A Survey of the Pathogenic Fungus, *Batrachochytrium dendrobatidis*, at the Smithsonian Environmental Research Center, Anne Arundel County, Maryland.

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Introduction

Batrachochytrium dendrobatidis (*Bd*) is a pathogenic species of chytrid fungus that produces flagellated, motile spores, which colonize keratinized epithelial cells in the skin of adult amphibians, and keratinized mouthparts of larval amphibians (Rachowicz and Vredenburg, 2004; Brutyn et al. 2012). It has been associated with declines and extirpations in over 200 amphibian species (Lips et al. 2006). Susceptibility to, and outcomes of *Bd* infection vary markedly both within and between species, ranging from no symptoms to mortality (Beebee and Griffiths 2005; Briggs et al. 2010). These inconsistencies are attributed to temperature (Berger et al. 2004), innate defenses (Harris et al. 2006; Woodhams et al. 2007), habitat (Kriger and Hero 2007; Rowley and Alford 2007), and host life history traits (Lips et al. 2003). Although *Bd* is found in a wide range of habitats and climates (Kilpatrick et al. 2010), catastrophic declines associated with *Bd* typically occur in cool, wet, and thermally consistent locations (Bielby et al. 2008; Murray et al. 2011; Berger et al. 2016). Such declines have been documented in Eastern Australia, Central America, and Europe (Berger et al. 1998; Lannoo et al. 2011; Johnson and Speare, 2003). In North America, observed declines have been restricted to the Western region of the United States (Longcore et al. 2007; Murray et al. 2009), with few *Bd*-related die-offs reported east of the Rocky Mountains (Petersen et al. 2016).

Studies in North America are generally sparse and opportunistic, showing inconsistencies in climatic determinants of *Bd* infectivity (Savage et al. 2011). Throughout the eastern United States, numerous studies (see Davidson and Chambers 2011; Huang and Wilson 2013; Lannoo et al. 2011; Longcore et al. 2007; Oullett et al. 2004; Peterson et al. 2016; Tupper et al. 2011) have identified the persistence of *Bd* in amphibians without associated population declines (Caruso and Lips 2013; Daszak et al. 2003; Muletz et al. 2014; Rothermel 2008). However, the mechanisms influencing

the virulence of *Bd*, are poorly understood. A better understanding of its distribution and prevalence among amphibians is necessary to assess the potential threat of *Bd* on local amphibian populations (Daszak et al. 2003). To the best of our knowledge, *Bd* data from the Mid-Atlantic are limited; there are only two prior *Bd* studies conducted in Maryland (Grant et al. 2008; Hossack et al. 2010), and none have focused solely on anurans. Here we assessed the prevalence of *Bd* in anuran species at the Smithsonian Environmental Research Center (SERC), in Anne Arundel County, Maryland. Our primary objective was to assess the prevalence of *Bd* among anurans, thus enhancing existing, but limited information on *Bd* in Maryland. We also aimed to determine if *Bd* was confined to anurans of particular ecological guilds (Kriger and Hero 2007; Longcore et al. 2007; Tupper et al. 2011).

Methods

We opportunistically sampled for *Bd* at the Smithsonian Environmental Research Center (hereafter SERC; 38°53'17.41"N 76°33'15.52 W), in Anne Arundel County, Maryland (for more about SERC see Tupper et al. 2016) between 27 March and 27 June 2014 and 2015. We hand captured (while wearing new nitrile gloves) anurans and assayed skin surfaces via methods described by Hyatt et al. (2007) using sterile dry swabs (no. MW113, Medical Wire and Equipment Company, Durham, NC). We placed skin swabs in 1.5 mL microcentrifuge tubes, which were then frozen until molecular analyses. We followed the Purification of Total DNA from Animal Tissues protocol (Qiagen® DNeasy Blood & Tissue Kit, Valencia, CA) to extract DNA. To detect *Bd*, we prepared a 20 µL PCR reaction with 10 µL Sso Advanced™ universal probes supermix (Bio-Rad, Hercules, CA), 200 nM each primer (ITS1-3Chytr and 5.8sChytr; Boyle et al. 2004), 250 nM MGB probe, and 2 µL of extracted DNA. We ran samples on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) at 95°C for 3 min, followed by 45 cycles of 95°C for 30-sec and 55°C for 45-sec. We used positive and negative controls in DNA extraction and PCR amplification. Our goal was to determine the presence (not infection intensity) of *Bd*. Therefore, only one standard concentration was required as a positive control. We conducted qPCR analyses in triplicate on each sample to confirm a positive result. We considered a sample positive if it began to fluoresce before 40 cycles of our PCR reaction on at least two occasions. We used a Fisher's Exact test (Zar 2005) to assess differences in *Bd* detection rates between two broad ecological guilds of anurans: terrestrial/arboreal and aquatic (Table 1; Tupper et al. 2011) in minitab version 17 (ww.minitab.com). All nomenclature corresponds with Crother (2012).

Results

We opportunistically sampled 116 individuals across 11 species for *Bd*. Sixty-three (54.3 %) of the samples were collected in 2014 and 53 (45.7 %) were collected in 2015. Due to the opportunistic sampling scheme, sampling was not consistent between months and the monthly sampling distribution was not consistent between years. When pooled across years, five (4.3 %), 47 (40.5 %), 25 (21.6 %) and 39 (33.6 %) of the samples were collected in March, April, May and June, respectively. Seven of the 11 (63.6 %) species sampled tested positive for *Bd*, but only 12 of 116 (10.3 %) of the total samples contained *Bd*. Nine (75 %) of the positive detections were from 2014, and three (25 %) were collected in 2015. Six (50 %), three (25 %), and three (25 %) of the *Bd* positive samples were collected in May, June, and April, respectively. The largest proportion of detections (25%) occurred in the Pickerel Frog (*Lithobates palustris*) and Southern Leopard Frog (*Lithobates sphenocephalus*). The Eastern Cricket Frog (*Acris crepitans*) had the next largest detection rate (22 %). *Bd* was also detected in the American Bullfrog (*Lithobates catesbeianus*; 16.7 %) and Green Frog (*Lithobates clamitans*; 10.5 %). We also identified *Bd* in Cope's Gray Treefrog (*Hyla chrysoscelis*) and American Toads (*Anaxyrus americanus*; < 1%). The Spring Peeper (*Pseudacris crucifer*), Eastern Spadefoot (*Scaphiopus holbrookii*), Wood Frog (*Lithobates sylvaticus*) and Gray Treefrog (*Hyla versicolor*) were

all *Bd* negative (Table 1). Significantly more detections of *Bd* occurred in the aquatic guild (16.9%; N = 59) than in the terrestrial and arboreal guild (0.04%; N = 57; Fisher's exact test $P < 0.05$).

Discussion

Although we detected *Bd* in 63.6 % of species sampled, the detection rate across individuals was low (10.3 %), and we did not observe visual signs of chytridiomycosis, the diseased state as a result of *Bd* infection (Muths et al. 2008). Our results are similar to studies conducted throughout the eastern United States, which have documented *Bd* in a wide range of amphibian species generally absent of clinical signs of chytridiomycosis (Grant et al. 2008; Pullen et al. 2010), and without associated catastrophic declines (Lannoo et al. 2011; Longcore et al. 2007; Petersen et al. 2016; Rothermel et al. 2008). Our work is also similar to other studies in that *Bd* was primarily detected in aquatic species but was not restricted to fully aquatic species (Longcore et al. 2007; Pearl et al. 2007; Rodriguez et al. 2009; Tupper et al. 2011). However, our results differ in terms of detection rates: we found one of the lowest detection rates across anuran species in the eastern United States (Davidson and Chambers 2011; Huang and Wilson 2013; Lannoo et al. 2011; Longcore et al. 2007; Petersen et al. 2016; Rothermel et al. 2008; Tupper et al. 2011; Table 2), the lowest in Maryland and, with the exception of Augustine and Neff (2016), the lowest detection rates when compared to similar studies conducted throughout Virginia. These comparisons include sites at the nearby Chesapeake and Ohio National Historic Park, Montgomery County, Maryland, and Huntley Meadows Park, Fairfax County, Virginia (Davidson and Chambers 2011; Grant et al. 2008; Goodman and Ararso 2012; Pullen et al. 2010; Hughey et al. 2014; Tupper et al. *in progress*). Results from Long Branch Nature Center, Arlington County, Virginia (Augustine and Neff 2016) must be interpreted cautiously, as the sample size was low (N = 25). In addition, sampling only spanned five genera and six species, with just three anuran species represented (N = 11).

Despite increased *Bd* sampling efforts in the eastern United States, certain areas still remain insufficiently surveyed (Olson et. al. 2013; GBDMP 2016). In addition to our study, only two other studies have investigated *Bd* in amphibians of Maryland (Grant et al. 2008; Hossack et al. 2010) and only Grant et al. (2008) sampled anurans. Grant et al. (2008) had a notably lower sample size (N = 53) than the present study, and only sampled three species (American Bullfrog, Pickerel Frog, and Green Frog) within a single genus (*Lithobates*). Therefore, our data add considerably to the understanding of *Bd* prevalence in Maryland across a wide range of anurans.

Although the presence of *Bd* throughout the eastern United States is well documented, it has yet to be associated with declines (Longcore et al. 2007; Grant et al. 2008; Pullen et al. 2010; Rothermel et al. 2008). Nevertheless, data implicate *Bd* as a primary source of certain dramatic amphibian declines worldwide (Berger et al. 1998; Collins and Crump 2009; Skerratt et al. 2007; Talley et al. 2015). The enigmatic nature of these declines stems from difficulties in demonstrating a causative link between disease and decline (Daszak et al. 2003). This difficulty is due to complex host-pathogen dynamics, and co-existing stressors (i.e. pollution, introduced species, increased UVB-radiation, and climate change) that may be implicated independently, or in conjunction with the pathogen (Beebee and Griffiths 2005). The extent to which each of these factors interacts with, and compounds the effects of *Bd* at the individual, community, and population level is not thoroughly understood, and hypotheses explaining these interactions are controversial (Blaustein et al. 2011). Our ability to interpret these complex interactions, and identify the potential threat of *Bd* on local amphibian populations relies heavily on continued surveillance, both at new and existing locations. We therefore recommend continued *Bd* monitoring using heightened biosecurity protocols (VHS 2016) at SERC and throughout the region.

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Table 1. Number and proportion of *Bd* positives by species at the Smithsonian Environmental Research Center. Superscript numbers following species are ecological guilds. 1 = terrestrial/ arboreal, and 2 = aquatic. N = sample size.

Species	N	*Bd* Positive	% Positive
American Bullfrog[2]	6	1	17
American Toad[1]	21	1	5
Cope's Gray Treefrog[1]	14	1	7
Eastern Cricket Frog[1]	18	4	22
Eastern Spadefoot[1]	8	0	0
Gray Treefrog[1]	1	0	0
Green Frog[2]	19	2	11
Pickerel Frog[2]	8	2	25
Southern Leopard Frog[2]	4	1	25
Spring Peeper[1]	13	0	0
Wood Frog[2]	4	0	0

Table 2. Proportion of *Bd* detections in anuran species sampled throughout Maryland and Virginia between 2008 and 2017. A range for % *Bd* Positive indicates that data were presented as percentages per species and site. * = Bedford, Campbell, Craig, Giles, Lynchburg City, Montgomery and Richmond City Counties. ** = Buckingham, Charles City, and Henrico Counties. N = Sample size.

Study	Location	N	% *Bd* Positive
Augustine & Neff 2016	Arlington County, VA	11	0
Davidson & Chambers 2011	Wise County, VA	41	14.6
Goodman & Ararso 2012	Prince Edward County, VA	103	7.8
Grant et al. 2008	Montgomery County, MD	53	17
Hughey et al. 2014	Throughout Western VA*	292	0-92
Present Study	Anne Arundel County, MD	116	10
Pullen et al. 2011	Throughout Central VA**	740	14.1

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