Detection of the Pathogenic Fungus, *Batrachochytrium dendrobatidis,* in Anurans of Huntley Meadows Park, Fairfax County, Virginia

Todd A. Tupper[1†*], Lauren D. Fuchs[2†], Caitlin O'Connor-Love[1]
Robert Aguilar[3] Christine Bozarth[1], David Fernandez[1]

[1]Northern Virginia Community College
Department of Math, Science, and Engineering
5000 Dawes Avenue
Alexandria, Virginia 22311

[2]George Mason University
Department of Systems Biology
10900 University Blvd
Manassas, VA 20110

[3]Smithsonian Environmental Research Center
Fish and Invertebrate Ecology Lab
647 Contees Wharf Road
Edgewater, Maryland 21037

*Corresponding author: ttupper@nvcc.edu
†Authors contributed equally to the development of the manuscript

Introduction

The chytrid fungus, *Batrachochytrium dendrobatidis* (hereafter *Bd*), has been identified as a proximate driver of amphibian population declines and extinctions worldwide (Lambertini et al., 2016; Lips et al., 2006; Olson et al., 2013). The pathogen is now widespread across much of North America, demonstrating a highly heterogeneous spatial distribution (Lannoo et al., 2011). *Bd*-related declines have been documented in several western states (Arizona, California and Colorado; Bradley et al., 2002; Briggs et al., 2005; Muths et al., 2003). Portions of the southwest and the eastern United States have reported high prevalence of the pathogen, but without concomitant declines (Lannoo et al., 2011; Petersen et al., 2016).

Bd is a pathogenic mycotic species, which produces flagellated, motile spores that colonize keratinized epithelial cells in the skin of adult amphibians, and keratinized mouthparts of larval amphibians (Brutyn et al., 2012). Clinical manifestations of *Bd* infection reflect the diseased state known as chytridiomycosis. Hyperplasia and keratosis in the diseased state interfere with cutaneous respiration and osmoregulation, and can be lethal (Berger et al., 1998; Kilpatrick et al., 2010; Rachowicz et al., 2006; Voyles et al., 2009). Susceptibility to the pathogen, and effects of the infection vary both within and among species, with cases ranging from asymptomatic to fatal (Beebee and Griffiths, 2005; Briggs et al, 2010; Savage et al., 2011). This variation has been attributed to differences in innate defenses and host life-history traits (Harris et al., 2006; Lips et al., 2003; Woodhams et al., 2007). Habitat and climatic conditions have been shown to significantly influence the virulence of *Bd* infection, with lethal outbreaks of chytridiomycosis most commonly associated with cooler, wetter, and thermally consistent environmental conditions (Berger et al., 2004; Bielby et al., 2008; Kriger and Hero, 2007; Murray et al., 2011; Rowly and Alford, 2007; Savage et al., 2011).

It is thought that *Bd* may be endemic to many regions of North America, including the eastern United States, where the pathogen has been present since the 1960's, and known to infect at least 48 amphibian species (Hughey et al., 2014; Lannoo et al., 2011; Longcore et al., 2007; Ouellet et al., 2005). In the Mid-Atlantic region, *Bd* has been shown to exist without associated declines (Goodman and Ararso, 2012; Grant et al., 2008; Hughey et al., 2014; Lannoo et al., 2011; Petersen et al., 2016; Pullen et al., 2010). Propagation of the fungus and/or virulence has been seemingly discouraged by local climatic conditions. However, synergistic interactions between a changing climate and a suite of anthropogenic stressors may function to alter infection dynamics, potentially inducing disease outbreaks and subsequent declines of local amphibian populations (Davidson et al., 2003; Longcore et al., 2007; Pounds et al., 2006). This latent threat highlights the need for long-term monitoring. Monitoring data are critical to identifying emerging patterns of *Bd* infectivity, understanding its effect on local amphibian populations, and are integral to management of natural areas containing amphibifauna (Olson et al., 2013).

Information on the prevalence of *Bd* in anuran species throughout Virginia is relatively limited. Although prior studies suggest that *Bd* is widespread in the state (Hughey et al., 2014), there appear to be inconsistent trends in rates of infection, with overall prevalence (across anuran species) ranging from as low as 8% (central Virginia; Goodman and Ararso, 2012) to as high as 35% (Hughey et al., 2014). Systematic *Bd* surveys have also been conducted in areas of western, southern and central Virginia (Goodman and Ararson, 2012; Gratwicke et al., 2011; Hughey et al., 2014; Pullen et al., 2010). To the best of our knowledge, only one other study has surveyed for *Bd* in Northern Virginia (Augustine and Neff, 2016). However, because of the low sample size (N = 25), and minimal representation of anuran species (three species; N = 11), supplemental data is necessary to more accurately assess *Bd* prevalence in this region of Virginia. Our primary objective was to determine whether *Bd* is present in this portion of the state, and if so, how its prevalence compares to other regions within Virginia and throughout the Mid-Atlantic region. Data from our study will aid in the development and implementation of disease management protocols on local and regional scales.

Methods

We collected samples at Huntley Meadows Park in Fairfax County, Virginia (Figure 1; 38°45'36.57" N -77°05'44.13" W). Huntley Meadows Park is approximately 577 ha and, other than a green corridor on its southeast side, is predominately surrounded by suburban developments (Figure. 1). Huntley Meadows Park is comprised of a large central wetland that is hydrologically connected to the majority of the park's other smaller wetlands, which range from early-successional, herbaceous open-canopy wetlands to later-successional hardwood swamps. Between 26 March and 5 June 2016, we opportunistically sampled anurans throughout Huntley Meadows Park. Adhering to biosecurity standards outlined by the Virginia Herpetological Society (VHS, 2016), we hand-captured anurans, and swabbed the skin surface with sterile dry swabs (no. MW113, Medical Wire and Equipment Company, Durham, NC). We stored the swabs in 1.5 mL microcentrifuge tubes and kept them frozen until molecular analyses. We followed the Purification of Total DNA from Animal Tissues protocol (Qiagen®, Valencia, CA) to elute DNA from each swab. We prepared a PCR master mix containing 10 µL Sso Advanced™ universal probes supermix (Bio-Rad, Hercules, CA), 200 nM of each primer (ITS1-3Chytr and 5.8sChytr; Boyle et al. 2004), 250 nM MGB probe, and sterile water. We combined 18 µL of the master mix and 2 µL of eluted DNA in a 96-well PCR plate. Positive and negative controls were included for both the DNA elusion and amplification. The positive control required a single, standard concentration as we were testing for presence of the *Bd* pathogen,

Figure 1. Huntley Meadows Park. Blue icons represent sampling locations. We swabbed multiple individuals at each sampling location. We presented the location without a +/- symbol because we found both *Bd* positive and negative individuals at most sampling locations.

and not zoospore load. To detect *Bd*, we used a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Samples were exposed to 95°C for three minutes, then run through 45 cycles of 95°C for 30-sec and 55°C for 45-sec. We performed three rounds of PCR per sample. Samples were considered positive if they fluoresced prior to the 40th cycle of the PCR reaction on at least two occasions.

We used multiple logistic regression to determine which variables were significant predictors of *Bd* infection. The predictors were day of year, sex, ecological guild, species and site. The sex variable was an ordinal variable consisting of three categories: male, female, juvenile. Therefore, it also was a proxy for age (adult/subadult). The ecological guild variable was also ordinal and consisted of two categories, terrestrial/arboreal (Eastern American Toad, Cope's Gray Treefrog Green Treefrog, Spring Peeper) and aquatic (American Bullfrog, Green Frog, Southern Leopard Frog). We assessed goodness-of-fit and predictive ability of the logistic regression model with Hosmer-Lemeshow and Somers' D statistics, respectively. We then used descriptive statistics, chi-square tests, and independent sample t-tests to further examine variables identified as significant by multiple logistic regression analysis. Zar (2009) was used to guide statistical analyses. Minitab version 17 was used for all statistical analyses (www.minitab.com), and

ArcGIS version 10.3 (www.ersi.com) and DivaGIS version 7.5.0 (www.diva-gis.org) were used to create maps. Nomenclature follows Crother (2012).

Results

We collected 100 samples between 26 March and 5 June 2016. While we were unable to sample equally among species, we sampled approximately evenly between ecological guilds. We collected the majority of samples in April and May (Tables 1 and 2). Multiple logistic regression

Table 1. Species sampled for *Bd* at Huntley Meadows Park. [1]= terrestrial/arboreal ecological guild. [2] = aquatic ecological guild.

Common Name	Scientific Name	N	# Positive	% Positive
Eastern American Toad[1]	*Anaxyrus americanus*	13	3	23
American Bullfrog[2]	*Lithobates catebeianus*	11	3	27
Cope's Gray Treefrog[1]	*Hyla chrysoscelis*	9	1	11
Green Frog[2]	*Lithobates clamitans*	19	7	37
Green Treefrog[1]	*Hyla cinerea*	11	1	9
Southern Leopard Frog[2]	*Lithobates sphenocephalus*	23	18	78
Spring Peeper[1]	*Pseudacris crucifer*	13	1	8

Table 2. Sampling distribution and number and proportion of *Bd* detections per month.

Month	N	# Positive	% Positive
March	9	8	88.9
April	22	13	59.1
May	52	11	21.2

indicated that sex (age); ($Z = 3.11$; $P < 0.05$), ecological guild ($Z = 3.02$; $P < 0.05$) and day of year ($Z = -2.42$; $P < 0.05$) were strong predictors of infection when considered together with each other and species, and site ($G = 48.1$; $df = 5$; $P < 0.001$; Hosmer-Lemeshow $P = 0.91$; Somers' $D = 0.78$; Table 3). Species and site were not indicated as significant. We identified

Table 3. Multiple logistic regression output. Coef = coefficient, SE = standard error.

Predictor	Coef	SE Coef	*Z*	*P*
Constant	-3.940	3.529	-1.12	
Day of Year	-0.042	0.018	-2.42	
Sex (age)	1.359	0.437	3.11	
Guild	2.629	0.869	3.02	
Species	0.230	0.199	1.16	
Site	0.133	0.097	1.38	

significantly higher infection rates in adult male and female anurans ($\chi^2 = 7.6$; df = 2; $P < 0.05$; Table 4) of the aquatic ecological guild ($\chi^2 = 17.9$; df = 1; $P < 0.001$ *see* Table 1) earlier in the season ($\chi^2 = 25.0$; df = 3; $P < 0.001$; *see* Table 2). Of the 99 testable samples, 34 were positive, yielding an overall infection rate of 34.3%.

Table 4. Number and proportion of *Bd* detections across sexes, and correspondingly, by age class.

Sex	N	# Positive	% Positive
Female	41	13	31.7
	15	1	6.7
Male	39	18	46.2

Discussion

To the best of our knowledge, our work is only the second study to document *Bd* in anurans of Northern Virginia. Because information on *Bd* in this region (in anurans) are so limited, our data are particularly valuable—especially considering that we were able to increase the sample size and expand upon the number of species sampled. Our findings, coupled with results from other Virginia studies, confirm that *Bd* is prevalent throughout the state (Davison and Chambers, 2011; Goodman and Ararson, 2012; Gratwicke et al., 2011; Hughey et al., 2014; Lannoo et al., 2011; Petersen et al., 2016; Pullen et al., 2010). With the exception of Hughey et al. (2014), the overall infection rate in anurans from our study (34.3%; Table 5) was higher than all other studies

Table 5. *Bd* prevalence rates (% *Bd* positive) for studies conducted throughout Virginia and Maryland.

Study	Location	N	% *Bd* Positive
Fuchs et al., 2017	Anne Arundel County MD	116	10
Davidson and Chambers, 2011	Wise County VA	41	14.6
Goodman and Ararso, 2012	Central VA	103	7.8
Grant et al., 2008	C&O National Park, MD	53	17
Hughey et al., 2014	Western VA	292	35
Pullen et al., 2011	Central VA	740	14.1
Tupper et al., 2017	Fairfax County VA	99	34.3

conducted in Virginia (Davison and Chambers, 2011; Goodman and Ararson, 2012; Gratwicke et al., 2011; Lannoo et al., 2011; Petersen et al., 2016; Pullen et al., 2010) and neighboring Maryland (Fuchs et al., 2017; Grant et al., 2008).

Interestingly, the overall infection rate reported in our study also falls among the highest relative to all studies conducted throughout the entire eastern United States (*see* Petersen et al., 2016 and Rothermel et al., 2008 for exceptions). Huntley Meadows Park, a natural depression surrounded by densely populated suburban development (dgif.virginia.gov), is the principle education park for Prince William, Fairfax, and Arlington Counties, and it is host to over 200,000 visitors annually (Kathleen O'Shea, pers. comm). These factors may expose the park's wildlife to a range of anthropogenic stressors, such as pollution (herbicides pesticides, fossil fuel runoff,

siltation), introduced predators (Northern Snakehead *Channa argus*; FOHMP, 2017), human interaction, and noise (Beebee and Griffiths, 2005; Blaustein et al., 2012; Pullen et al., 2008). Exposure to anthropogenic stressors can reduce immune function in hosts and consequently increase disease prevalence (Bruno et al., 2003; Carey et al., 1999; Daszak et al. 1999, 2001; Hoverman et al., 2011; Rohr et al., 2008). It is possible that anthropogenic stressors could have contributed to the relatively high rate of infection found in our study. However, further inquiry would be necessary to assess that statement.

Multiple logistic regression analysis indicated that three variables contributed significantly to infection: day of year, sex (age class), and ecological guild. We found *Bd* to be more prevalent in earlier months of sampling. This finding parallels various temperate regions in situ studies that show infection rates declining as the season progresses and warms (Hughey et al., 2014; Kinney et al., 2011; Longcore et al., 2007; Muths et al., 2008; Petersen et al., 2016). This trend is likely because *Bd* produces zoospores (in vitro) between 4 and 25°C and its pathogenicity declines when temperatures are above 23°C (Berger et al., 2004; Lamirande and Nichols, 2002; Piotrowski et al., 2004; Woodhams et al., 2003).

Timing of sampling may have influenced our finding of lower infection rates among juveniles and metamorphs, in comparison to both male and female adult anurans. Sampling for subadults was greater during periods when metamorphosis and dispersal occurred (*see* Wright and Wright, 1949 and http://www.virginiaherpetologicalsociety.com/). Thus, 87% of our juvenile samples were from June (a warmer month that would likely yield fewer *Bd* positive samples) and predominantly from Spring Peeper (85%), a species that has often demonstrated lower *Bd* infection rates than other anurans (Fuchs et al., 2017; Longcore et al., 2007; Rothermel et al., 2008; Tupper et al., 2011).

We found *Bd* to be more prevalent in aquatic species (53.8% overall, with the highest infection rate in Southern Leopard Frog) than in terrestrial/arboreal species (13%). Similar patterns in infectivity rates across ecological guilds have previously been reported (Kriger and Hero, 2007; Longcore et al., 2007; Tupper et al., 2011). Water is an effective medium for transmission of *Bd* zoospores (Kolby et al., 2015), and it is thought that the lower degree of thermal variability in aquatic environments, relative to terrestrial environments, may be critical to the pathogen's ability to more readily infect aquatic or semi-aquatic amphibians (Chatfield et al., 2012; Kriger and Hero, 2007; Moffitt et al., 2015; Weldon et al., 2004). Although *Bd* is more prevalent in aquatic species, it is not confined to aquatic guilds, as we detected it in the American Toad, Cope's Gray Treefrog, Green Treefrog and Spring Peeper. Additionally, numerous studies have found it in terrestrial/arboreal species (Berger et al., 2005; Daszak et al., 2003; Lannoo et al., 2011; Longcore et al., 2007; Oullett et al., 2005; Rothermel et al., 2008).

Previous studies conducted throughout the Mid-Atlantic region indicate a widespread occurrence of *Bd* in multiple anuran species without associated signs of chytridiomycosis (Fuchs et al., 2017; Grant et al., 2008; Pullen et al., 2010) and without related population declines (Petersen et al. 2016; Longcore et al. 2007; Lannoo et al. 2011; Rothermel et al. 2008). While we also did not observe symptoms of chytridiomycosis, it is thought that cases of the disease may be increasing locally or invading previously uninhabited regions (Daszak et al., 2003). Climate change may shift environmental conditions in favor of the pathogen. Consequently, the potential for interactions between the host and pathogen, coupled with a suite of anthropogenic factors, may influence outcomes of infection in unexpected ways (Davidson et al., 2003; Longcore et al., 2007; Pounds et al., 2006). We therefore recommend continued disease monitoring in the region,

especially where the pathogen is known to exist, and in locations where host immunity may be compromised by anthropogenic stressors. Huntley Meadows Park is one such location, and serves as a critical refuge for wildlife inhabiting increasingly urbanized Northern Virginia. We encourage VHS members to follow Huntley Meadows Park biosecurity protocols to decrease the potential for disease transmission. If visiting the park, please inquire about sanitation stations at the at the visitor center before walking trails

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