

PRESENCE AND PREVALENCE OF BD (*BATRACHOCHYTRIUM DENDROBATIDIS*) IN CENTRAL PENNSYLVANIAN WOODLAND VERNAL POOLS

KRISTIN S. COURY^{1,2,4}, BRENNAN A. NEAL^{1,3}, MICHAEL J. SHIN¹, AND ERIK D. LINDQUIST¹

¹ Department of Biological Sciences, Messiah College, Mechanicsburg, PA 17055, USA

² Current Address: Robins Nature & Visitor Center, Maymont Foundation, Richmond, VA 23220

³ Current Address: Department of Sciences, Crossroads Christian Academy, Corozal, Panama City, Republic of Panama

⁴ Corresponding Author: ks1478@messiah.edu

ABSTRACT: *Batrachochytrium dendrobatidis* (*Bd*), a virulent chytrid fungus responsible for dramatic amphibian declines, has been detected in the northwestern and southeastern regions of Pennsylvania. However, little environmental *Bd* testing has been performed in central Pennsylvania, particularly in the unique and speciose habitats of woodland vernal pools. Our study included sampling in four vernal pools over a period of three months during amphibian breeding periods. Skin swabs were taken from three caudate and two anuran species, during the course of late winter and spring migrations ($n = 143$). Low *Bd* zoospore equivalent loads were detected in only a few individuals, in three of the five species but in all four vernal pools sampled. No significant trends were seen between zoospore loads and ambient temperature or migration timing across the species sampled.

INTRODUCTION

Batrachochytrium dendrobatidis (*Bd*) is a fungus belonging to the order Chytridiales that has been responsible for catastrophic declines in amphibian populations within the last several years (Piotrowski et al. 2004, Hyatt et al. 2007). Mobile zoospores enable *Bd* to acquire and infect new hosts rapidly via water sources, in which it can survive independently for a limited time (Piotrowski et al. 2004). *Bd* infection can cause thickening of the skin, inhibition of gas and waste exchange, and often results in death (Voyles et al. 2007). Rapid onset and high infection rates of *Bd* have rendered amphibians as one of the most threatened vertebrate groups on the planet (Fisher et al. 2009, Hyatt et al. 2007).

Historically, *Bd* research has focused on amphibian communities residing outside of North America (Padgett-Flohr, 2008). Within the United States, only two studies have specifically focused on amphibians in the state of Pennsylvania. In these accounts of *Bd* in Pennsylvania, the focus has been on species that utilize lotic water systems in northern regions of the state. While *Bd* has been confirmed in two salamander species: *Notophthalmus viridescens* and *Cryptobranchus alleganiensis* (Regester et al. 2012 and Groner and Relyea 2010), there have

been conflicting results concerning the state of infection in these regions with studies showing both positive and negative results for *Bd* screening (Glennay, et. a 2010).

Currently, there are no studies that have focused on the lentic water systems or terrestrial amphibians of Central Pennsylvania. However, there have been both positive and negative results from studies in the US focusing on Ambystomatid salamanders (Davidson et al. 2003, Richards-Hrdlicka et al. 2013, Rothermel et al. 2008) and no positive *Bd* results for *Pseudacris crucifer* (Longcore et al. 2007, Richards-Hrdlicka et al. 2013, Groner and Relyea 2010). In addition, there has been little research focusing on *Bd* infection in vernal pool habitats around the United States, which are critical habitats for the complex lifecycles of many species of amphibians (Lindquist et al. 2013).

The South Mountain region of central Pennsylvania is characterized by ephemeral woodland vernal pools, and provides a unique habitat for a wide variety of amphibians, including Ambystomatid salamanders (Lindquist et al. 2013). Vernal pools in particular serve as a hub of breeding activity for many species of amphibians, and act as important habitat links for the maintenance of the forest ecosystem and nutrient cycling (Lindquist et al.

Table 1: Positive Individual Zoospore Equivalent Load and Replicate Count. Positive *Bd* primer amplification among individuals with respective replicate amplification. AmbJef = *Ambystoma jeffersonianum*; AmbMac = *Ambystoma maculatum*; PseCru = *Pseudacris crucifer*. *PseCru 9 excluded from further analysis as it is a clear outlier.

Individual	Average Zoospore Equivalent Load	Replicate Count
AmbJef 12	0.25	1
AmbJef 16	0.38	3
AmbJef 24	0.15	1
AmbJef 27	0.65	1
AmbMac 1	0.61	3
AmbMac 7	1.04	1
AmbMac 8	0.30	2
AmbMac 12	0.44	1
AmbMac 14	1.04	1
AmbMac 16	0.22	1
AmbMac 17	0.23	1
PseCru 1	1.62	3
PseCru 3	1.11	3
PseCru 4	4.13	3
PseCru 5	1.04	3
PseCru 9	11438.3*	1
PseCru 20	18.14	3
PseCru 32	0.32	3
PseCru 33	1.78	3

2013). During the hydrological period in these vernal pools, hundreds to thousands of individuals of numerous species can frequent a given pool, making both interspecific and intraspecific *Bd* transmission a possibility. Therefore, determining the presence and prevalence of *Bd* in these unique habitats is the first step in understanding of amphibian disease ecology in central Pennsylvania's woodland vernal pools: either to project the possible impact of *Bd* on these ecosystems, or to gain greater understanding of the disease's function in this environment.

Our study focused on determining the presence and prevalence of *Bd* in south-central Pennsylvanian vernal pools, using standard techniques with qPCR analysis. Due to the unique hydrologic cycle of vernal pools, presence of the disease in this environment would suggest either that *Bd* persists in seasonally dry pools or that *Bd* is harbored in the skin of infected adults and passed terrestrially amongst reservoir species when pools are dry. Both scenarios imply acutely adaptive disease physiology for yearly *Bd* persistence in woodland vernal pools or among the fauna that migrate to and from these breeding sites.

Furthermore, we investigated potential correlations between *Bd* zoospore load and ambient temperature; including daily high/low air temperatures and pool water temperatures. Due to positive *Bd* results being found in other parts of the state, we hypothesized that *Bd* would be present in Pennsylvania woodland vernal pools. Additionally, we hypothesized that zoospore load numbers would increase as ambient temperatures reached optimal *Bd* growth conditions (17-23°C).

MATERIALS AND METHODS

During the spring of 2013, samples were taken from four locally known vernal pool sites within the South Mountain physiographic province of south-central Pennsylvania. Sites were delineated by a minimum of five

miles separating pools, ensuring that no sites were reasonably accessible by the same amphibian breeding populations. Two of the four sites included multiple pools within 50 meters of one another, all easily accessible by breeding individuals from the same populations, and therefore, disease conditions.

Field Sampling – Prior to the survey, sites for sampling were selected based on known amphibian presence, accessibility, and proximity. Sampling was conducted throughout February, March, and April of 2013 during nights of low or falling barometric pressure, in order to coincide with amphibian migrations and to maximize sample size. The species collected included three caudate and two anuran species: *N. viridescens* (Red-spotted Newt), *Ambystoma jeffersonianum* (Jefferson Salamander), *A. maculatum* (Spotted Salamander), *Lithobates sylvaticus* (Wood Frog), and *P. crucifer* (Spring Peeper). A predetermined minimum target sample size of 30 individuals was set for each species, although our ideal sample size was not achieved among *N. viridescens*, *L. sylvaticus*, and *A. jeffersonianum*. Study subjects were handled individually with new nitrile gloves and stored in new Ziploc® bags. Samples were transported to the laboratory for further processing. Sex, weight, SVL, ventral skin swabs, a toe clipping, and general observations were taken during the processing period for each individual. Our swabbing protocol modeled the methods published by Hyatt et al. (2007), with modifications to maximize sample DNA, including an increase in number of swabs at each location (5-10) and swabbing along the vent and costal grooves of urodeles. Each was returned to its respective site within 24 hours of capture and processing. Swabs and toe clips were stored at -20°C until DNA extraction was performed. Toe clips were retained for further testing unrelated to this study, but served primarily as field markers to prevent re-capture of individuals.

Extraction and qPCR Protocol– DNA was extracted from the skin swabs using QIAGEN QIAamp DNA mini kit reagents and following a modified version of the "DNA Purification from Buccal Swabs (Spin Protocol)" protocol from the QIAamp DNA Mini and Blood Mini Handbook (June 2012). Modifications included: use of 200µl ATL in step 2, incubation of sample for 1hr at 56°C with 10" pulse-vortexing every 10' during step 3, using 200µl Buffer AL in step 5, incubation of sample at 70°C for 30' with 10" pulse-vortexing every 10' in step 6, and application of 40µl of Buffer AE in step 14. (Bletz, unpublished). The remainder of the protocol was followed as published. qPCR was conducted using ABI StepOne Plus™ Real-time qPCR machine and 2X Taqman Master Mix (Applied Biosystems) and custom target primers and probes as per Boyle et al. (2004). The QIAGEN QIAamp DNA mini kit was selected for its ease-of-use, accessibility, and cost-effectiveness.

Thermocycling for genomic DNA extracted from sample swabs included 40 cycles with an annealing temperature of 60°C. Individual sample *Bd* zoospore load was quantified using a standard curve constructed with known *Bd* zoospore equivalents. A total of 143 samples were collected, extracted, and tested in triplicate during qPCR.

RESULTS

B. dendrobatidis presence was confirmed in at least one species found in each of the four vernal pools. Of the five amphibian species studied, *Bd* was detected among three species, each with fairly narrow zoospore equivalent (ZE) ranges: *A. jeffersonianum* (0.15-0.65 ZE detected), *A. maculatum* (0.22-1.04 ZE detected), and *P.*

crucifer (0.3-4.1 ZE detected) (Table 1). The standard curve was created using five known ZE amounts: 10,000, 1,000, 100, 10, and 1 ZE. Using Ct values of the known standards, a logarithmic fit ($p < 0.01$) was then applied to the mean of the triplicate Ct values of the sampled individuals. The subsequent ZE value was then considered representative of the individual's disease status. Of the 143 individuals sampled, 19 individuals registered detectable zoospore DNA during qPCR analysis (Table 1). An individual was considered positive when at least one of the triplicate wells amplified *Bd* DNA. The prevalence rate among all samples was 13.2%, while species prevalence rates were highest among *P. crucifer* (21.62%).

Comparison of the relationship between water temperature data with individual zoospore load was considered insignificant ($p = 0.24$), as were relationships between zoospore load and daily high ($p = 0.24$) and low ($p = 0.39$) air temperatures, as recorded by the NOAA station in Harrisburg, PA.

DISCUSSION

As a result of this study, *B. dendrobatidis* is now known to exist in Central Pennsylvanian woodland vernal pools. Although three of the five species studied had small sample sizes ($n < 30$), the presence detected in our environmental testing gives appreciable data to confirm a new location for the fungus' range in Pennsylvania. Samples amplifying ChytrMGB2 primers exhibited extremely low zoospore equivalents with some individuals only reporting *Bd* presence in 1-2 replicate wells. None of the individuals captured and tested exhibited any classic signs of chytridiomycosis, the disease associated with *Bd* infection (i.e.: reddened seat patch/vent, laborious walking/hopping, struggle in righting oneself), affirming our laboratory results of exceedingly low levels of *Bd* among the populations studied. Furthermore, in light of Vredenburg's 10000 ZE rule, our data affirm that no individuals sampled were fatally infected. Despite a modest representative sample, we are confident that the data confirm that *Bd* is present in Central Pennsylvanian woodland vernal pools.

There was no significant correlation detected between ambient temperatures and zoospore load. Instead, the zoospore levels appeared to remain fairly steady despite increasingly favorable temperatures throughout our field season. The low, consistent zoospore levels suggest that additional limiting factors appear to be at play in the *Bd* lifecycle, inhibiting the collapse of the amphibian community so often associated with *Bd* detection and chytridiomycosis. Environmental temperature is known to be a primary factor influencing the success of *Bd* colonization, reproduction, and subsequent chytridiomycosis (Savage et al. 2011). The most devastating amphibian declines have occurred in cool, montane environments with very stable, moderate climates that coincide with, and fluctuate within, the range of optimal temperature for *Bd* growth and reproduction (Woodhams et al. 2008).

Previous study has shown that individuals from a variety of adult North American frogs are able to survive and "clear" *Bd* infection under laboratory temperature treatments (Chatfield and Richards-Zawacki 2011; Andre et al. 2008; Retallick and Miera 2007). Another study found that as little as 16 hours of exposure to 37°C temperatures could eradicate the pathogen from an individual (Woodhams et al. 2003). It is possible that seasonal extremes in the tested environment prevent lethal infection among the studied amphibians, allowing them to survive with sub-lethal infection or enabling populations to

be "seasonally cured" of the infection from year to year (Kriger and Hero 2006). Furthermore, previous study has postulated that elevated temperature may not only compromise *Bd*'s function, but may also influence the host's resistance to *Bd* infection, enabling the individual's immune system to clear the infection (Andre et al. 2008). Central Pennsylvanian climate fluctuates dramatically throughout the year: temperatures and water presence range beyond the range of tolerance for most *Bd* strains, potentially reducing environmental zoospore load and enabling amphibian survival with sub-lethal infections.

It is important to note that our sampling methods solely examined *Bd* among adult, breeding individuals. It is possible that *Bd* infection and individual mortality rates may be higher among larvae within the vernal pools. Previous study has shown that metamorphosing individuals are more susceptible to lethal infection, due to the presence of keratinized tissues associated with skeletal development (Venesky et al. 2010). It is possible that larvae and juveniles may be the more affected amphibian demographic within this ecosystem. Adults with sub-lethal infections that enter the pools during spring migrations may negatively affect the more vulnerable, developing residents of the pools by providing a consistent source of fresh zoospores throughout breeding season, while surviving with minimal infection. Further study of population trends within these environments may indicate whether *Bd* is negatively affecting population numbers, or whether *Bd* functions as a more ecologically integrated, selective pressure that persists in an otherwise healthy community (Rosenblum et al. 2013).

The narrow seasonal window for ideal *Bd* growth compounded with the ephemeral nature of vernal pools appears to yield a tempered infection rate among local, adult amphibians. While the exact reason remains unclear, this phenomenon is potentially influenced by compromised *Bd* function, lesser strain virulence, improved host immune response, or a combination of these factors. Consequently, it appears that the temperature-hydroperiod-infection relationship may be more complicated in this dynamic, temperate ecosystem than previously thought, showing the need for more study in the prevalence of *Bd* in this unique habitat.

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