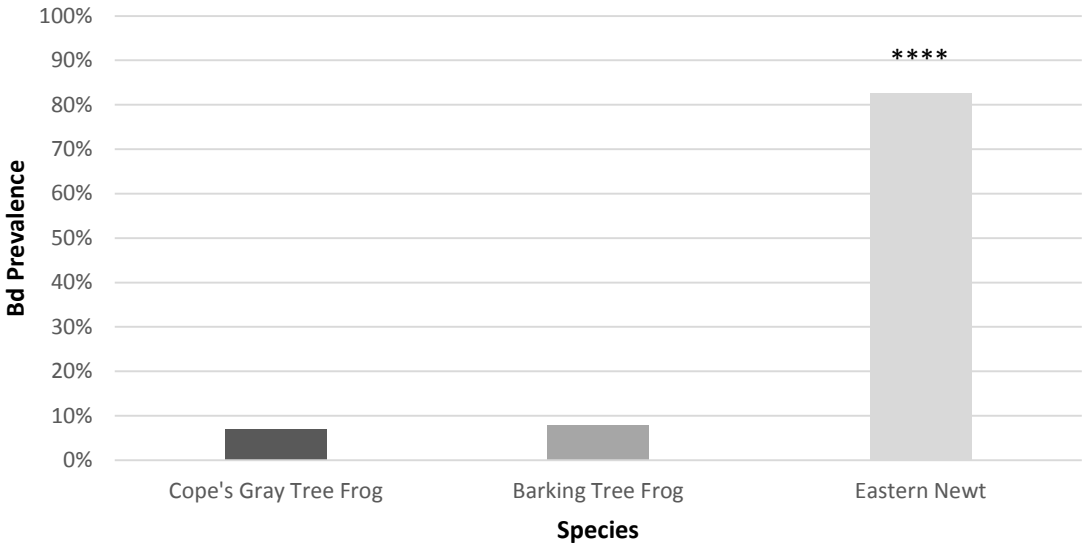

Figure 3. Prevalence of Bd on the skin of Cope's Gray Tree Frogs (*Hyla chrysoscelis*), Barking Tree Frogs (*Hyla gratiosa*), and Eastern Newts (*Notophthalmus viridescens*) sampled at Arnold Air Force Base in middle Tennessee from March 2017 to August 2018.

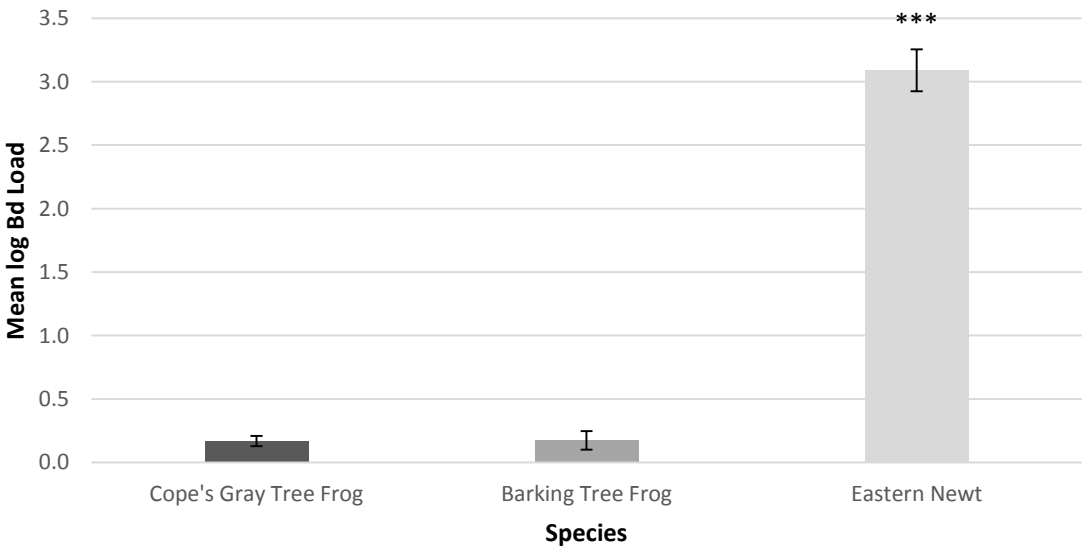

Figure 4. Mean log Bd load of Cope's Gray Tree Frogs (*Hyla chrysoscelis*), Barking Tree Frogs (*Hyla gratiosa*), and Eastern Newts (*Notophthalmus viridescens*) sampled at Arnold Air Force Base in middle Tennessee from March 2017 to August 2018. (Error bars = standard error)

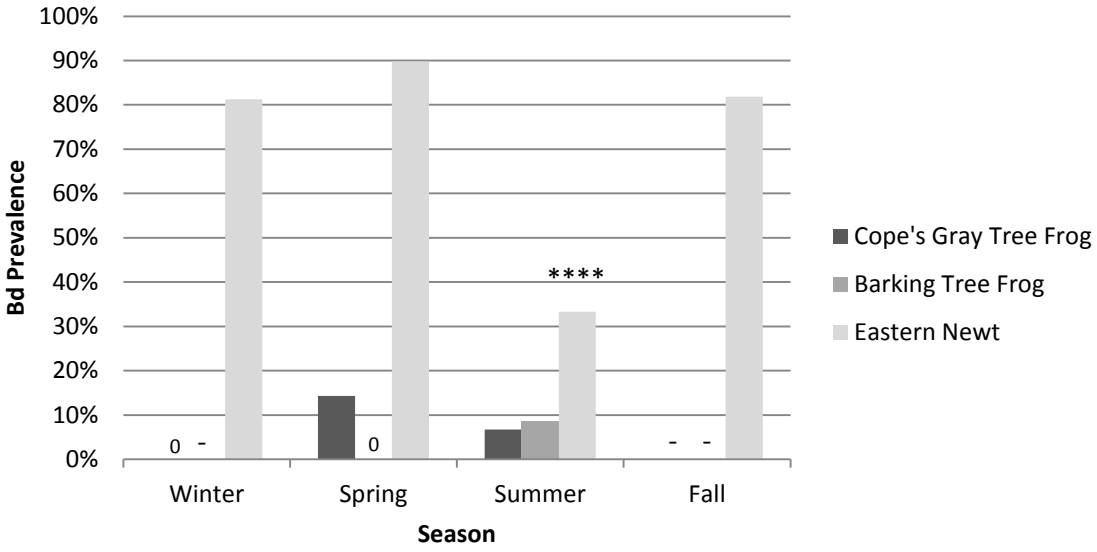

Figure 5. Seasonal shifts in Bd prevalence on the skin of Cope's Gray Tree Frogs (*Hyla chrysoscelis*), Barking Tree Frogs (*Hyla gratiosa*), and Eastern Newts (*Notophthalmus viridescens*) sampled at Arnold Air Force Base in middle Tennessee from March 2017 to August 2018.

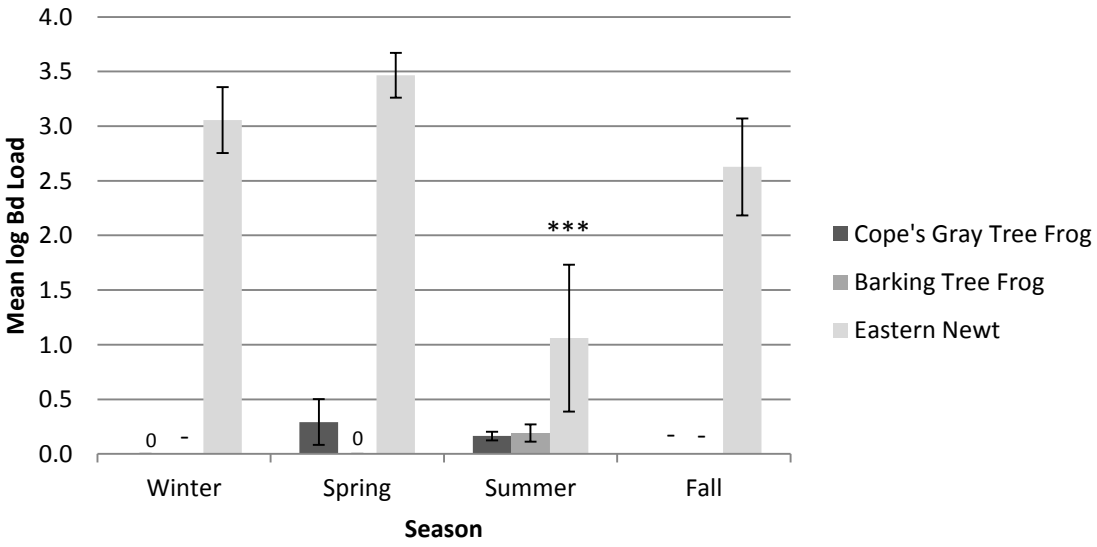

Figure 6. Seasonal shifts in mean log Bd load of Cope's Gray Tree Frogs (*Hyla chrysoscelis*), Barking Tree Frogs (*Hyla gratiosa*), and Eastern Newts (*Notophthalmus viridescens*) sampled at Arnold Air Force Base in middle Tennessee from March 2017 to August 2018. (Error bars = standard error)

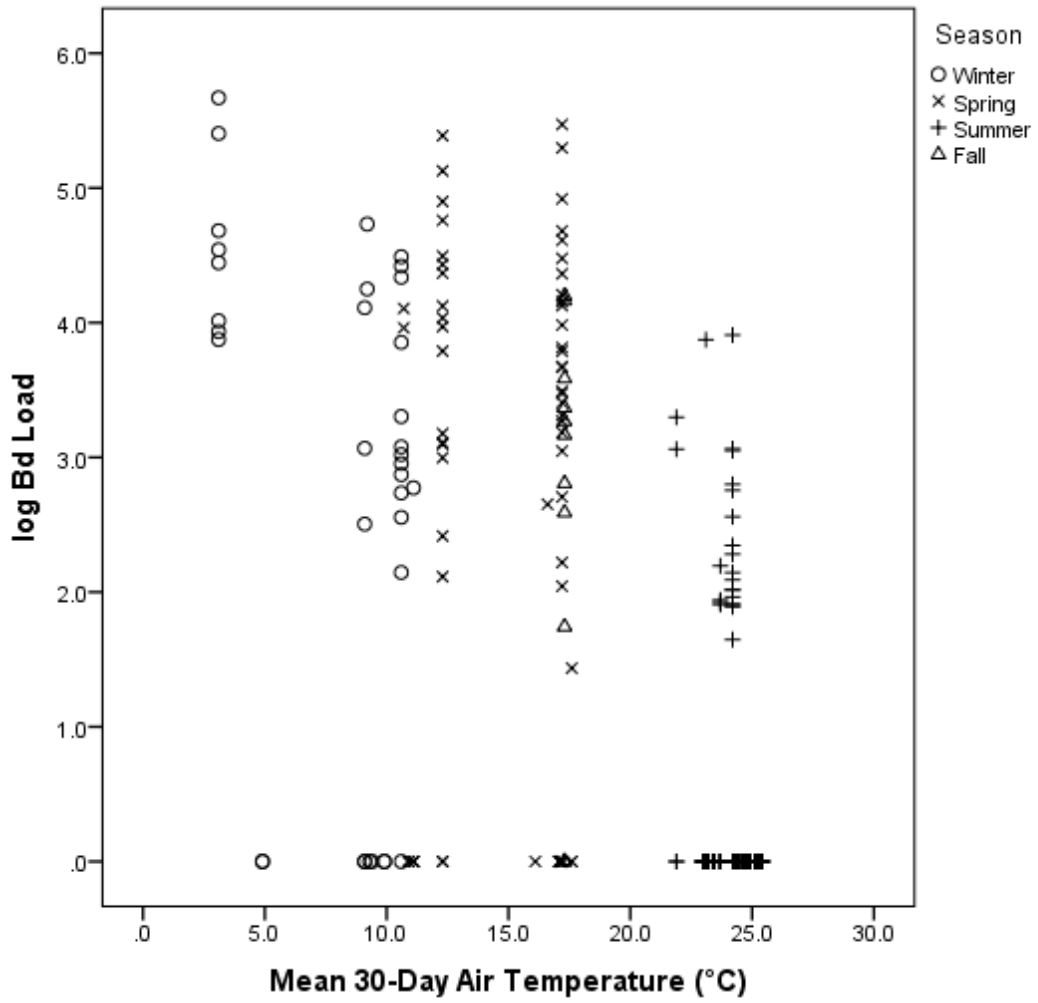

Figure 7. Scatter plot of negative relationship between mean air temperature for 30 days prior to sampling and log Bd load of Cope's Gray Tree Frogs (*Hyla chrysoscelis*), Barking Tree Frogs (*Hyla gratiosa*), and Eastern Newts (*Notophthalmus viridescens*) sampled at Arnold Air Force Base in middle Tennessee from March 2017 to August 2018.

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