ASPECTS OF HERPETOFAUNAL DISEASES AT TWO NATURAL AREAS IN MARYLAND AND VIRGINIA

by

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ABSTRACT

ASPECTS OF HERPETOFAUNAL DISEASES AT TWO NATURAL AREAS IN MARYLAND AND VIRGINIA

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Over the past several decades, amphibians and reptiles have been notably impacted by emerging infectious pathogens. Anuran populations have experienced significant morbidity and mortality events as the result of two pathogens in particular: *Batrachochytrium dendrobatidus* (*Bd*), and Frog-Virus 3 (FV-3). Reptiles have also been threatened by infectious pathogens, namely, *Ophidiomyces ophidiicola* (*Oo*), which has been attributed to the declines of several snake populations. Here, I present the results of investigations on presence, prevalence and co-occurrence of *Bd* and FV-3 in anurans, and the occurrence of *Oo* in snakes in Maryland (at the Smithsonian Environmental Research Center; SERC) and Virginia (at Huntley Meadows Park; HMP). Results from these investigations indicated that *Bd* is present in both Maryland (10.3%) and Virginia (34.3%). However, FV-3 was not detected in either state. In our investigation of *Oo* in snakes of Maryland and Virginia, we found an overall infection rate of 55.7%. Our results demonstrate that infectious herpetofaunal pathogens are present at both of our study sites,

which emphasizes the need for ongoing disease monitoring at local and regional scales, and the administration of enhanced biosecurity protocols at these sites.

CHAPTER I

PREFACE

Biodiversity loss is often the result of complex interactions between a variety of biotic and abiotic stressors that may drive population declines. One major factor that has demonstrated a capacity to negatively influence biodiversity is infectious disease (Blaustein et al., 2018). Emerging infectious pathogens have been implicated in wildlife epidemics worldwide (Allender et al., 2015). The deleterious effects of these pathogenic agents have also become increasingly apparent among amphibians and reptiles (collectively, herpetofauna; Skerratt et al., 2007; Robert, 2010; Clark, 2011; Lorch et al., 2016). Compounding the impacts due to habitat loss, pollution, and other anthropogenic influences, the rise in these emerging infectious diseases represents a significant threat to herpetofaunal biodiversity both at a global and local level (Gibbons et al., 2000; North et al., 2015; Rosa et al., 2017).

ANURAN DISEASES:

Over the past several decades, amphibians have experienced globally-occurring mass mortality events attributed to two pathogens in particular: *Batrachochytrium dendrobatidis* (etiological agent of Chytridiomycosis) and Ranaviruses, specifically *Ranavirus* type-species Frog Virus 3 (hereafter FV 3; Chinchar, 2002; Fox et al., 2006; Lips et al., 2006; Haislip et al., 2011; Echaubard et al., 2016). Data reflecting the

distribution, prevalence and population-level impacts of these diseases on local amphibian populations are relatively limited, and improved disease monitoring efforts are warranted. The goal of our research was to assess the presence and prevalence of *Bd* and FV-3 in local anuran populations, thereby contributing to our limited understanding of these pathogens in the Mid-Atlantic region. This work was prompted by an observation of a potentially symptomatic Northern Watersnake (*Nerodia sipedon*) at the Smithsonian Environmental Research Center, and by a mass die off of Wood Frogs (*Lithobates sylvaticus*) just 18 km from Huntley Meadows Park (Fairfax County Park Authority, 2015; Fuchs et al., 2018).

The second, third and fourth chapters of this thesis present the results of a three-year investigation on *Bd* and FV-3 in anuran populations at two natural areas in Maryland and Virginia. Each of the three chapters consists of a manuscript that was published in the journal, *Catesbeiana*. Chapter two (Fuchs et al., 2017a) documents the presence and prevalence of *Bd* at the Smithsonian Environmental Research Center in Anne Arundel County, Maryland (38°53'17.41" N 76°33'15.52 W; Figure 2.1), where *Bd* has been previously confirmed (Tupper et al., 2016). The third chapter (Fuchs et al., 2017b) reports on the pathogen at Huntley Meadows Park in Fairfax County, Virginia (38°45'36.57" N -77°05'44.13" W; Figure 5.1). Chapter four is an investigation of the co-occurrence of *Bd* and FV-3 at these same study sites. The results from this study were also published in *Catesbeiana* (Fuchs et al., 2018). The language (e.g. the use of the term "our" instead of "my"), citations, headers, tables, figures and references for these are presented in the format specified by this journal.

SNAKE FUNGAL DISEASE:

Infectious disease has also been identified as a threat to several reptile taxa, especially snakes (Todd et al., 2010). Since the early 2000s, many free-ranging snake populations have experienced often-severe declines attributable to a mycotic infection known as snake fungal disease (SFD; Clark et al., 2011; Allender et al., 2015). SFD can occur as a result of infection by a fungal pathogen known as *Ophidiomyces ophidiicola* (*Oo*; Allender et al., 2015; Paré and Sigler, 2016). Due to its relatively recent emergence (Burbrink et al., 2017), the host range and geographic distribution of this pathogen are still unclear (Guthrie et al., 2016).

Over the past decade, reports of SFD have become increasingly common, with cases of infection documented in at least 20 eastern states, including Maryland and Virginia (Allender et al., 2015; Guthrie et al., 2016; Tupper et al., 2015, 2018). The rising incidence of SFD poses an added challenge for snake conservation. Yet, despite growing reports of the disease, information on SFD in the Mid-Atlantic is still sparse, and increased efforts to monitor for the *Oo* pathogen are necessary. The main objective of our study was to assess the presence and prevalence of *Oo* at the Smithsonian Environmental Research Center and at Huntley Meadows Park. Like chapters two and three, this work was also developed in response to anecdotal observations of potentially symptomatic individuals at both sites (Tupper et al., 2015; 2018). Results from this study are currently in preparation for publication in the journal, *Amphibian & Reptile Conservation*, and are presented in the fourth chapter of this thesis. The language, formatting and citation style of chapter four follow the requirements of *Amphibian & Reptile Conservation.*

LITERATURE CITED

Allender, M. C, D. B. Raudabaugh, F.H. Gleason, and A. N. Millier. 2015. The natural history, ecology, and epidemiology of *Ophidiomyces ophiodiicola* and its potential impact on free-ranging snake populations. fungal ecol. 17:187-196.

Blaustein A. R., B. A. Han, R. A. Relyea, P. T. Johnson, J. C. Buck, S. S. Gervasi, and L. B. Kats. 2011. The complexity of amphibian population declines: understanding the role of cofactors in driving amphibian losses. Annals of the New York Academy of Sciences 1223: 108–119. doi: 10.1111/j.1749–6632.2010.05909.x.

Burbrink, F.T., J.M Lorch, and K.R. Lips. 2017. Host susceptibility to snake fungal disease is highly dispersed across phylogenetic and functional trait space. Science Advances 3: e1701387.

Chinchar, V.G. 2002. Ranaviruses (family *Iridoviridae*): emerging cold-blooded killers. Archives of Virology 147: 447-470

Clark, R. W., M. N. Marchand, B. J. Clifford, R. Stechert, and S. Stephens. 2011. Decline of an isolated timber rattlesnake (*Crotalus horridus*) population: interactions between climate change, disease, and loss of genetic diversity. Biol. Conserv. 144:886-891.

Echaubard, P., B.D. Pauli, V. L. Trudeau, and D. Lesbarrères. 2016. Ranavirus infection in northern leopard frogs: the timing and number of exposures matter. Journal of Zoology 298: 30-36.

Fairfax County Park Authority. 2015. Ranavirus, amphibians, and how to keep them apart. https://ourstoriesandperspectives.com/2015/09/14/ranavirus-amphibians-and-how-to-keep-themapart/ (Accessed March 2018).

Fox, S.F., A.L. Greer, R. Torres-Cervantes, and J. P. Collins. 2006. First case of ranavirus associated morbidity and mortality in natural populations of a South American frog, *Atelognathus patagonicus*. Diseases of Aquatic Organisms 72: 87–92. PLoS ONE 6(7): e22307.

Fuchs, L. F., T. A. Tupper, T. Sephapur, R. Aguilar, and C. A. Bozarth. 2017a. A survey of the pathogenic fungus, *Batrachochytrium dendrobatidus*, at the Smithsonian

Environmental Research Center, Anne Arundel County, Maryland. Catesbeiana 37: 32-40.

Fuchs, L. F., T. A. Tupper, C. O'Connor-Love, R. Aguilar, C. Bozarth, and D. Fernandez. 2017b. Detection of the pathogenic fungus, *Batrachochytrium dendrobatidis*, in anurans of Huntley Meadows Park, Fairfax County, Virginia. Catesbeiana 37: 109-120.

Fuchs, L. F., T. A. Tupper, C. A. Bozarth, D. Fernandez, and R. Aguilar. 2018. An investigation of co-infection by *Batrachochytrium dendrobatidis* and *Ranavirus* (FV3) in anurans of two natural areas in Anne Arundel County, Maryland and Fairfax County, Virginia, USA. Catesbeiana 38: 45-55.

Gibbons, J.W., D.E. Scott, T.J. Ryan, K.A. Buhlmann, T.D. Tuberville, B.S. Metts, J.L. Greene, T. Mills, Y. Leiden, S. Poppy, and C.T. Winne. 2000. The global decline of reptiles, Déjá Vu amphibians. BioScience 50: 653-666.

Guthrie, A.L., S. Knowles, A.E. Ballmann, and J.M. Lorch. 2016. Detection of snake fungal disease due to *Ophidiomyces ophiodiicola* in Virginia, USA. Journal of Wildlife Diseases 52:143-149.

Haislip, N.A., M.J. Gray, J.T. Hoverman, and D.L. Miller. 2011. Development and disease: how susceptibility to an emerging pathogen changes through anuran development. PLoS ONE6(7): e22307. doi:10.1371/journal.pone.0022307.t001.

Lips, K.R., F. Brem, R. Brenes, J.D. Reeve, R.A. Alford, J. Voyles, C. Carey, L. Livo, A.P. Pessier, and J.P. Collins. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proceedings of the National Academy of Sciences of the United States of America 103: 3165–3170.

Lorch, J. M., S. Knowles, J. S. Lankton, K. Michell, J. L. Edwards, J. M. Kapfer, R. A. Staffen, E. R. Wild, K. Z. Schmidt, A. E. Ballmann, D. Blodgett, T. M., Farrell, B. M. Glorioso, L. A. Last, S. J. Price, K. L. Schuler, C. E. Smith, J. F. X. Wellehan Jr., and D. S. Blehart. 2016. Snake fungal disease: an emerging threat to wild snakes. Phil. Trans. R. Soc. B 371:20150457.

North, A.C., D.J. Hodgson, S.J. Price, A.G.F. Griffith. 2015. Anthropogenic and ecological drivers of amphibian disease (Ranavirosis). PLoS ONE 10(6): e0127037.doi:10.1371/journal.pone.0127037.

Paré, J.A. & L. Sigler. 2016. An overview of reptile fungal pathogens in the genera *Nannizziopsis*, *Paranannizziopsis*, and *Ophidiomyces*. Journal of Herpetological Medicine and Surgery 26:46-53.

Robert, J. 2010. Emerging ranaviral infectious diseases and amphibian decline. Diversity 2:314-330.

Rosa, G.M., J. Sabino-Pinto, T.G. Laurentino, F. Pasman, R. Rebelo, R.A. Griffiths, A.C. Stöhr, R.E. Marschang, S.J. Price, T.W.J. Garner, and J. Bosch. 2017. Impact of asynchronous emergence of two lethal pathogens on amphibian assemblages. Scientific Reports 7:43260.doi: 10.1038/srep43260.

Skerratt, L.F., L. Berger, R. Speare, S. Cashins, K. R. McDonald, A.D. Phillott, H. B. Hines, and N. Kenyon. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. EcoHealth 4: 125–134.

Todd, B.D., J.D. Willson, and J.W. Gibbons. 2010. The global status of reptiles and causes of their decline. In: Sparling D.W., C.A. Bishop, and S. Krest (Eds). Ecotoxicology of Amphibians and Reptiles, Second Edition, pp47-67. CRC Press, Pensacola, FL, USA.

Tupper, T.A., R. Aguilar, A. Ferrara, and C. Hay. 2015. Field Notes: Snake fungal disease. Catesbeiana. 35(1).

Tupper, T.A., R. Aguilar, T. Sepahpur, C. Hay, and S. Ebrahimi. 2016. A survey of amphibians at the Smithsonian Environmental Research Center, Anne Arundel County, Maryland. Banisteria 46: 12–24.

CHAPTER II.

A SURVEY OF THE PATHOGENIC FUNGUS, *BATRACHOCHYTRIUM DENDROBATIDIS*, AT THE SMITHSONIAN ENVIRONMENTAL RESEARCH CENTER, ANNE ARUNDEL COUNTY, MARYLAND

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 2.1; 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 2.1). 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 2.2), 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.

Table 2.1. Number and proportion of *Bd* detections by species at the Smithsonian Environmental Research Center. Superscript numbers following species are ecological guilds. 1 = terrestrial/arboreal, and 2 = aquatic.

Species	**# Sampled**	***Bd* Detections**	**% Detected**
American Bullfrog[2]	6	1	0.17
American Toad[1]	21	1	0.05
Cope's Gray Treefrog[1]	14	1	0.07
Eastern Cricket Frog[1]	18	4	0.22
Eastern Spadefoot[1]	8	0	0
Gray Treefrog[1]	1	0	0
Green Frog[2]	19	2	0.11
Pickerel Frog[2]	8	2	0.25
Southern Leopard Frog[2]	4	1	0.25
Spring Peeper[1]	13	0	0
Wood Frog[2]	4	0	0

Table 2.2. 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

LITERATURE CITED

Augustine, L., and M. Neff. 2016. Determining the prevalence of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) and Ranavirus at Long Branch Nature Center in Arlington, Virginia. Catesbeiana 36: 35–42.

Beebee, T. J. C., and R. A. Griffiths. 2005. The amphibian decline crisis: A watershed for conservation biology? Biological Conservation 125: 271–281.

Berger, L., R. Speare, P. Daszak, D. E. Green, A. A. Cunningham, C. L. Goggin, R. Slocombe, M. A. Ragan, A. D. Hyatt, K. R. McDonald, H. B. Hines, K. R. Lips, G. Marantelli, and H. Parkes.1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proceedings of the National Academy of Sciences of the United States of America 95, 9031–9036.

Berger, L., R. Speare, H. B. Hines, G. Marantelli, A. D. Hyatt, K. R. McDonald, L. F. Skerratt, V. Olsen, J. M. Clarke, G. Gillespie, M. Mahony, N. Sheppard, C. Williams, and M. J. Tyler. 2004. Effect of season and temperature on mortality in amphibians due to chytridiomycosis. Australian Veterinary Journal 82: 31–36.

Berger, L., A. A. Roberts, J. Voyles, J. E. Longcore, K. A. Murray, L. F. Skerratt. 2016. History and recent progress on chytridiomycosis in amphibians. Fungal Ecology 19: 89–99.

Bielby, J., N. Cooper, A. A. Cunningham, T. W. J. Garner, and A. Purvis. 2008. Predicting susceptibility to future declines in the world's frogs. Conservation Letters 1: 82–90.

Blaustein A. R., B. A. Han, R. A. Relyea, P. T. Johnson, J. C. Buck, S. S. Gervasi, and L. B. Kats. 2011. The complexity of amphibian population declines: understanding the role of cofactors in driving amphibian losses. Annals of the New York Academy of Sciences 1223: 108–119. doi: 10.1111/j.1749–6632.2010.05909.x.

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

Briggs, C. J., A. R. Knapp, and V. T. Vredenburg. 2010. Enzootic and epizootic dynamics of the chtryid fungal pathogen of amphibians. Proceedings of the National Academy of Sciences of the United States of America 107: 9695–9700. http://doi.org/10.1073/pnas.0912886107

Brutyn, M., K. D'Herde, M. Dhaenens, P. Van Rooij, E. Verbrugghee, A. D. Hyatt, S. Croubels, D. Deforce, R. Ducatelle, F. Haesebrouck, A. Martel, and F. Pasmans. 2012. *Batrachochytrium dendrobatidis* zoospore secretions rapidly disturb intercellular junctions in frog skin. Fungal Genetics and Biology 49: 830–837.

Caruso, N. M., and K. R. Lips. 2013. Truly enigmatic declines in terrestrial salamander populations in Great Smoky Mountains National Park. Diversity and Distributions 19: 38–38.

Collins, J. P., and M. L. Crump. 2009. Extinction in our times: global amphibian declines. Oxford University Press, Oxford.

Crother, B. I. 2012. Scientific and standard English names of amphibians and reptiles of North America North of Mexico, pp. 1–92, SSAR Herpetological Circular 39.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2003. Infectious disease and amphibian population declines. Diversity and Distributions 9: 141–150.

Davidson, S. R. and D. L. Chambers. 2011. Occurrence of *Batrachochytrium dendrobatidis* in amphibians of Wise County, Virginia, USA. Herpetological Review 42: 214–216.

GBDMP (Global *Bd*-Mapping Project- *Bd*-Maps). 2016. http://www.bdmaps.net/surveillance/s_country.asp. (Accessed 3 June 2016).

Goodman, R.M., and Y.T Ararso. 2012. Survey of ranvirus and the fungus *Batrachochytrium denodrobatidis* in frogs of central Virginia, USA. Herpetological Review 43:78–80.

Grant, E. H. C., L. L. Bailey, J. L. Ware, and K. L. Duncan. 2008. Prevalence of the amphibian pathogen *Batrachochytrium dendrobatidis* in stream and wetland populations in Maryland, USA. Applied Herpetology 5: 233–241.

Harris, R. N., T. Y. James, A. Lauer, M. A. Simon, and A. Patel. 2006. Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. EcoHealth 3:53–56.

Hossack, B. R., M. J. Adams, E. H. C. Grant, C. A. Pearl, J. B. Bettaso, W. J. Barichivich, W. H. Lowe, K. True, J. L. Ware, and P. S. Corn. 2010. Low prevalence of chytrid fungus (*Batrachochytrium denodrobatidis*) in amphibians of U.S. headwater streams. Journal of Herpetology 44: 253–260.

Huang, R. and L. A. Wilson. 2013. *Batrachochytrium denodrobatidis* in amphibians of the Piedmont and Blue Ridge provinces in northern Georgia, USA. Herpetological Review 44: 95–98.

Hughey, M.C., M.H. Becker, J.B. Walke, M.C. Swartwout, and L. K. Belden. 2014. *Batrachochytrium denodrobatidis* in Virginia amphibians within and among site variation in infection. Herpetological Review 45:428–438.

Hyatt, A. D., D. G. Boyle, V. Olsen, D. B. Boyle, L. Berger, D. Obendorf, A. Dalton, K. Kriger, M. Hero, H. Hines, R. Phillott, R.Campbell, G. Marantelli, F. Gleason, and A. Colling. 2007. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms 73:175–192.

Kilpatrick, A. M., C. J. Briggs, and P. Daszak. 2010. The Ecology and impact of chytridiomycosis: an emerging disease of amphibians. Trends in Ecology and Evolution 25: 109–118.

Kriger, K. M., and J M. Hero. 2007. The chytrid fungus *Batrachochytrium dendrobatidis* is non-randomly distributed across amphibian breeding habitats. Divers Distrib. 13:781–788.

Lannoo, M. J., C. Petersen, R. E. Lovich, P. Nanjappa, C. Phillips, J. C. Mitchell, and I. Macallister. 2011. Do frogs get their kicks on Route 66? Continental U.S. Transect reveals spatial and temporal patterns of *Batrachochytrium dendrobatidis* infection. PLoS ONE 6: e22211. doi: 10.1371/journal.pone.0022211

Lips, K. R., J. D. Reeve, and L. R. Witters. 2003. Ecological traits predicting amphibian population declines in Central America. Conservation Biology 17:1078–1088.

Lips, K. R., F. Brem, R. Brenes, J. D. Reeve, R. A. Alford, J. Voyles, C. Carey, L. Livo, A.P. Pessier, and J. P. Collins. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proceedings of the National Academy of Sciences of the United States of America 103: 3165–3170.

Longcore, J. R., J. E. Longcore, A. P. Pessier, and W. A. Halteman. 2007. Chytridiomycosis widespread in anurans of northeastern United States. Journal of Wildlife Management 71: 435–444.

Muletz, C., N. M. Caruso, R. C. Fleischer, R. W. McDiarmid, and K. R. Lips. 2014. Unexpected rarity of the pathogen *Batrachochytrium dendrobatidis* in Appalachian *Plethodon* salamanders: 1957–2011. PLoS ONE 9(8): e103728. doi: 10.1371/journal.pone.0103728

Murray, K. A., D. Rosauer, H. McCallum, and L. F. Skerratt. 2011. Integrating species traits with extrinsic threats: closing the gap between predicting and preventing species declines. Proceedings of the Royal Society B: Biological Sciences 278: 1515–1523.

Muths, E., D. S. Pilliod, and L. J. Livo. 2008. Distribution and environmental limitations of an amphibian pathogen in the Rocky Mountains, USA. Biological Conservation 141: 1484–1492.

Olson, D. H., D. M. Aanensen, K. L. Ronnenberg, C. I. Powell, S. F. Walker, J. Bielby, T. W. J. Gerner, G. Weaver, The Bd Mapping Group, and M. C. Fisher. 2013. Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. PLoS ONE 8(2): e56802. doi: 10.1371/journal.pone.0056802.

Oullet, M., I. Mikaelian, B. D. Pauli, J. Rodriguez, and D. M. Green. 2005. Historical evidence of widespread chytrid infection in North American amphibian populations. Conservation Biology 19: 1431–1440.

Pearl, C. A., E. L. Bull, D. E. Green, J. Bowerman, M. J. Adams, A. Hyatt, and W. H. Wente. 2007. Occurrence of the amphibian pathogen *Batrachochytrium dendrobatidis* in the Pacific Northwest. Journal of Herpetology. 41:145–149.

Petersen, C. E., R. E. Lovich, C. A. Phillips, M. J. Dreslik, and M. J. Lannoo. 2016. Prevalence and seasonality of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* along widely separated longitudes across the United States. EcoHealth pp 1-15. DOI 10.1007/s10393-016-1101–4.

Pullen, K. D., A. M. Best, and J. L. Ware. 2010. Amphibian pathogen *Batrachochytrium dendrobatidis* prevalence is correlated with season and not urbanization in central Virginia. Diseases of Aquatic Organisms 91: 9–16.

Rodriguez, E. M., T. Gamble, M. V. Hirt, and S. Cotner. 2009. Presence of *Batrachochytrium dendrobatidis* at the headwaters of the Mississippi River, Itasca State Park, Minnesota, USA. Herpetological Review 40:48–50.

Rothermel, B. B., S. C. Walls, J. C. Mitchell, C. K. Dodd Jr., L. K. Irwin, D. E. Green, V. M. Vasquez, J. W. Petranka, and D. J. Stevenson. 2008. Widespread occurrence of the amphibian chytrid fungus *Batrachochytrium deondrobatidis* in the southeastern USA. Diseases of Aquatic Organisms 82: 3–18.

Rowley, J. J. and R. A. Alford. 2007. Behaviour of Australian ranforest stream frogs may affects the transmission of chytridiomycosis. Diseases of Aquatic Organisms 77: 1–9.

Savage, A. E., M. J. Sredl, and K. R. Zamudio. 2011. Disease dynamics vary spatially and temporally in a North American amphibian. Biological Conservation 144: 1910–1915.

Skerratt, L.F., L. Berger, R. Speare, S. Cashins, K. R. McDonald, A.D. Phillott, H. B. Hines, and N. Kenyon. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. EcoHealth 4: 125–134.

Talley, B. L., C. R. Muletz, V. T. Vredenburg, R. C. Fleischer, and K. R. Lips. 2015. A century of *Batrachochytrium dendrobatidis* in Illinois amphibians (1888–1989). Biological Conservation 182: 254–261.

Tupper, T.A. J.W. Streicher, S.E. Greenspan, B.C. Timm. 2011 and R.P Cook. Detection of *Batrachochytrium dendrobatidis* in anurans of Cape Cod National Seashore, Barnstable County, Massachusetts, USA. Herpetological Review 42: 62–65.

Tupper, T. A., R. Aguilar, T. Sepahpur, C. Hay, and S. Ebrahimi. 2016. A survey of amphibians at the Smithsonian Environmental Research Center, Anne Arundel County, Maryland. Banisteria 46: 12–24.

Tupper, T.A., L.D. Fuchs, C. Love, D. Lawlor and C.A. Bozarth. In progress. A survey of *Batrachochytrium dendrobatidis* at Huntley Meadows Park in Fairfax County, Virginia. Catesbieana 0:0–00.

VHS (Virginia Herpetological Society). 2016. Infectious diseases of our native herps and disinfection protocols. http://virginiaherpetologicalsociety.com/disease/index.htm. Accessed May 2016.

Woodhams, D. C., K. Ardipradja, R. A. Alford, G. Marantelli, L. K. Reinert, and L. A. Rollins-Smith. 2007. Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. Animal Conservation 10: 409–417.

Zar, J. H. 2010. Biostatistical Analysis. 5th Edition. Prentice Hall, New York, NY. 960 pp.

CHAPTER III.

DETECTION OF THE PATHOGENIC FUNGUS, BATRACHOCHYTRIUM DENDROBATIDIS, IN ANURANS OF HUNTLEY MEADOWS PARK, FAIRFAX COUNTY, VIRGINIA

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 (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. 3.1). 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, 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 3.1 and 3.2). Multiple logistic regression 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 3.3). Species and site were not indicated as significant. We identified significantly higher infection rates in adult male and female anurans ($\chi^2 = 7.6$; df = 2; $P < 0.05$; Table 3.4) of the aquatic ecological guild ($\chi 2 = 17.9$; df = 1; $P < 0.001$ *see* Table 3.1) earlier in the season ($\chi 2 = 25.0$; df = 3; $P < 0.001$; *see* Table 3.2). Of the 99 testable samples, 34 were positive, yielding an overall infection rate of 34.3%.

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 3.5) was higher than all other studies 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.

Figure 3.1. Huntley Meadows Park. Blue icons represent sampling locations. We swabbed multiple individuals at each sampling location. We only presented the location without a +/- symbol because we found both *Bd* positive and negative individuals at most sampling locations.

Table 3.1. 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 cinereal*	11	1	9
Southern Leopard Frog[2]	*Lithobates sphenocephalus*	23	18	78
Spring Peeper[1]	*Pseudacris crucifer*	13	1	8

Table 3.2. 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

Table 3.3. Multiple logistic regression output. Coef = coefficient

Predictor	Coef	*P*
Day of Year	-0.042	0.016
Sex (age)	1.359	0.002
Guild	2.629	0.002
Species	0.230	0.248
Site	0.133	0.168

Table 3.4. Number and proportion of *Bd* detections across sexes, and correspondingly, by age class.

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

Table 3.5. *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

LITERATURE CITED

Augustine, L., and M. Neff. 2016. Determining the prevalence of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) and Ranavirus at Long Branch Nature Center in Arlington, Virginia. Catesbeiana 36: 35–42.

Beebee, T. J. C., and R. A. Griffiths. 2005. The amphibian decline crisis: A watershed for conservation biology? Biological Conservation 125: 271–281.

Berger, L., R. Speare, P. Daszak, D. E. Green, A. A. Cunningham, C. L. Goggin, R. Slocombe, M. A. Ragan, A. D. Hyatt, K. R. McDonald, H. B. Hines, K. R. Lips, G. Marantelli, and H. Parkes. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proceedings of the National Academy of Sciences of the United States of America 95, 9031–9036.

Berger, L., R. Speare, H. B. Hines, G. Marantelli, A. D. Hyatt, K. R. McDonald, L. F. Skerratt, V. Olsen, J. M. Clarke, G. Gillespie, M. Mahony, N. Sheppard, C. Williams, and M. J. Tyler. 2004. Effect of season and temperature on mortality in amphibians due to chytridiomycosis. Australian Veterinary Journal 82: 31–36.

Berger, L., R. Speare, and L. F. Skerratt,. 2005. Distribution of Batrachochytrium dendrobatidis and pathology in the skin of green tree frogs *Litoria caerulea* with severe chytridiomycosis. Diseases of Aquatic Organisms 68, 65–70.

Bielby, J., N. Cooper, A. A. Cunningham, T. W. J. Garner, and A. Purvis. 2008. Predicting susceptibility to future declines in the world's frogs. Conservation Letters 1: 82–90.

Blaustein A. R., S. S. Gervasi, P. T. J. Johnson, J. T. Hoverman, L. K. Belden, et al. (2012) Ecophysiology meets conservation: understanding the role of disease in amphibian population declines. Philosophical Transactions of the Royal Society B: Biological Sciences 367: 1688–1707.

Bradley, G. A., P. C. Rosen, M. J. Srdl, T. R. Jones, and J. E. Longcore. 2002. Chytridiomycosis in native Arizona frogs. Journal of Wildlife Diseases 38:206–

212.

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

Briggs, C. J., A. R. Knapp, and V. T. Vredenburg. 2010. Enzootic and epizootic dynamics of the chtryid fungal pathogen of amphibians. Proceedings of the National Academy of Sciences of the United States of America 107: 9695–9700. http://doi.org/10.1073/pnas.0912886107.

Bruno, J. F., L. E. Petes, C. D. Harvell, and A. Hettinger. 2003. Nutrient enrichment can increase the severity of coral diseases. Ecology Letters 6:1056–1061.

Brutyn, M., K. D'Herde, M. Dhaenens, P. Van Rooij, E. Verbrugghee, A. D. Hyatt, S. Croubels,D. Deforce, R. Ducatelle, F. Haesebrouck, A. Martel, and F. Pasmans. 2012. *Batrachochytrium dendrobatidis* zoospore secretions rapidly disturb intercellular junctions in frog skin. Fungal Genetics and Biology 49: 830–837.

Carey, C., N. Cohen, and L. Rollins-Smith. 1999. Amphibian declines: an immunological perspective. Developmental and Comparative Immunology 23:459–472.

Chatfield, M. W., H. P. Moler, and C. L. Richards-Zawacki. 2012. The amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, in fully aquatic salamanders from southeastern North America. PLoS ONE 7:e44821.

Crother, B. I. 2012. Scientific and standard English names of amphibians and reptiles of North America North of Mexico, pp. 1–92, SSAR Herpetological Circular 39.

Daszak, P., L. Berger, A. A. Cunningham A. D. Hyatt, D. E. Green, and R. Speare. 1999. Emerging infectious diseases and amphibian population declines. Emerging Infectious Diseases 5:735-748.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2001. Anthropogenic environmental change and the emergence of infectious diseases in wildlife. Acta Tropica 78:103–116.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2003. Infectious disease and amphibian population declines. Diversity and Distributions 9: 141–150.

Davidson, E. W., M. Parris, J. P. Collins, J. E. Longcore, A. P. Pessier, and J. Brunner. 2003. Pathogenicity and trans- mission of chytridiomycosis in tiger salamanders (Amby- lostoma tigrinum). Copeia 2003:601-607.

Davidson, S. R. and D. L. Chambers. 2011. Occurrence of *Batrachochytrium dendrobatidis* in amphibians of Wise County, Virginia, USA. Herpetological Review 42: 214–216.

FOHMP (Friends of Huntley Meadows Park). 2017. Northern Snakehead Fish: Huntley Meadows Park. http://www.friendsofhuntleymeadows.org/snakehead%202011.html. Accessed May 2017.

Fuchs, L. F., T. A. Tupper, T. Sepapur, R. Aguilar, and C. A. Bozarth. 2017. A Survey of the Pathogenic Fungus, *Batrachochytrium dendrobatidis*, at the Smithsonian Environmental Research Center, Anne Arundel County, Maryland. Catesbeiana 37: 32-39.

Goodman, R.M., and Y.T Ararso. 2012. Survey of ranvirus and the fungus *Batrachochytrium denodrobatidis* in frogs of central Virginia, USA. Herpetological Review 43:78–80.

Grant, E. H. C., L. L. Bailey, J. L. Ware, and K. L. Duncan. 2008. Prevalence of the amphibian pathogen *Batrachochytrium dendrobatidis* in stream and wetland populations in Maryland, USA. Applied Herpetology 5: 233–241.

Gratwicke, B., M. Evans, E. H. C. Grant, J. Greathouse, W. J. McShea, N. Rotzel, and R. C. Fleischer. 2011. Low prevalence of Batrachochytrium dendrobatidis detected in Appalachian salamanders from Warren County, Virginia, USA. Herpetological Review 42:217-219.

Harris, R. N., T. Y. James, A. Lauer, M. A. Simon, and A. Patel. 2006. Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. EcoHealth 3:53–56.

Hoverman, J. T., M. J. Gray, N. A. Haislip, and D. L. Miller. 2011. Phylogeny, life history, and ecology contribute to differences in amphibian susceptibility to ranaviruses. EcoHealth 8:301-319.

Hughey, M.C., M.H. Becker, J.B. Walke, M.C. Swartwout, and L. K. Belden. 2014. *Batrachochytrium denodrobatidis* in Virginia amphibians within and among site variation in infection. Herpetological Review 45:428–438.

Kilpatrick, A. M., C. J. Briggs, and P. Daszak. 2010. The ecology and impact of chytridiomycosis: an emerging disease of amphibians. Trends in Ecology and Evolution 25:109–118.

Kinney VC, J. L. Heemeyer, A. P. Pessier, and M. J. Lannoo. 2011. Seasonal pattern of

Batrachochytrium dendrobatidis infection and mortality in *Lithobates areolatus*: Affirmation of Vredenburg's ''10,000 zoospore rule''. PLoS ONE 6(3):e16708. doi:10.1371/ journal.pone.0016708.

Kriger, K. M., and J M. Hero. 2007. The chytrid fungus *Batrachochytrium dendrobatidis* is non-randomly distributed across amphibian breeding habitats. Divers Distrib. 13:781–788.

Lambertini, C., C. G. Becker, T. S. Jenkinson, D. Rodriguez, D. da Silva Leite, T. Y. James, K. R. Zamudio, and L. F. Toledo. 2016. Local phenotypic variation in amphibian-killing fungus predicts infection dynamics. Fungal Ecology 20: 15-21.

Lamirande, E. W., and D. K. Nichols. 2002. Effects of host age on susceptibility to cutaneous chytridiomycosis in blue-and- yellow poison dart frogs (*Dendrobates tinctorius*). In: Proceedings of the Sixth International Symposium on the Pathology of Reptiles and Amphibians, St. Paul, MN, USA.

Lannoo, M. J., C. Petersen, R. E. Lovich, P. Nanjappa, C. Phillips, J. C. Mitchell, and I. Macallister. 2011. Do frogs get their kicks on Route 66? Continental U.S. Transect reveals spatial and temporal patterns of *Batrachochytrium dendrobatidis* infection. PLoS ONE 6: e22211. doi: 10.1371/journal.pone.0022211.

Lips, K. R., J. D. Reeve, and L. R. Witters. 2003. Ecological traits predicting amphibian population declines in Central America. Conservation Biology 17:1078–1088.

Lips, K. R., F. Brem, R. Brenes, J. D. Reeve, R. A. Alford, J. Voyles, C. Carey, L. Livo, A.P. Pessier, and J. P. Collins. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proceedings of the National Academy of Sciences of the United States of America 103: 3165–3170.

Longcore, J. R., J. E. Longcore, A. P. Pessier, and W. A. Halteman. 2007. Chytridiomycosis widespread in anurans of northeastern United States. Journal of Wildlife Management 71: 435–444.

Moffitt, D., L. A. Williams, A. Hastings, M. W. Pugh, M. M. Gangloff, and L. Siefferman. 2015. Low prevalence of the amphibian pathogen *Batrachochytrium dendrobatidis* in the southern Appalachian Mountains. Herpetological Conservation and Biology 10:123-136.

Murray, K. A., D. Rosauer, H. McCallum, and L. F. Skerratt. 2011. Integrating species traits with extrinsic threats: closing the gap between predicting and preventing species declines. Proceedings of the Royal Society B: Biological Sciences 278:

1515–1523.

Muths, E., P. S. Corn, A. P. Pessier, and D. E. Green. 2003. Evidence for disease-related amphibian decline in Colorado. Biological Conservation 110: 357–365.

Muths, E., D. S. Pilliod, and L. J. Livo. 2008. Distribution and environmental limitations of an amphibian pathogen in the Rocky Mountains, USA. Biological Conservation 141: 1484–1492.

Olson, D. H., D. M. Aanensen, K. L. Ronnenberg, C. I. Powell, S. F. Walker, J. Bielby, T. W. J. Gerner, G. Weaver, The Bd Mapping Group, and M. C. Fisher. 2013. Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. PLoS ONE 8(2): e56802. doi: 10.1371/journal.pone.0056802.

Oullet, M., I. Mikaelian, B. D. Pauli, J. Rodriguez, and D. M. Green. 2005. Historical evidence of widespread chytrid infection in North American amphibian populations. Conservation Biology 19: 1431–1440.

Petersen, C. E., R. E. Lovich, C. A. Phillips, M. J. Dreslik, and M. J. Lannoo. 2016. Prevalence and seasonality of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* along widely separated longitudes across the United States. EcoHealth pp 1-15. DOI 10.1007/ s10393-016-1101–4.

Piotrowski, J. S., S. L. Annis, and J. E. Longcore. 2004. Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. Mycologia 96:9–15.

Pounds, J. A., M. R. Bustamante, L. A. Coloma, J. A. Consuegra, M. P. L. Fogden, P. N. Foster, E. La Marca, K. L. Masters, A. Merino-Viteri, R. Puschendorf, S. R. Ron, G. A. Sanchez-Azofeifa, C. J. Still, and B. E. Young. 2006. Widespread amphibian extinctions from epidemic disease driven by global warming. Nature 439:161–167.

Pullen, K. D., A. M. Best, and J. L. Ware. 2010. Amphibian pathogen *Batrachochytrium dendrobatidis* prevalence is correlated with season and not urbanization in central Virginia. Diseases of Aquatic Organisms 91: 9–16.

Rachowicz LJ, et al. (2006) Emerging infectious disease as a proximate cause of amphibian mass mortality. Ecology 87:1671–1683.

Rohr J. R., A. M. Schotthoefer, T. R. Raffel, H. J. Carrick, N. Halstead, J. T. Hoverman JT, et al. 2008. Agrochemicals increase trematode infections in a declining amphibian species. Nature 455:U1235– U1250.

Rothermel, B. B., S. C. Walls, J. C. Mitchell, C. K. Dodd Jr., L. K. Irwin, D. E. Green, V. M. Vasquez, J. W. Petranka, and D. J. Stevenson. 2008. Widespread occurrence of

the amphibian chytrid fungus *Batrachochytrium deondrobatidis* in the southeastern USA. Diseases of Aquatic Organisms 82: 3–18.

Rowley, J. J. and R. A. Alford. 2007. Behaviour of Australian ranforest stream frogs may affects the transmission of chytridiomycosis. Diseases of Aquatic Organisms 77: 1–9.

Savage, A. E., M. J. Sredl, and K. R. Zamudio. 2011. Disease dynamics vary spatially and temporally in a North American amphibians. Biological Conservation 144: 1910–1915.

VDGIF (Virginia Department of Game and Inland Fisheries). 2017. CMN04 Huntley Meadows Park. https://www.dgif.virginia.gov/vbwt/sites/huntley-meadows-park/. Accessed May 2017.

VHS (Virginia Herpetological Society). 2016. Infectious diseases of our native herps and disinfection protocols. http://virginiaherpetologicalsociety.com/disease/index.htm. Accessed May 2017.

Voyles J, et al. (2009) Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. Science 326:582–585.

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

Woodhams, D. C., R. A. Alford, and G. Marantelli. 2003. Emerging disease of amphibians cured by elevated body temperature. Diseases of Aquatic Organisms 55:65–67.

Woodhams, D. C., K. Ardipradja, R. A. Alford, G. Marantelli, L. K. Reinert, and L. A. Rollins- Smith. 2007. Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. Animal Conservation 10: 409–417.

Wright, A. H., and A. A. Wright. 1949. Handbook of Frogs and Toads of the United States and Canada. Third Edition. Ithaca, New York, U.S.A.: Comstock Publishing Associates.

Zar, J.H. 2009. Biostatistical Analysis. 5 edition. Pearson, Upper Saddle River, N.J. 960 pp.

CHAPTER IV.

AN INVESTIGATION OF CO-INFECTION BY *BATRACHOCHYTRIUM DENDROBATIDIS* AND *RANAVIRUS* (FV3) IN ANURANS OF TWO NATURAL AREAS IN ANNE ARUNDEL COUNTY, MARYLAND AND FAIRFAX COUNTY, VIRGINIA, USA

INTRODUCTION

Since the 1980s, amphibian populations have experienced global population declines and extinctions (Skerratt et al., 2007; Robert, 2010). Enigmatic events, including the emergence and spread of infectious diseases, are associated with many of these recent declines (Daszak et al., 2003; Stuart et al., 2004; Bielby et al., 2008; Olson et al., 2013). Globally-occurring mass mortality events of amphibians have been associated with two pathogens in particular: the chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), and ranaviruses (specifically *Ranavirus* type species Frog Virus 3 [hereafter FV3]; Chinchar, 2002; Fox et al., 2006; Lips et al., 2006; Haislip et al., 2011; Echaubard et al., 2016). Both *Bd* and the ranaviruses are listed as notifiable pathogens by the World Organization for Animal Health (OIE, 2008; Echaubard et al., 2016).

Bd and ranaviruses are both known to occur globally, and across broad geographic and host ranges (Schock et al., 2010; Bancroft et al., 2011; Miller et al., 2011). Data indicate that *Bd* is widespread and often highly prevalent in the mid-Atlantic United States and that it often occurs without concomitant population declines (Longcore et al., 2007;

Grant et al., 2008; Rothermel et al., 2008; Pullen et al., 2010; Lannoo et al., 2011; Petersen et al., 2016; Fuchs et al., 2017; Tupper et al., 2017). Conversely, data on FV3 in mid-Atlantic amphibians are limited. However, FV3 infections in this region have been documented, and have also been associated with localized mortality events and declines (Petranka et al., 2003; Harp and Petranka, 2006; Schock et al., 2009; Davidson & Chambers, 2011; Hoverman et al., 2012; Fairfax County Park Authority, 2015; Duffus et al., 2015). Although deficiencies in FV3 data may be due to sparse sampling, it may also reflect the disease's biology. FV3 is highly virulent and can cause > 90% mortality in some cases (Green et al., 2002). This virulence can make sub-lethal infections difficult to detect, as deceased frogs may be less conspicuous and/or available for sampling (Harp and Petranka, 2006).

Bd and *Ranavirus* FV3 have been found to co-occur and co-infect individual hosts in various habitats (Whitfield et al., 2013; Warne et al., 2016; Rosa et al., 2017). Throughout North America, co-occurrence of *Bd* and FV3 has been reported in a number of aquatic communities. However, concurrent infections within an individual host has not yet been demonstrated *in situ* (Fox et al., 2006; Hoverman et al., 2012; Souza et al., 2012; Whitfield et al., 2013). Although data on concurrent infection are limited, it is suspected that the pathogens interact synergistically, promoting greater infection intensity and disease progression within the host due to the initial immunocompromising-effects from the primary invading pathogen (Garyfer et al., 2012; Warne et al., 2016). The objective of our study was to determine whether infection by these pathogens (both *Bd* and FV3) was occurring concurrently in anurans at two natural areas in Virginia and Maryland where *Bd*

is known to occur (*see* Fuchs et al., 2017; Tupper et al., 2017). We also aimed to provide baseline data on rates of FV3 infection across anuran species of the mid-Atlantic that could be incorporated into a growing body of literature on FV3 infection.

METHODS

We collected samples at the Smithsonian Environmental Research Center (hereafter SERC; 38°53'17.41"N 76°33'15.52 W) in Anne Arundel County, MD (for more about SERC, *see* Tupper et al., 2016), and at Huntley Meadows Park (hereafter HMP; 38°45'36.57" N - 77°05'44.13" W) in Fairfax County, Virginia (for more about HMP, *see* Tupper et al., 2017) between 13 March and 25 September 2016. Following Virginia Herpetological Society biosecurity protocols (VHS, 2016), we opportunistically hand-captured and sampled anurans at various locations throughout each study site. To sample for FV3, we chose a minimally-invasive method that has previously been used to detect the virus (Driskell et al., 2009; Gray et al., 2009; Pessier and Mendelson, 2010; Miller et al., 2015). This method consisted of collecting epithelial cells of the oropharyngeal region by circling the swab along the tongue, roof and sides of the mouth, and the pharynx (San Diego Zoo ICR, 2016). All swabs were stored in 1.5 mL microcentrifuge tubes and kept frozen until molecular analyses could be performed. To sample for *Bd*, we swabbed several skin surfaces (following methods of Hyatt et al., 2007) using a sterile dry swab (no. MW113, Medical Wire and Equipment Company, Durham, NC).

DNA was eluted from each swab using the Purification of Total DNA from Animal Tissues protocol (Qiagen®, Valencia, CA). To assay for FV3, we prepared a PCR master mix which contained 10µL SSo Advanced™ universal probes supermix (Bio-Rad, Hercules, CA), 2µL eluted DNA, 0.6 µL forward primer, 1.8µL reverse primer (Mao et al., 1996), 0.5 µL MGB probe, and 5.1µL sterile water, for a 20µL reaction total (Brunner and Collins, 2009). We included DNA elusion and amplification positive and negative controls with each PCR run. We amplified the DNA using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). We ran samples at 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds, 54°C for 30 seconds, and 72°C for 15 seconds. We performed at least two rounds of PCR on each sample; samples with inconsistent results were run through a third cycle. We considered any sample that fluoresced prior to the 40th cycle of the PCR reaction on at least two occasions to be positive. The *Bd* PCR techniques followed methods described by Boyle et al. (2004). For a more detailed description of these methods, *see* Fuchs et al. (2017) and Tupper et al. (2017).

To determine the total rates of infection across both sites, we calculated the proportion of *Bd* and FV3 positive samples for five grouping variables: anuran species, sampling month, ecological guild, and sex and age classes (*see* Tupper et al., 2017). Statistical analyses were completed in Minitab version 18 (www.minitab.com).

RESULTS

We collected samples from a total of 100 anurans at HMP, and 88 anurans at SERC between 13 March and 25 September 2016. We sampled for FV3 in 170 anurans; all samples tested negative for the virus. Of the 186 testable *Bd* samples, 59 tested positive for an overall infection rate of 31.7% (Table 4.1). We found that the highest *Bd* infection rates occur in March and April (59.3% and 59.1%, respectively; Table 4.2), in male adults (43.7%; Table 4.3), and in Southern Leopard (*Lithobates sphenocephalus*; 66.7%) and Pickerel (*Lithobates palustris*; 63.6%; Table 4.1) Frogs. Anurans sampled in the aquatic guild resulted in a higher percent of *Bd* positive individuals (40.3%) than in the terrestrial/arboreal guild (17.9%; Table 4.3).

DISCUSSION

Ranavirus infections, including FV3, have been documented in Maryland and Virginia (Davidson and Chambers, 2011; Hamed et al., 2013; Scott et al., 2016), however, FV3 was not detected in any of our samples. Our results reflect one of two possibilities, either FV3 was not present at either location, or the pathogen was present, but we were unsuccessful in detecting it due to our sampling techniques. In order to limit stress to the animal, we chose the least intrusive sampling method shown to detect FV3, (oropharyngeal swabbing; *see* Driskell et al., 2009; Pessier and Mendelson, 2010; Miller et al., 2015; San Diego Zoo ICR, 2016). Despite being less invasive, swabbing is also less reliable than lethal techniques, such as liver tissue sampling, and may produce more false negatives than other non-lethal techniques, such as toe and tail clips (Miller et al., 2008; Gray et al., 2012;

Forzán et al., 2017). Therefore, it is possible that even if an anuran was carrying the virus, our sampling method may not have been sensitive enough to detect it.

Though *Ranavirus* has low host specificity, larvae and metamorphs of certain species show far greater susceptibility to infection than others (Daszak et al., 1999; Brunner et al., 2004; Robert et al., 2005; Robert, 2010; Lesbarréres et al., 2012; North et al., 2015). For instance, larval and recently metamorphosed Wood Frogs (*Lithobates sylvaticus*), Gopher Frogs (*Lithobates capito*) and Eastern Spadefoot Toads (*Scaphiopus holbrookii*) are among the most susceptible to FV3 (Goodman and Araraso, 2009; Haislip et al., 2011; Hoverman et al., 2011; Miller et al., 2011; Lesbarréres et al., 2012; Earl and Gray, 2014; Forzán et al., 2017). Because anurans in our study were captured opportunistically, we were unable to adequately sample the most susceptible species. While oropharyngeal swabbing can effectively detect *Ranavirus* in tadpoles (Gray et al., 2012; Kolby et al., 2015), this method would likely damage oropharyngeal tissues in smaller anurans. Therefore, we chose to restrict our sampling to metamorphosed anurans.

Although we did not detect FV3 at either of our sampling sites, *Ranavirus* has been documented in both Virginia and Maryland (Davidson and Chambers, 2011; Hamed et al., 2013; Smith et al., 2016). Additionally, FV3 has been confirmed as the likely cause of a recent Wood Frog tadpoles die-off at the nearby Old Colchester Park and Preserve in Fairfax County, VA (Fairfax County Park Authority, 2015). We therefore suggest continued FV3 monitoring at HMP and SERC and recommend that future studies focus sampling efforts on larval Wood Frog and Eastern Spadefoot Toad (while minimizing harm to anurans). We also suggest continued monitoring for *Bd*, which is already known to be

prevalent at both locations (Fuchs et al., 2017; Tupper et al., 2017). Our study confirms that *Bd* remains prevalent at both SERC and HMP, with an overall infection rate that is among the highest in the region (31.72%; *see also* Hughey et al., 2014). Notably, a post-hoc analysis of data collected over a three-year period (2014-2016) revealed a nearly-20% increase in the infection rate of *Bd* at SERC (10.3%-29.2%; Tupper and Fuchs, unpublished data; Fuchs et al., 2017). Continued monitoring of both pathogens will facilitate more informed management decisions, and will allows us to better understand the effects of their interactions within anuran hosts.

Table 4.1. Proportion of *Bd* and *Ranavirus* FV3 positive samples by species. AMTO = Eastern American Toad (*Anaxyrus americanus*); BUFR = American Bull Frog (*Lithobates catesbeianus*); CGTF = Cope's Gray Tree Frog (*Hyla chrysoscelis*); CRFR=Eastern Cricket Frog (*Acris crepitans*); GRFR = Green Frog (*Lithobates clamitans*); GTFR = Green Tree Frog (*Hyla 46inereal*); PIFR = Pickerel Frog (*Lithobates palustris*); SLFR = Southern Leopard Frog (*Lithobates sphenocephalus*); SPPE = Spring Peeper (*Pseudacris crucifer*); WOFR = Wood Frog (*Lithobates sylvaticus*). * = Aquatic guild. Species without an asterisk indicates terrestrial/arboreal guild.

Species	**N (FV3)**	**% FV3 Positive**	**N (*Bd*)**	**% *Bd* Positive**
AMTO	26	0	26	26.9
BUFR*	26	0	25	24
CRFR*	13	0	17	5.9
CGTF	12	0	12	16.7
GRFR*	34	0	33	36.4
GTFR	12	0	12	8.3
PIFR*	10	0	11	63.6
SLFR*	31	0	33	66.7
SPPE	5	0	16	6.3
WOFR*	1	0	1	0
Total	170	0	186	31.7

Table 4.2. Proportion of FV3 and *Bd* positive individuals grouped by sampling month.

Month	**N (FV3)**	**% FV3 Positive**	**N (*Bd*)**	**% *Bd* Positive**
MARCH	22	0	27	59.3
APRIL	22	0	22	59.1
MAY	75	0	74	27.0
JUNE	12	0	24	12.5
JULY	5	0	5	20.0
AUG	5	0	5	0.0
SEPT	29	0	29	24.1

Table 4.3. Proportion of FV3 and *Bd* positive individuals grouped by sex (and by default, age class) and ecological guild.

Sex/Guild	N (FV3)	% FV3 Positive	N (*Bd*)	% *Bd* Positive
Female	66	0	64	26.6
Juvenile	34	0	51	23.5
Male	70	0	71	43.7
Aquatic	114	0	119	40.3
Terrestrial/arboreal	56	0	67	17.9

LITERATURE CITED

Bancroft, B. A., B. A. Han, C. L. Searle, L. M. Biga, D. H. Olson, L. B. Kats, J. J. Lawler, and A. R. Blaustein. 2011. Species-level correlates of susceptibility to the pathogenic amphibian fungus *Batrachochytrium dendrobatidis* in the Unites States. Biodiversity and Conservation 20: 1911-1920.

Bielby, J., N. Cooper, A. A. Cunningham, T. W. J. Garner, and A. Purvis. 2008. Predicting susceptibility to future declines in the world's frogs. Conservation Letters 1: 82–90.

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

Brunner, J. L., D. M. Schock, E. W. Davidson, and J. P. Collins. 2004. Intraspecific reservoirs: complex life history and the persistence of a lethal ranavirus. Ecology 85:560–566.

Chinchar, V. G. 2002. Ranaviruses (family *Iridoviridae*): emerging cold-blooded killers. Archives of Virology 147: 447-470.

Daszak, P., L. Berger, A. A. Cunningham A. D. Hyatt, D. E. Green, and R. Speare. 1999. Emerging infectious diseases and amphibian population declines. Emerging Infectious Diseases 5:735-748.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2003. Infectious disease and amphibian population declines. Diversity and Distributions 9: 141–150.

Davidson, S. R. A. and D. L. Chambers. 2011. Ranavirus prevalence in amphibian populations of Wise County, Virginia, USA. Herpetological Review 42: 214–215.

Driskell E. A., D. L. Miller, S. L. Swist, and Z. Z. Gyimesi. (2009) PCR detection of ranavirus in adult anurans from the Louisville Zoological Gardens. Journal of Zoo and Wildlife Medicine 40:559–563.

Duffus ALJ, Waltzek TB, Stöhr AC, Allender MC, Gotesman M, Whittington RJ, Hick P, Hines MK, Marschang RE. 2015. Distribution and host range of ranaviruses, p. 9–57. In Gray MJ, Chinchar VG (ed), *Ranaviruses*: lethal pathogens of ectothermic vertebrates. Springer, New York. Doi:10.1007/978-3-319-13755-1_2.

Earl, J. E. and M. J. Gray. 2014. Introduction of ranavirus to isolated wood frog populations could cause local extinction. EcoHealth 11: 581-592.

Echaubard, P., B. D. Pauli, V. L. Trudeau, and D. Lesbarrères. 2016. Ranavirus infection in northern leopard frogs: the timing and number of exposures matter. Journal of Zoology 298: 30-36.

Fairfax County Park Authority. 2015. Ranavirus, amphibians, and how to keep them apart. https://ourstoriesandperspectives.com/2015/09/14/ranavirus-amphibians-and-how-to-keep-them-apart/ (Accessed March 2018).

Forzán, M. J., K. M. Jones, E. Ariel, R. J. Whittington, J. Wood, J. J. F. Markham, and P. Y. Daoust. 2017. Pathogenesis of Frog Virus 3 (*Ranavirus*, *Iridoviridae*) infection in wood frogs (*Rana sylvatica*). Veterinary Pathology 54: 531-548.

Fox, S. F., A. L. Greer, R. Torres-Cervantes, and J. P. Collins. 2006. First case of ranavirus associated morbidity and mortality in natural populations of a South American frog, *Atelognathus patagonicus*. Diseases of Aquatic Organisms 72: 87–92. PloS ONE 6(7): e22307.

Fuchs, L. F., T. A. Tupper, T. Sepapur, R. Aguilar, and C. A. Bozarth. 2017. A Survey of the Pathogenic Fungus, *Batrachochytrium dendrobatidis*, at the Smithsonian Environmental Research Center, Anne Arundel County, Maryland. Catesbeiana 37: 32-39.

Grant, E. H. C., L. L. Bailey, J. L. Ware, and K. L. Duncan. 2008. Prevalence of the amphibian pathogen *Batrachochytrium dendrobatidis* in stream and wetland populations in Maryland, USA. Applied Herpetology 5: 233–241.

Grayfer, L., F. D. J. Andino, G. Chen, G. V. Chinchar, and J. Robert. 2012. Immune evasion strategies of ranaviruses and innate immune responses to these emerging pathogens. Viruses 4: 1075-1092.

Gray M. J., D. L. Miller, J. T. Hoverman. 2009. Ecology and pathology of amphibian ranaviruses. Diseases of Aquatic Organisms 87: 243–266.

Gray, M. J., D. M. Miller, and J. T. Hoverman. 2012. Reliability of non-lethal surveillance methods for detecting ranavirus infection. Diseases of Aquatic Organisms 99: 1-6.

Green D. E., K. A. Converse, and A. K. Schrader. 2002. Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996-2001. Annals of the New York Academy of Sciences 969: 323–339.

Haislip, N. A., M. J. Gray, J. T. Hoverman, and D. L. Miller. 2011. Development and disease: how susceptibility to an emerging pathogen changes through anuran development. PloS ONE6(7): e22307. Doi:10.1371/journal.pone.0022307.t001.

Hamed, M. K., M. J. Gray, and D. L. Miller DL. 2013. First report of ranavirus in plethodontid salamanders from the Mount Rogers National Recreation Area, Virginia, USA. Herpetological Review 44: 455–457.

Harp, E. M. and J. W. Petranka. 2006. Ranavirus in wood frogs (*Rana sylvatica*): potential sources of transmission within and between ponds. Journal of Wildlife Diseases 42: 307-318.

Hoverman, J. T., M. J. Gray, N. A. Haislip, and D. L. Miller. 2011. Phylogeny, life history, and ecology contribute to differences in amphibian susceptibility to ranaviruses. EcoHealth 8: 301-319.

Hoverman, J. T., M. J. Gray, D. L. Miller, and N. A. Haislip. 2012. Widespread occurrence of ranavirus in pond-breeding amphibian populations. EcoHealth 9: 36-48.

Hyatt, A. D., D. G. Boyle, V. Olsen, D. B. Boyle, L. Berger, D. Obendorf, A. Dalton, K. Kriger, M. Hero, H. Hines, R. Phillott, R.Campbell, G. Marantelli, F. Gleason, and A. Colling. 2007. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms 73:175–192.

Kolby, J. E., K. M. Smith, S. D. Ramirez, F. Rabemananjara, A. P. Pessier, J. L. Brunner, C. S. Goldberd, L. Berger, L. F. Skerratt. 2015. Rapid response to evaluate the presence of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) and ranavirus in wild amphibian populations in Madagascar. PloS ONE10(6): e0125330.doi:10.1371/journal.pone.0125330.

Lannoo, M. J., C. Petersen, R. E. Lovich, P. Nanjappa, C. Phillips, J. C. Mitchell, and I. Macallister. 2011. Do frogs get their kicks on Route 66? Continental U.S. Transect reveals spatial and temporal patterns of *Batrachochytrium dendrobatidis* infection. PloS ONE 6: e22211. Doi: 10.1371/journal.pone.0022211.

Lesbarréres, D., A. Blaseiro, J. Brunner, V. G. Chinchar, A. Duffus, J. Kerby, D. L. Miller, J. Robert, D. M. Schock, T. Waltzek, and M. J. Gray. 2012. Ranavirus: past, present and future. Biology Letters 8: 481-483.

Lips, K. R., F. Brem, R. Brenes, J. D. Reeve, R. A. Alford, J. Voyles, C. Carey, L. Livo, A. P. Pessier, and J. P. Collins. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proceedings of the National Academy of Sciences of the United States of America 103: 3165–3170.

Longcore, J. R., J. E. Longcore, A. P. Pessier, and W. A. Halteman. 2007. Chytridiomycosis widespread in anurans of northeastern United States. Journal of Wildlife Management 71: 435–444.

Mao, J., T. N. Tham, G. A. Gentry, A. Aubertin, and V. G. Chinchar. 1996. Cloning, sequence analysis, and expression of the major capsid protein of the Iridovirus Frog Virus 3. Virology 216: 431-436.

Miller D. L., S. Rajeev, M. Brookins, J. Cook, L. Whittington, and C. A. Baldwin. 2008. Concurrent infection with Ranavirus, *Batrachochytrium dendrobatidis*, and Aeromonas in a captive amphibian colony. Journal of Zoo and Wildlife Medicine 39: 445–449.

Miller, D., M. Gray, and A. Storfer. 2011. Ecopathology of ranaviruses infecting amphibians. Viruses 3: 2351-2373.

Miller D. L., A. P. Pessier, P. Hick, and R. J. Whittington. 2015. Comparative pathology of ranaviruses and diagnostic techniques. In: Gray MJ, Chinchar VG (eds) Ranaviruses: lethal pathogens of ectothermic vertebrates. Springer, New York.

North, A. C., D. J. Hodgson, S. J. Price, A. G. F. Griffith. 2015. Anthropogenic and ecological drivers of amphibian disease (Ranavirosis). PloS ONE 10(6): e0127037.doi:10.1371/journal.pone.0127037.

OIE (2008) OIE Aquatic Animal Health Code, Chapter 2.4.2. OIE, Paris. www.oie.int/eng/51iner/fcode/en_chapitre_ 2.4.2.htm. Accessed November 2018.

Olson, D. H., D. M. Aanensen, K. L. Ronnenberg, C. I. Powell, S. F. Walker, J. Bielby, T. W. J. Gerner, G. Weaver, The *Bd* Mapping Group, and M. C. Fisher. 2013. Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. PloS ONE 8(2): e56802. Doi: 10.1371/journal.pone.0056802.

Pessier A. P. and J. R. Mendelson. 2010. A manual for control of infectious diseases in amphibian survival assurance colonies and reintroduction programs. IUCN/SSC Conservation Breeding Specialist Group, Apple Valley, MN.

Petersen, C. E., R. E. Lovich, C. A. Phillips, M. J. Dreslik, and M. J. Lannoo. 2016. Prevalence and seasonality of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* along widely separated longitudes across the United States. EcoHealth pp 1-15. DOI 10.1007/ s10393-016-1101–4.

Petranka, J. W., S. S. Murray, and C. A. Kennedy. 2003. Responses of amphibians to restoration of a southern Appalachian wetland: perturbations confound post-restoration assessment. Wetlands 23: 278–290.

Pullen, K. D., A. M. Best, and J. L. Ware. 2010. Amphibian pathogen *Batrachochytrium dendrobatidis* prevalence is correlated with season and not urbanization in central Virginia. Diseases of Aquatic Organisms 91: 9–16.

Robert, K., H. Morales, W. Buck, N. Cohen, S. Marr, and J. Gantress. 2005. Adaptive immunity and histopathology in frog virus 3-infected Xenopus. Virology 332: 667–675.

Robert, J. 2010. Emerging ranaviral infectious diseases and amphibian decline. Diversity 2:314-330.

Rosa, G. M., J. Sabino-Pinto, T. G. Laurentino, F. Pasman, R. Rebelo, R. A. Griffiths, A. C. Stöhr, R. E. Marschang, S. J. Price, T. W. J. Garner, and J. Bosch. 2017. Impact of asynchronous emergence of two lethal pathogens on amphibian assemblages. Scientific Reports 7:43260.doi: 10.1038/srep43260.

Rothermel, B. B., S. C. Walls, J. C. Mitchell, C. K. Dodd Jr., L. K. Irwin, D. E. Green, V. M. Vasquez, J. W. Petranka, and D. J. Stevenson. 2008. Widespread occurrence of the amphibian chytrid fungus *Batrachochytrium deondrobatidis* in the southeastern USA. Diseases of Aquatic Organisms 82: 3–18.

San Diego Zoo Institute for Conservation Research. 2016. Sampling protocol for ranaviruses. http://admininstitute.sandiegozoo.org/sites/default/files/Ranavirus%20Guidelines%202016.pdf. Accessed February 2018.

Schock, D. M., T. K. Bollinger, and J. P. Collins. 2009. Mortality rates differ among amphibian populations exposed to three strains of a lethal ranavirus. EcoHealth 6: 438-448.

Schock, D. M., G. R. Ruthig, J. P. Collins, S. J. Kutz, S. Carriere, R. J. Gau, A. M. Veitch, N. C. Larter, D. P. Tate, G. Guthrie, D. G. Allaire, and R. A. Popko. 2010. Amphibian chytrid fungus and ranaviruses in the northwest territories, Canada. Diseases of Aquatic Organisms 92: 231-240.

Skerratt, L. F., L. Berger, R. Speare, S. Cashins, K. R. McDonald, A.D. Phillott, H. B. Hines, and N. Kenyon. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. EcoHealth 4: 125–134.

Smith, S. A., K. J. Monsen-Collar, D. E. Green, H. S. Niederriter, M. L. Hall, K. A. Terrell, K. D. Gipe, C. A. Urban, C. A. Patterson, R. A. Siegel, B. Zarate, J. D. Kleopfer, E. H. Campbell-Grant, and C. P. Driscoll. 2016. Detecting the extent of mortality events from *Ranavirus* in amphibians of the northeastern U.S. Report to the Northeast Association of Fish and Wildlife Agencies for Regional Conservation Needs.

Souza, M. J., M. J. Gray, P. Colclough, and D. L. Miller. 2012. Prevalence of infection by *Batrachochytrium dendrobatidis* and *Ranavirus* in eastern Hellbenders

(*Cryptobranchus alleganeiensis alleganeiensis*). Journal of Wildlife Diseases 48:560–566.

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

Tupper, T. A., L. D. Fuchs, C. O'Connor-Love, R. Aguilar, C. Bozarth, and D. Fernandez. 2017. Detection of the pathogenic fungus, *Batrachochytrium dendrobatidis*, in anurans of Huntley Meadows Park, Fairfax County, Virginia. Catesbeiana 37: 109-120.

VHS (Virginia Herpetological Society). 2016. Infectious diseases of our native herps and disinfection protocols. http://virginiaherpetologicalsociety.com/disease/index.htm. Accessed May 2016.

Warne, R. W., B. LaBumbard, S. Lagrange, V. T. Vredenburg, and A. Catenazzi. 2016. Co-infection by chytrid fungus and ranaviruses in wild and harvested frogs in the tropical Andes. PloS ONE 11(1): e0145864.doi:10.1371/journal.pone.0145864.

Whitfield, S. M., E. Geerdes, I. Chacon, E. B. Rodriguez, R. J. Jimenez, M. A. Donnelly, and K. L. Kerby. 2013. Infection and co-infection by the amphibian chytrid fungus and ranavirus in wild Costa Rican frogs. Diseases of Aquatic Organisms 104: 173-178.

Zar, J.H. 2009. Biostatistical Analysis. 5th edition. Pearson, Upper Saddle River, N.J. 960 pp.

CHAPTER V.

DETECTION OF *OPHIDIOMYCES OPHIDIICOLA* AT TWO NATURAL AREAS IN ANNE ARUNDEL COUNTY, MARYLAND AND FAIRFAX COUNTY, VIRGINIA

Introduction

Snake fungal disease (SFD) has emerged as a growing threat to snakes throughout much of North America (Dolinski et al. 2014; Allender et al. 2015; Lorch et al. 2016; Paré and Sigler 2016), and has been associated with widespread morbidity and mortality in numerous species (Guthrie et al. 2016; Lorch et al. 2016; Stengle 2018). The disease is attributed to *Ophidiomyces ophidiicola* (*Oo*), a mycotic pathogen known only to infect snakes (Allender et al. 2015; Paré and Sigler 2016). Clinical manifestations of infection typically include scabs, crusty scales, superficial pustules, subcutaneous nodules, dysecdysis, and ocular cloudiness (Dolinski et al., 2014; McBride et al., 2015; Tetzlaff et al. 2015). SFD infections are generally chronic and mortalities likely result from secondary complications, such as impaired sensory capabilities or altered thermoregulatory behaviors (Lorch et al. 2015, 2016). Since 2006, SFD has become increasingly documented, with cases of infection reported in at least 20 eastern states including Maryland, Virginia, and West Virginia (Allender et al. 2015; Guthrie et al. 2016; Tupper et al. 2015, 2018, WVDNR). Despite growing reports of SFD throughout the Mid-Atlantic, systematic studies are limited. Compounded by the impacts of habitat loss, pollution, and other

anthropogenic influences, the rising incidence of SFD poses an added challenge for snake conservation, underscoring the significance of ongoing disease monitoring (Franklinos et al. 2017; Kucherenko et al. 2018). The primary objective of this study was to assess the presence and prevalence of *Oo* at two natural areas in Maryland and Virginia. Results of this study contribute to our understanding of the distribution and prevalence of SFD in the region.

Materials and Methods

We opportunistically sampled for *Oo* in snakes from Huntley Meadows Park (HMP; 38°45'36.57" N -77°05'44.13" W; Fig. 1) in Fairfax County, Virginia between 22 April and 21 June, 2018, and at the Smithsonian Environmental Research Center (SERC; 38°53'17.41"N 76°33'15.52 W; Fig. 2) in Anne Arundel County, Maryland between 31 March and 9 October, 2018. We hand-captured (wearing sterile nitrile gloves) and visually inspected snakes for symptoms of SFD (Allender et al. 2011; Clark et al. 2011). Then, using a modified protocol developed by Allender et al. (2016) we collected skin cells with sterile dry swabs (no. MW113, Medical Wire and Equipment Company, Durham, NC), from all craniofacial and ventral body scales. We swabbed each region five times. For symptomatic snakes, we also swabbed lesions, pustules, nodules or displaced scales (Allender et al. 2011; 2016). We stored swabs in sterile 1.5 mL microcentrifuge tubes and kept them frozen until molecular analyses. Prior to release, we determined the snake's sex via cloacal probing (*see* Blanchard and Finster 1933). We also measured (snout-to-vent and total length [cm]), weighed (g), and photographed each snake to help differentiate

similarly sized conspecifics. To limit SFD transmission, we employed aseptic techniques and followed appropriate biosecurity protocols (*see* Rzadkowska et al. 2016; VHS 2016).

To assay for *Oo*, we eluted swabbed DNA using the Purification of Total DNA from Animal Tissues Protocol (Qiagen®, Valencia, CA). Following the methods described by Allender et al. (2015), we combined 2.5 µL of eluted DNA with 12.5 µL Sso Advanced™ universal probes supermix (Bio-Rad, Hercules, CA) 1.25 µL of a combined target-specific primer (OphioITS-F and OphioITS-R)-probe, and water, creating a 25 µL reaction. We amplified the DNA via qPCR using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). DNA was amplified using the following cycling parameters: 1 cycle at 50˚C for 2 minutes, 1 cycle at 95˚C for 10 minutes, 40 cycles of 95˚C for 15 seconds, and 60˚C for 60 seconds, followed by a final cycle at 72˚C for 10 minutes. We used positive and negative controls in DNA extraction and amplification and performed up to five rounds of qPCR for each sample. A sample was considered positive if at least three of the replicates had a lower cycle threshold (C_t) than the lowest detected standard dilution of the positive control (Allender et al., 2016).

We plotted sampling sites within our study areas (indicating locations of positive and negative samples; Figures 5.1 and 5.2) with ESRI ArcMap (version 10.6). Snake nomenclature corresponds with Crother et al. (2017). Tables and descriptive statistics were completed with Microsoft Excel for Office 365 (Microsoft Corp., Washington, USA), and Minitab v. 16 (Minitab Inc., Pennsylvania, USA).

Results

We captured and swabbed 60 snakes (35 from HMP and 25 from SERC) across nine species (Table 5.1). Northern Watersnake (*Nerodia sipedon*) comprised the largest proportion (N = 26; 43.3%) of our captures. Eastern Ratsnake (*Pantherophis alleghaniensis*; N = 10), Common Ribbonsnake (*Thamnophis sauritus*; N = 9) and Eastern Wormsnake (*Carphophis amoenus*; N = 7) were also well-represented, comprising 16.7%, 15% and 11% of the total snake sample, respectively. Northern Black Racer (*Coluber constrictor*; N = 2), Eastern Kingsnake (*Lampropeltis getula*; N = 1), Northern Ring-necked snake (*Diadophis punctatus*; N = 1), Eastern Gartersnake (*Thamnophis sirtalis*; (N = 2), and Dekay's Brownsnake (*Storeria dekayi*; N = 2) were all sparsely represented. We detected *Oo* in 33 snakes and in a shed skin of a Northern Black Racer, yielding an overall infection rate of 55.7%. More than half of the positive samples (55.9%) were from a single species—Northern Watersnake. Of the nine species sampled, Northern Watersnake had the highest infection rate (73.1%), followed by Eastern Ratsnake (70%). Northern Black Racer, Eastern Wormsnake, and Common Ribbonsnake were positive in 66.7%, 28.6%, and 11.1% of samples, respectively. We only sampled one Eastern Kingsnake and Northern Ring-necked snake; both were positive. Dekay's Brownsnake was positive in one of two samples and Eastern Gartersnake was the only species that did not test positive for *Oo*. Twenty-five of the 35 (71.4%) symptomatic snakes tested positive for *Oo* and nine of the 26 (34.6%) asymptomatic individuals were *Oo* positive (Table 5.2). Prevalence varied between study locations, with 34.6% of snakes testing positive at HMP and 84.6% at SERC.

Discussion

Although *Oo* has previously been documented in Maryland and Virginia (Guthrie et al. 2016; Tupper et al. 2018), our work is one of only two studies (*see* Guthrie et al. 2016) to systematically investigate SFD in these states. In Maryland, observations of fungal dermatitis have been reported from the Smithsonian Environmental Research Center (SERC) since 2014 (Tupper et al. 2015), with *Oo* recently being confirmed as the etiological agent of a dermal infection in Northern Watersnake (Tupper et al. 2018). Our results add four new species (Eastern Wormsnake, Northern Black Racer, Northern Ring-necked snake, and Eastern Ratsnake) to the pathogen's documented host range in Maryland, which previously included Northern Watersnake (Tupper et al. 2018) and Timber Rattlesnake (*Crotalus horridus;* unconfirmed; Tupper et al. 2017). In eastern Virginia, Guthrie et al. (2016) documented *Oo* in four (symptomatic) species: Northern Watersnake (N = 3), Rainbow Snake (*Farancia erytrogramma;* N = 1), Northern Black Racer (N = 2), and Brown Watersnake (*Nerodia taxispilota;* N = 2). We add four new host species to the list of *Oo* positive species occurring in Virginia: Eastern Kingsnake, Eastern Ratsnake, Dekay's Brownsnake and Common Ribbonsnake).

Our overall infection rate of 57.4% falls among the highest (*except see* McKenzie et al. 2018) across the eastern and midwestern United States (Smeenk et al. 2016; Allender et al. 2016). Prevalence of *Oo* throughout the region appears to be highly variable, with rates of infection as low as 0% and 4.9% in Ohio and Michigan, respectively (Smeenk et al. 2016; Allender et al. 2016), and up to nearly 62% in eastern Kentucky (McKenzie et al.

2018). We interpret these rates cautiously, however, taking into consideration the variation in methodologies between studies (McCoy et al. 2017; Grisnik et al. 2018; Hileman et al. 2018; McKenzie et al. 2018). For instance, the number of sterile dry swab applicators used per snake has been shown to influence detection accuracy, with use of only one applicator greatly increasing the probability of obtaining false-negative results (Hileman et al., 2018). Our results support this concept, with 16 (47.1%) of the 34 positive snakes testing positive for *Oo* in only one of two swabs (nine from the craniofacial swab only, seven from the body swab only). Underestimation of infection may also occur when diagnostic tests are limited to visibly symptomatic snakes (*see* Guthrie et al. 2016). While clinical signs have been associated with a higher probability of PCR-positive results, studies have also demonstrated that approximately 12% of snakes without clinical signs will test positive for *Oo* (Allender et al. 2016; Bohuski et al. 2015; Hileman et al. 2018; McKenzie et al. 2018). Our data support prior work as only 34.6% of our positive samples were from asymptomatic snakes. Detection of *Oo* in asymptomatic snakes has been attributed to several possible factors. In some cases, symptomatic snakes may be misclassified if clinical signs are subtle and overlooked during visual inspection (Grisnik et al. 2018; Hileman et al. 2018). Snakes that are in fact asymptomatic may either be in the early stages of infection, or potentially asymptomatic carriers of infection (Lorch et al. 2016).

It is still unclear how susceptibility and severity of infection differs between species (Grisnik et al. 2018), but species composition of our sample may partly explain both the prevalence and relative high proportion of positive asymptomatic snakes in our study. We detected *Oo* in eight of the nine species sampled in this study, which was not surprising

given that each of these species has previously tested positive for the pathogen in the eastern and mid-western United States (Lorch et al. 2016; Persons et al. 2017; Grisnik et al. 2018; McKenzie et al. 2018). However, because of low sample sizes in certain species it was difficult to properly assess how each of these species contributed to the overall infection rate. We found *Oo* to be most prevalent in Northern Watersnake, with an infection rate of 73%. This species represented nearly 43% of our total sample and thus had a strong influence on overall prevalence (55.7%). Prior studies with similar proportions of aquatic specie have also demonstrated relatively high infection rates among Northern Watersnakes, and other species with aquatic affiliations. However, this trend in infectivity may in part reflect the habitat preferences of the pathogen (Lorch et al. 2016; McKenzie et al. 2018), rather than a particular susceptibility to the pathogen. Further work is needed to elucidate these relationships.

Results from our study confirm that *Oo* is present and relatively prevalent in both Maryland and Virginia, and that infection is often accompanied by clinical manifestations consistent with SFD. Currently, efforts to monitor *Oo* are limited. The geographic distribution and host range of the pathogen are still largely unknown (Burbrink et al. 2017), and it is thought that SFD may be more widely distributed than these documented cases suggest (USGS, 2018). It has been proposed that (even when collected systematically) biased detection methods may result in underestimations of prevalence within a population (Grisnik et al. 2018; Hileman et al., 2018). This potential for inaccurate assessments highlights the need for more systematic and standardized sampling efforts and diagnostic protocols. Based on the increasing number of reports of SFD throughout the eastern United

States, we suspect *Oo* is highly infectious and pathogenic. We therefore suggest increased efforts to sample for and monitor *Oo* throughout the Mid-Atlantic region. Additionally, it is crucial that enhanced biosecurity protocols be implemented to limit disease transmission in the region.

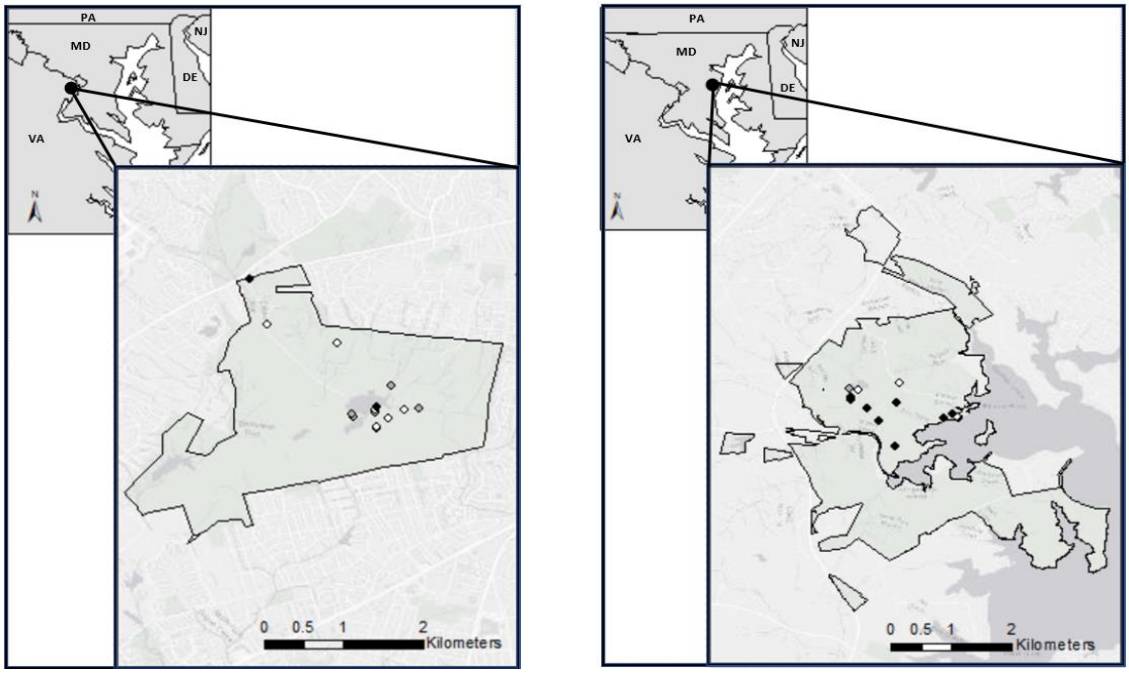

Fig. 5.1. (Left). Location of HMP snake capture locations. Black markers = all positive samples; white markers = all negative samples; gray markers = positive and negative samples.

Fig. 5.2. (Right). Location of SERC snake capture locations. Black markers = all positive samples; white markers = all negative samples; gray markers = positive and negative samples.

Table 5.1. Snake morphometrics. KG = kilograms, CM = centimeters, M = male, F = female, NS = not sexed, SVL = snout-to-vent length. TL = total length, MIN = minimum, MAX = maximum, $\bar{X}$ = mean SE = standard error.

SPECIES	N	WEIGHT (KG)				SVL (CM)				TL (CM)			
		MIN	MAX	$\bar{X}$	SE	MIN	MAX	$\bar{X}$	SE	MIN	MAX	$\bar{X}$	SE
EASTERN WORMSNAKE	-	-	-	-	-	-	-	-	-	-	-	-	-
F	4	0.005	0.007	0.006	0	14.8	27.3	20.5	2.98	18	30.1	24.1	3.04
M	3	0.005	0.02	0.01	0.01	15.8	21	18.9	1.58	18.7	26.2	23.1	2.26
NORTHERN BLACK RACER	-	-	-	-	-	-	-	-	-	-	-	-	-
F	1	-	-	-	-	-	-	-	-	-	-	-	-
M	1	0.008	0.4	0.2	0.3	30.2	94.6	62.4	45.5	43.3	123	83.3	56.5
NORTHERN RING-NECKED SNAKE	-	-	-	-	-	-	-	-	-	-	-	-	-
F	1	0.05	0.05	0.05	-	16.1	16.1	16.1	-	21.6	21.6	21.6	-
EASTERN KINGSNAKE	-	-	-	-	-	-	-	-	-	-	-	-	-
F	1	0.2	0.2	0.1	-	55.4	55.4	55.4	-	58.5	58.5	58.5	-
NORTHERN WATERSNAKE	-	-	-	-	-	-	-	-	-	-	-	-	-
F	8	0.08	0.5	0.3	0.05	43	78.8	62.6	4.37	57.3	101	76.6	4.79
M	17	0.01	1	0.3	0.06	22.5	95.5	55.8	4	28.5	116	72.1	4.56
NS	1	-	-	-	-	-	-	-	-	-	-	-	-
EASTERN RATSNAKE	-	-	-	-	-	-	-	-	-	-	-	-	-
F	6	0.3	0.7	0.5	0.07	87.8	127	106	5.58	98.4	144	121	6.32
M	3	0.4	0.7	0.6	0.1	96.8	134	116	10.7	125	148	139	7.21
NS	1	0.02	0.02	0.02	-	48.8	48.8	48.8	-	50.9	50.9	50.9	-
DEKAY'S BROWNSNAKE	-	-	-	-	-	-	-	-	-	-	-	-	-
F	1	0.04	0.04	0.04	-	16.2	16.2	16.2	-	19.1	19.1	19.1	-
M	1	0.005	0.005	0.005	-	14	14	14	-	19.8	19.8	19.8	-
COMMON RIBBONSNAKE	-	-	-	-	-	-	-	-	-	-	-	-	-
F	2	0.08	0.4	0.3	0.2	50.2	55	52.6	2.4	50.6	77	63.8	13.2
M	6	0.006	0.3	0.1	0.04	26.7	44.7	36.7	2.73	39.2	66.5	56.7	4.16
NS	1	0.1	0.1	0.1	-	50.8	50.8	50.8	-	-	-	-	-
EASTERN GARTERSNAKE	-	-	-	-	-	-	-	-	-	-	-	-	-
F	1	0.2	0.2	0.2	-	44	44	44	-	57.5	57.5	57.5	-
M	1	0.07	0.07	0.07	-	45.3	45.3	45.3	-	69.8	69.8	69.8	-

Table 5.2. Prevalence by species. S/+ = symptomatic and positive, A/+ = asymptomatic and positive, S/- = Symptomatic and Negative.

Species	N	Pos	Species Prevalence (%)	Overall Prevalence (%)	S/+	A/+
Eastern Wormsnake	7	2	28.6	5.9	0	2
Northern Black Racer	3	2	66.7	5.9	2	0
Northern Ring-Necked Snake	1	1	100	2.9	0	1
Eastern Kingsnake	1	1	100	2.9	1	0
Northern Watersnake	26	19	73.1	55.9	15	4
Eastern Ratsnake	10	7	70	20.6	5	2
Dekay's Brownsnake	2	1	50	2.9	1	0
Common Ribbonsnake	9	1	11.1	2.9	1	0
Eastern Gartersnake	2	0	0	0	0	0
Total	61	34	-	55.7	25	9

LITERATURE CITED

Allender MC, Dreslik M, Wylie S, Philips C, Wylie DB, Maddox C, Delaney MA, Kinsel MJ. 2011. *Chrysosporium* sp. infection in eastern Massasauga rattlesnakes. *Emerging Infectious Diseases* 17: 2383-2384.

Allender MC, Raudabaugh DB, Gleason FH, Millier AN. 2015. The natural history, ecology, and epidemiology of *Ophidiomyces ophiodiicola* and its potential impact on free-ranging snake populations. *Fungal Ecology* 17: 187-196.

Allender MC, Hileman ET, Moore J, Tetzlaff S. 2016. Detection of *ophidiomyces*, the causative agent of snake fungal disease, in the eastern massasauga (*Sistrurus catenatus*) in Michigan, USA, 2014. *Journal of Wildlife Diseases* 52: 694-698.

Blanchard FN, Finster EB. 1933. A method of marking live snakes for future recognition, with a discussion of some problems and results. *Ecology* 14: 334-347.

Bohuski E, Lorch JM, Griffin KM, Blehert DS. 2015. Taqman real-time polymerase chain reaction for detection of *Ophidiomyces ophidiodiicola*, the fungus associated with snake fungal disease. *BMC Veterinary Research* 11: 95.

Burbrink FT., Lorch JM, Lips KR. 2017. Host susceptibility to snake fungal disease is highly dispersed across phylogenetic and functional trait space. *Science Advances* 3: e1701387.

Clark RW, Marchand MN, Clifford BJ, Stechert R, Stephens S. 2011. Decline of an isolated timber rattlesnake (*Crotalus horridus*) population: interactions between climate change, disease, and loss of genetic diversity. *Biological Conservation* 144: 886-891.

Crother BI. 2017. Scientific and standard English names of Amphibians and Reptiles of North America north of Mexico, with comments regarding confidence in our understanding. *SSAR Herpetological Circular* 43: 59-80.

Dolinski AC, Allender MC, Hsiao V, Maddox CW. 2014. Systemic *Ophidiomyces ophiodiicola* infection in a free-ranging plains garter snake (*Thamnophis radix*). *Journal of Herpetological Medicine and Surgery* 24: 7-10.

Franklinos LHV, Lorch JM, Bohuski E, Fernandez JR, Wright ON, Fitzpatrick L, Petrovan S, Durrant C, Linton C, Baláž V, et al. 2017. Emerging fungal pathogen *Ophidiomyces ophidiicola* in wild European snakes. *Scientific Reports* 7: 3844. DOI:10.1038/s41598-017-03352-1

Grisnik M, Leys JE, Bryan D, Hardman RH, Miller DL, Cobb VA, Ogle C, Simpson C, Campbell JR, Applegate RD, et al. 2018. Host and geographic range of snake fungal disease in Tennessee, USA. *Herpetological Review* 49: 682-690.

Guthrie AL, Knowles S, Ballmann AE, Lorch JM. 2016. Detection of snake fungal disease due to *Ophidiomyces ophiodiicola* in Virginia, USA. *Journal of Wildlife Diseases* 52: 143-149.

Hileman ET, Allender MC, Bradke DR, Faust LJ, Moore JA, Ravesi MJ, Tetzlaff SJ. 2018. Estimation of *Ophidiomyces* prevalence to evaluate snake fungal disease risk. *Journal of Wildlife Management* 82:173–181; doi: 10.1002/jwmg.21345.

Kucherenko A, Herman JE, Everham III EM, Urakawa H. 2018. Terrestrial snake environmental DNA accumulation and degradation dynamics and its environmental application. *Herpetologica* 74: 38-49.

Latney LV, Wellehan J. 2013. Selected emerging infectious diseases of squamata. *Veterinary Clinics of North America - Exotic Animal Practice* 16: 319-338.

Lorch JM, Lankton J, Werner K, Falendysz EA, Mccurley K, Blehart DS. 2015. Experimental infection of snakes with *Ophidiomyces ophiodiicola* causes pathological changes that typify snake fungal disease. *MBio* 6: e01534-15.

Lorch JM, Knowles S, Lankton JS, Michell K, Edwards JL, Kapfer JM, Staffen RA, Wild ER, Schmidt KZ, Ballmann AE, et al. 2016. Snake fungal disease: an emerging threat to wild snakes. *Philosophical transactions of the Royal Society of London, Section B, Biological sciences* 371: 20150457.

Mcbride MP, Wojick KB, Georoff TA, Kimbro J, Garner MM, Wang X, Chidress AL, Wellehan Jr. JFX. 2015. *Ophidiomyces ophiodiicola* dermatitis in eight free-ranging timber rattlesnakes C*rotalus horridus*) from Massachusetts. *Journal of Zoo and Wildlife Medicine* 46:86-94.

Mccoy CM, Lind CM, Farrell TM. 2017. Environmental and physiological correlates of the severity of clinical signs of snake fungal disease in a populations of pigmy rattlesnakes, *Sistrurus miliarius*. *Conservation Physiology* 5(1): cow077; doi: 10.1093/conphys/cow077.

Mckenzie JM, Price SJ, Fleckenstein JL, Drayer AN, Connette GM, Bohuski E, Lorch JM. 2018. Field diagnostics and seasonality of *Ophidiomyces ophidiicola* in wild snake populations. *Ecohealth* doi:10.1007/s10393-018-1384-8.

Mitchell MA, Tully Jr. T. 2009. Snakes. Pp. 136-163 In: *Manual of Exotic Pet Practice*. Elsevier Inc., USA.

Paré JA**,** Sigler L. 2016. An overview of reptile fungal pathogens in the genera *Nannizziopsis*, *Paranannizziopsis*, and *Ophidiomyces*. *Journal of Herpetological Medicine and Surgery* 26: 46-53.

Persons TB, Stengle AG, Yorks DT, deMaynadier PG. 2017. Snake fungal disease from Maine, USA, and an update of documented occurrences in the United States. *Herpetological Review* 48: 62-63.

Rzadkowska M, Allender MC, O'Dell M, Maddox C. 2016. Evaluation of common disinfectants effective against *Ophidiomyces ophiodiicola*, the causative agent of snake fungal disease. *Journal of Wildlife Diseases* 52: 759-762.

Smeenk NA, Lipps Jr. GJ, Allender MC, Backus K, Freeman M, Hribar D, Parsley M. 2016. Snake fungal disease, *Ophidiomyces ophiodiicola*, in Ohio, USA. *Herpetological Review* 47: 592-594.

Stengle A. 2018. Habitat selection, connectivity and population genetics of a timber rattlesnake (*Crotalus horridus*) metapopulation in southwestern Massachusetts and New England. Ph.D. Dissertation, University of Massachusetts, Amherst, Department of Organismic and Evolutionary Biology.

Tetzlaff S, Allender MC, Ravesi M, Smith J, Kingsbury B. 2015. First report of snake fungal disease from Michigan, USA involving massasaugas, *Sistrurus catenatus* (Rafinesque 1818). *Herpetology Notes* 8: 31-33.

Tupper TA, Aguilar R, Ferrara A, Hay C. 2015. Field notes: Snake fungal disease. *Catesbeiana*. 35: 36-37.

Tupper TA, Fuchs LD, Shaffer DL, Aguilar R. 2017. Snake fungal disease, *Crotalus horridus*. *Catesbeiana* 37: 128-130.

Tupper TA, Aguilar R, Fuchs LD. 2018. Field notes: snake fungal disease: MD. *Catesbeiana* 38: 78-79.

USGS (United States Geological Survey). Available: https://www.nwhc.usgs.gov/disease_information/other_diseases/snake_fungal_disease.jsp. [Accessed January 2019].

VHS (Virginia Herpetological Society). Disinfection protocol. Available: http://www.virginiaherpetologicalsociety.com/research/disease/index.htm. [Accessed March, 2019].

WVDNR (West Virginia Division of Natural Resources). 2017. Snake fungal disease detected in West Virginia's snakes. Available: http://www.wvdnr.gov/2017news/17news098.shtm. [Accessed February 2019].

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