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Amphibian pathogen *Batrachochytrium dendrobatidis* prevalence is correlated with season and not urbanization in central Virginia

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ABSTRACT: The global amphibian pathogen *Batrachochytrium dendrobatidis* (*Bd*) has been documented among many species throughout the United States, though cases of chytridiomycosis, the resulting disease, have occurred mostly on the west coast. We conducted a 2 yr survey of amphibians along an urban gradient in Virginia, USA, to test whether *Bd* prevalence among the amphibians sampled varied with urbanization and/or season. A total of 867 adult amphibians from 13 species and 49 tadpoles from 3 species were tested for *Bd*. The level of urbanization was based on surrounding human population density and anthropogenic disturbance. *Bd* was detected in 6 species. *Bd* prevalence was not found to vary with increases in urbanization, but did vary with season. Prevalence peaked in the spring at 45%, when temperatures were between 14 and 25°C, and dropped to below 2% in the autumn. Results from this survey support the hypothesis that *Bd* is endemic to the studied sites in Virginia. The present study, in concurrence with previous research by other investigators, shows that *Bd* is affected strongly by weather patterns. Urbanization, defined by human population density, appeared to have minimal impact on the prevalence of *Bd.* In addition to understanding the geographic distribution of *Bd,* it is important to understand factors that affect its prevalence if we are to develop approaches to managing this emerging disease.

KEY WORDS: *Batrachochytrium dendrobatidis* · Amphibian disease · Season · Urbanization

INTRODUCTION

Batrachochytrium dendrobatidis (*Bd*), which causes the fungal infection chytridiomycosis, was first diagnosed as a causative agent of certain amphibian population declines in Australia and Panama in 1998 (Berger et al. 1998). It has since been found among dead, moribund, and even healthy individuals in North, Central, and South America; Africa; and Europe (Speare & Berger 2004); as well as Indonesia (Kusrini et al. 2008) and Japan (Goka et al. 2009).

It infects the keratinized epidermis of amphibians leading to hyperkeratosis. Though external signs can include lethargy and abnormal posture, often individuals show no clinical signs of the disease, making it difficult to diagnose in the field (Densmore & Green 2007).

Bd is widely distributed throughout North and South America (Ouellet et al. 2005, Picco & Collins 2007). It occurs throughout the United States, particularly in the northeast (Longcore et al. 2007, Campbell Grant et al. 2008), the southeast (Green & Dodd 2007, Peterson et al. 2007, Rothermel et al. 2008), and the west (Cummer et al. 2005, Pearl et al. 2007). *Bd* has been detected in a wide array of amphibian species, including *Ambystoma maculatum*, *Notophthalmus viridescens*, *Lithobates catesbeianus*, *L. clamitans*, *L. palustris*, *L. pipiens*, *L. sylvatica*, and *Hyla versicolor* (Ouellet et al. 2005, Longcore et al. 2007, Peterson et al. 2007).

Certain environmental factors are associated with *Bd* prevalence and infection rates, including specific seasons (McDonald et al. 2005). The optimal growth for the fungus occurs between 17 and 25°C in a laboratory

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setting, and the fungus will die if temperatures rise above 29°C or fall below 0°C (Piotrowski et al. 2004). In a long-term monitoring study of *Bd* among 4 frog species in North Queensland, Australia, McDonald et al. (2005) reported that *Bd* prevalence was significantly associated with the season, being highest during the winter (defined as May through September). Kriger & Hero (2006) observed that the prevalence of *Bd* peaked when temperatures were between 12.3 and 19.4°C in Queensland Australia, and Pearl et al. (2007) found that *Bd* detection was higher during winter and spring in Oregon, USA. Mortality due to chytridiomycosis also increased in winter (Berger et al. 2004). Fewer studies have been conducted on habitat associations of *Bd*. One such study, by Kriger & Hero (2007), found *Bd* presence solely among amphibians that bred at permanent ponds versus ephemeral ponds.

Currently the spread of *Bd* to naive amphibian populations around the world is considered to be the major driver of the emergence of this disease and associated massive amphibian declines (Skerratt et al. 2007, Padgett-Flohr & Hopkins 2009). In addition, increases in habitat loss and degradation may weaken an individual's immune system, which in turn may cause them to be more likely to succumb to the infection (Carey et al. 1999, Daszak et al. 2001). Urban settings are of particular concern when considering this hypothesis, since these areas are highly susceptible to multiple stressors. The amphibians that reside in urbanized zones can be exposed to pollution from cars, trains, urban run-off, and atmospheric deposition (Sanzo & Hecnar 2006, Karraker et al. 2008). They are also subjected to smaller habitats and more physical disturbance (Mazerolle et al. 2005, Eigenbrod et al. 2008).

The purpose of the present study was to survey for *Bd* in central Virginia, where *Bd* has been documented in several species (Rothermel et al. 2008), but where a systematic survey has not been conducted. The objectives of the present study were to survey for the presence of *Bd* DNA throughout a 2 yr period along a gradient of urbanization to provide evidence whether *Bd* prevalence in central Virginia is associated with (1) more urbanized habitats, defined as high human population densities, (2) certain species, and (3) seasons.

MATERIALS AND METHODS

Sites. Amphibians were sampled at 6 sites along a gradient of urbanization in 2007 and 2008. Populations were sampled at each site twice a month March through June, and August through November. The 6 sites, from highly urban to least urban, according to surrounding population densities and relative onsite anthropogenic disturbance are described below.

Site 1: The Buttermilk Trail of the James River Park system (BT): This site is a public park that consists of a thin strip of land located between the James River and a high-density housing area. Located in downtown Richmond, VA, USA, at the fall line, this area has high physical disturbance from visitors, cars, and trains that run through the site. The approximate surrounding population density is 1426 persons per km^2 (EPA 2008). Amphibian species we identified at this site were *Anaxyrus americanus*, *A. fowleri*, *Acris crepitans*, *Lithobates catesbeianus*, *L. sphenocephalus*, and *Plethodon cinereus.*

Site 2: Bryan Park (BP): This site is a 106 ha public park in Richmond, located at the intersection of Interstates 95 and 64. Of the total park acreage, 66.8 ha are forested. The waterways within the park have been designated as impaired (EPA 2008). Waterways that are impaired do not meet the water quality standards of the Clean Water Act and therefore do not support at least one of its intended uses as defined by the Clean Water Act (EPA 2008). In addition, *Bd* has already been documented among *Lithobates catesbeianus* tadpoles within the site (Mitchell & Green 2002). The approximate surrounding population density is 1014 persons per km^2 (EPA 2008). The species that we identified at this site were *Anaxyrus americanus*, *A. fowleri*, *Lithobates catesbeianus*, *L. clamitans*, and *L. sphenocephalus.*

Site 3: The wetlands of the James River Park system (W): This site is also a public park located within the city of Richmond, approximately 16.2 ha in size along the James River. The park is situated between a large neighborhood, a golf course, and another public portion of the James River Park System called Pony Pasture. The approximate surrounding population density is 733 persons per km^2 (EPA 2008). The species identified at this site were *Acris crepitans*, *Anaxyrus americanus*, *Anaxyrus fowleri*, *Pseudacris crucifer*, *Hyla cinerea*, *H. versicolor*, *Lithobates clamitans*, *L. sphenocephalus*, *L. catesbeianus*, *Notophthalmus viridescens*, *Ambystoma maculatum*, and *Ambystoma opacum.*

Site 4: Harrison Lake National Fish Hatchery (HL): This 161.9 ha site in Charles City County, VA, consists of 20 small fish ponds as well as a lake and forested creek. The approximate surrounding population density is 23 persons per km^2 (EPA 2008). The species identified at this site were *Anaxyrus americanus*, *A. fowleri*, *Acris crepitans*, *Hyla cinerea*, *H. versicolor*, *Lithobates sphenocephalus*, *L. catesbeianus*, and *Pseudacris crucifer.*

Site 5: The Virginia Commonwealth University Inger and Walter Rice Center (RC): This site is a 138.4 ha tract of land along the James River in Charles City County, VA, used for research and educational purposes by Virginia Commonwealth University. It

consists of both hardwood and pine forests as well as tidal and non-tidal wetlands. The approximate surrounding population density is 14 persons per km^2 (EPA 2008). The species identified at this site were *Anaxyrus fowleri, Acris crepitans, Anaxyrus americanus, Lithobates catesbeianus, L. clamitans, L. sphenocephalus, Pseudacris crucifer, Ambystoma maculatum,* and *Ambystoma opacum.*

Site 6: Chickahominy Wildlife Management Area (CH): This site, managed by the Virginia Department of Game and Inland Fisheries for upland species, consists of 2111 ha bordering the Chickahominy River in Charles City County, VA. Morris Creek, flowing through the southern portion of the property, provides wetland habitat amidst the mixed hardwood and pine forest. The approximate surrounding population density is 7 persons per km^2 (EPA 2008). The species identified at this site were *Anaxyrus americanus, A. fowleri, Acris crepitans, Lithobates clamitans, L. sphenocephalus,* and *L. catesbeianus.*

Amphibian sampling. At each site, stream transects were conducted by walking along the edge of the stream, and pond transects were conducted by circumnavigating the pond's edge. Terrestrial transects were undertaken by moving through the site and inverting logs, rocks, and other cover. All species were captured opportunistically at each site using dip nets and hands. In addition, drift fences were used at Site 5 in September and October to capture *Ambystoma opacum* migrating into the vernal pools to breed. Funnel traps were used at Site 5 and Site 3 in February through March in order to capture *A. maculatum,* as they also migrated into the pools to breed. The funnel traps were baited with glow sticks, a reported amphibian attractant (Grayson & Roe 2007). All individuals included in the analysis were identified by genus and species.

All juveniles and adults captured were swabbed approximately 20 times with a sterile fine tip swab (Medical Wire and Equipment MW100-100) on the lower half of the underside of the abdomen and the ventral thighs of the rear legs, and each tadpole was swabbed on the keratinized mouth parts to check for presence of *Bd*. Swabs were stored at 4°C. A new pair of sterile, powder-free nitrile gloves was used to handle each individual captured to avoid cross contamination. Field equipment, including boots and nets, was sterilized with a 5% bleach solution between surveys to prevent transmission of pathogens between sites.

Laboratory analysis of swabs and dead or moribund individuals. DNA was extracted from the swabs to check for *Bd* using a SETS tube system and Prepman Ultra (Applied Biosystems). DNA isolated from the swabs was analyzed using a PCR analysis with *Bd*-specific primers Bd1a and Bd2a, which amplified a 300 bp fragment (Annis et al. 2004). The PCR consisted of a 25 µl reaction that included 0.5 µl of each primer (10 µM Bd1a and 14 µM Bd2a), 12.5 µl Hotstar Plus Master Mix (Qiagen), 11 µl water, and 0.5 µl DNA template. The thermocycler protocol included denaturing at 95°C for 15 min, 35 cycles of 95°C for 45 sec, 60°C for 45 sec, and 72°C for 1 min, followed by extension at 72°C for 10 min. The final products were resolved on a 1.2% agarose gel. Both positive and negative controls were used in each PCR assay. Original positive controls were from *Bufo boreas*, donated by Pisces Molecular. After the first sampling season, positive samples from the survey were used as positive controls.

Any individuals found dead or showing clinical signs were brought back to the lab. Individuals were euthanized using a 2 mg ml^{-1} solution of MS-222 and preserved in 70% ethanol. Every euthanized individual was necropsied, and skin was removed for histopathological analysis. Tissue was embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) by the Division of Surgical Pathology Laboratory at Virginia Commonwealth University School of Medicine in Richmond.

Data analysis. To analyze the prevalence of *Bd* among amphibians, percentages of positive individuals were compared among sites, species, and seasons using logistic regression. Score confidence intervals were used to describe the prevalence of *Bd* within each of the classifications. Each of the classifications of individuals was first examined with a bivariate analysis, and then all of the classification variables were considered in a multiple logistic regression model.

To test whether *Bd* prevalence was correlated with urbanization intensity, the urban and rural prevalence was compared in the logistic regression model after covarying out the effects of species groups and seasons. Tests were considered significant if $p < 0.05$. All statistical tests were computed using SAS software (JMP version 8.0.2, SAS Institute). It is important to note that the absence of a positive sample within a population does not mean that the disease or pathogen is absent from that population, only that it was not detected among the sampled individuals. However, even in small samples, the upper limit of the 95% confidence interval is considered an estimate of highest prevalence of the pathogen.

RESULTS

The study results will first review the relationships between *Bd* prevalence and each of the sample characteristics (urban versus rural sites, species, and yearly or seasonal variations).

Batrachochytrium dendrobatidis was detected at all of the sites. Of the total 916 individuals that were iden-

tified and sampled for the *Bd* fungus at all of the sites combined, 11.9% were positive. *Bd* prevalence did differ significantly among the 6 sites (likelihood ratio χ^2 = 15.3, df = 5, p = 0.0091; Table 1). Within the 3 urban sites, there was a significant difference in *Bd* prevalence (χ^2 = 14.8, df = 2, p = 0.006). BT exhibited only a 1.3% prevalence (95% CI = 0.2 to 6.8) whereas W exhibited the highest overall prevalence (15.7%, 95% CI = 11.3 to 21.4). There was no significant difference between the 3 rural sites (likelihood ratio χ^2 = 0.2, df = 2, p = 0.9). In the rural sites, *Bd* prevalence was 13.9% (95% CI = 11.2 to 17.1)

Omitting all of the other classification variables, *Bd* prevalence did not differ significantly in the urban versus the rural sites (p > 0.6).

Although *Bd* was detected on over 10% of the individuals at 5 of the 6 sites, no specimens exhibited signs of chytridiomycosis. Of the 18 specimens in which we

Table 1. *Batrachochytrium dendrobatidis.* Prevalence for the sampling sites and seasons for each genus and species identified. Mean = prevalence estimate based upon number of samples (n), of which *x* were positive. 95% CI = 95% score confidence interval. p crude = comparison of groups ignoring all other grouping (predictor) variables using a likelihood-ratio χ-squared test from logistic regression. p adjusted = comparison of groups while adjusting for all other grouping variables using a likelihood-ratio χ-squared test from multiple logistic regression. ns = not significant. na = not applicable since the salamanders were not included in the adjusted analysis

Predictors	Prevalence			p	
	Mean (%)	95% CI	(*x*/n)	Crude	Adjusted
Sites				0.0091	0.0400
Urban	10.4	(7.7, 14.0)	37/355	0.0006	<0.0001
Buttermilk Trail (BT)	1.3	(0.2, 6.8)	1/79		
Bryan Park (BP)	10.4	(4.5, 22.2)	5/48		
The wetlands (W)	15.7	(11.3, 21.4)	31/198		
Rural	13.9	(11.2, 17.1)	72/519	0.9024	0.0060
Harrison Lake National Fish Hatchery (HL)	12.3	(9.1, 16.5)	37/300		
Rice Center (RC)	11.3	(7.4, 17.0)	19/168		
Chickahominy (CH)	13.0	(9.4, 22.9)	16/123		
Species				<0.0001	<0.0001
Frogs and toads (metamorphed)	14.1	(11.7, 16.7)	104/740	<0.0001	
Acris crepitans	20.4	(16.7, 24.6)	80/393		
Anaxyrus americanus	6.3	(1.1, 28.3)	1/16		
Anaxyrus fowleri	0.0	(0.0, 5.1)	0/71		
Hyla cinerea	0.0	(0.0, 7.1)	0/50		
Hyla versicolor	0.0	(0.0, 24.2)	0/12		
Lithobates catesbeianus	8.0	(3.7, 16.4)	6/75		
Lithobates clamitans	5.9	(1.0, 27.0)	1/17		
Lithobates sphenocephalus	12.5	(6.9, 21.5)	10/80		
Pseudacris crucifer	23.1	(2.1, 24.1)	6/26		
Tadpoles	10.2	(4.4, 21.8)	5/49	0.1024	
Lithobates catesbeianus	100.0	(89.3, 100.0)	32/32		
Lithobates clamitans	0.0	(0.0, 25.9)	0/11		
Lithobates sphenocephalus	0.0	(0.0, 39.0)	0/6		
Salamanders	0.0	(0.0, 2.9)	0/127	ns	na
Ambystoma maculatum	0.0	(0.0, 12.1)	0/28		
Ambystoma opacum	0.0	(0.0, 8.6)	0/41		
Notophthalmus viridescens	0.0	(0.0, 19.4)	0/7		
Plethodon cinereus	0.0	(0.0, 35.4)	0/5		
Seasons					
2007	8.9	(6.6, 11.9)	40/451	0.0072	0.6197
2008	14.8	(11.9, 18.3)	69/466		
March–June	20.2	(16.9, 24.0)	101/499	<0.0001	<0.0001
March	15.7	(9.0, 26.0)	11/70		
April	19.7	(13.5, 27.8)	23/117		
May	35.3	(27.8, 43.6)	48/136		
June	10.8	(7.0, 16.2)	19/176		
Aug–Nov	1.9	(1.0, 3.7)	8/417	0.1538	0.4251
August	0.0	(0.0, 4.3)	0/85		
September	2.7	(1.2, 5.7)	6/224		
October	2.7	(0.7, 9.2)	2/75		
November	0.0	(0.0, 10.4)	0/33		

were able to examine skin histologically for chytrid thalli, none had clinical chytridiomycosis.

Infection with *Bd* was detected in adult *Acris crepitans* (20.4%), *Lithobates catesbeianus* (8.0%), *L. sphenocephalus* (12.5%), *L. clamitans* (5.9%), *Pseudacris crucifer* (23.1%), and *Anaxyrus americanus* (6.3%) during the 2 yr study. The prevalence of *Bd* did vary significantly among these adult species for 2007 and 2008 combined ($\chi^2 = 14.8$, df = 2, p = 0.0006). Only one *A. americanus* was found positive during the study. This individual was captured in May 2008 next to one of the rearing ponds at HL. Only one *L. clamitans* found in May 2007 was positive for *Bd* during the study. In 2008, 23% of *P. crucifer* were positive for *Bd*. The differences between these adult species did not vary year by year (p > 0.9). No *Bd* was detected in the *Anaxyrus fowleri, Hyla cinerea*, or *H. versicolor.*

A total of 49 tadpoles was analyzed. *Bd* was detected in all of the 32 *Lithobates catesbeianus* tadpoles, and no *Bd* was detected in the 11 *L. clamitans* or 6 *L. sphenocephalus* tadpoles tested.

Four salamander species were captured (n = 136) and assayed for *Bd*. *Ambystoma maculatum* (28 adults), *A. opacum* (41 adults), *Notophthalmus viridescens* (7 adults), and *Plethodon cinereus* (51 adults) were uniformly negative for *Bd*.

There was seasonal variation in *Bd* prevalence among the individuals analyzed ($\chi^2 = 7.2$, p < 0.0072; Table 1). There were higher percentages of positive *Bd* specimens in 2008 than in 2007 (approximately 15 versus 9%) and in late winter/spring when the average temperatures were between 14 and 25°C. In May, when the average temperatures for both 2007 and 2008 were 19 and 18°C, respectively, *Bd* percentages were highest at about 45%. In the late summer/autumn, percentages were much lower, at only 2%. Fig. 1 further illustrates that seasonal variation in *Bd* prevalence was different in the 6 sampling sites. In Site 1, individuals were sampled in the months of April, May, August, and September, but only one individual was *Bd*-positive. Seasonal prevalence was highest in May in Sites 2, 3, 5, and 6, but Site 4 was highest earlier in the year (March and April).

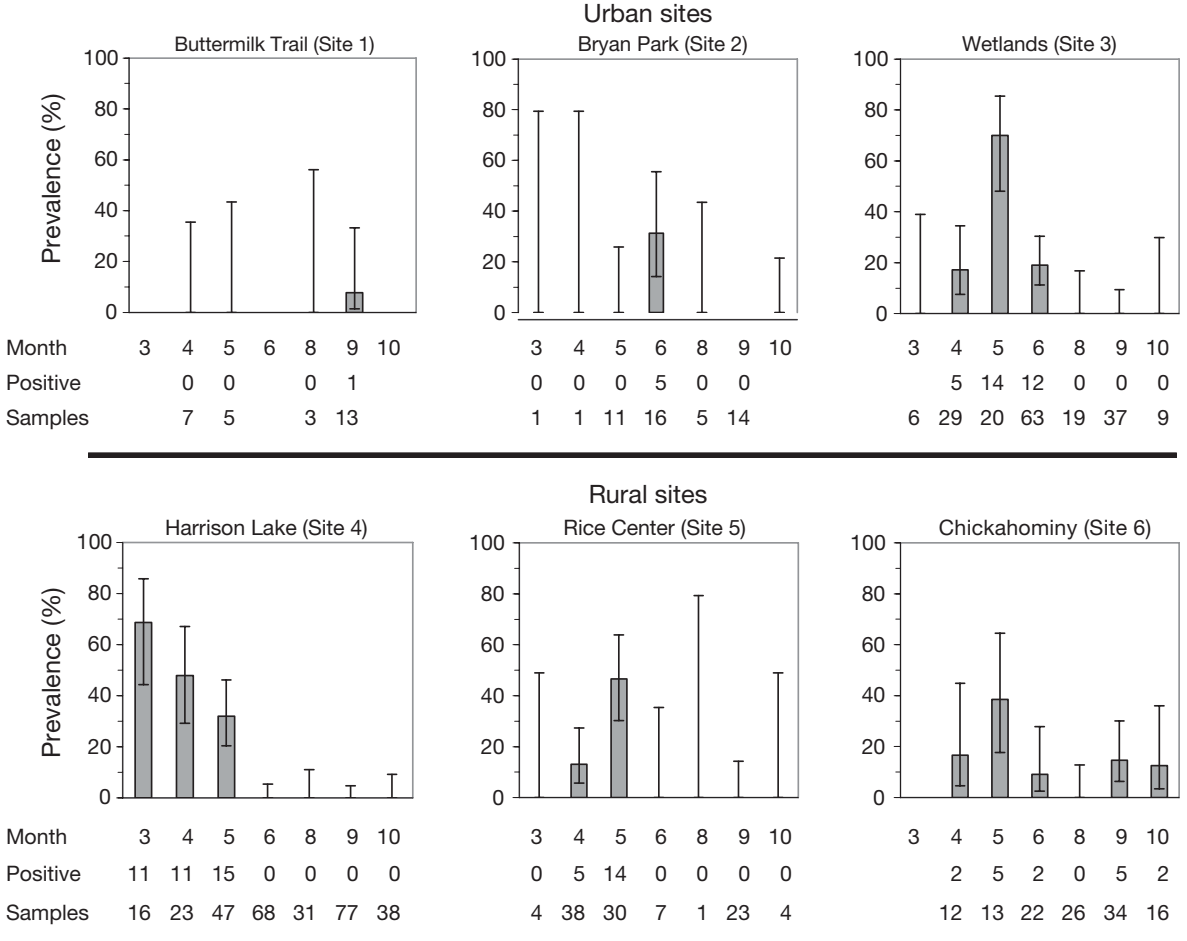

Fig. 1. Seasonal variation of *Batrachochytrium dendrobatidis* within each sampling site. Error bars are 95% confidence intervals

DISCUSSION

In support of other studies throughout the eastern half of the United States, our results show that *Bd* is prevalent among multiple species throughout the surveyed areas of Virginia. From the present study there are 2 key findings: (1) *Bd* varied with season but (2) did not vary with increasing urbanization at the 6 sites studied. Since *Bd* was found throughout the sites without any signs of disease, this suggests that the species in these areas are resistant to the pathogen. However, there is minimal data available on the incidence of *Bd* in central Virginia, as no extensive studies of this region have occurred. Only one study of *Bd* in central Virginia is available (Mitchell & Green 2002). Thus it is difficult to know how long *Bd* has been present in these areas and whether or not massive die-offs occurred in the past. Other studies along the eastern half of the United States have found results similar to ours. Steiner & Lehtinen (2008) found *Bd* in *Acris crepitans* in the midwest without any cases of chytridiomycosis. *Bd* has also been found in *Lithobates sphenocephalus*, *L. catesbeianus*, and *L. clamitans* (among other species) along the east coast without disease (Longcore et al. 2007, Peterson et al. 2007, Rothermel et al. 2008). Ouellet et al. (2005) found cases of *Bd* infection as far back as 1960 in multiple species of anurans and salamanders in North America.

Bd and urbanization

It is important in cases of emerging diseases not only to determine where a pathogen is located, but to understand the type of habitats with which it is associated. Previous research has attempted to identify the habitat requirements of *Bd*. Woodhams & Alford (2005) demonstrated that environmental conditions affected chytridiomycosis in frogs within tropical Queensland Australia; infection prevalence at the examined sites was greater in cooler seasons and at higher elevation. Kriger & Hero (2007) found an association between *Bd* and permanent water bodies, although *Bd* has been found occasionally among completely terrestrial species (Cummer et al. 2005, Ouellet et al. 2005). In a world largely influenced by the human presence, we also need to question whether habitat alteration and anthropogenically influenced lands have an effect on *Bd* prevalence. During our survey of central Virginia, we found that *Bd* prevalence did not vary along a range of urbanization. For the present study, urbanization specifically referred to population density and general anthropogenic influence including physical disturbance. According to our findings, it appears that *Bd* is evenly distributed across the studied habitats, despite urbanization variations along the gradient. This is consistent with a recent study in Ontario, Canada, that found no relationship between *Bd* among *Lithobates clamitans* and 4 measures of human habitat modification: human disturbance, distance to road, distance to industry, and distance to housing (St. Amour et al. 2008). In stating our results, it is important to be cautious in interpreting them, as less-urbanized areas can also be subjected to other anthropogenic disturbances that can have negative impacts on amphibian populations (McCoy et al. 2008), such as excess inputs of nitrogen (Rouse et al. 1999), pesticides (Christin et al. 2003), herbicides (Rohr et al. 2006), and other chemical contaminants (Sparling et al. 2000). In addition, there are multiple other variations among the 6 sites, such as presence of agricultural activity, plant population density, and river or pond access, which may also impact *Bd* prevalence. Thus the apparent lack of impact of urbanization on *Bd* incidence in our study must be recognized as based only on the limited definition of human population density. For the purposes of this study, only 6 sites were surveyed due to the frequency of visits. It is essential to recall that this survey covered only 2 years; in order to truly understand the dynamics of *Bd* there is need for a longer term survey. It would be essential for future studies to include more sites over a wider geographic range to extend our knowledge of *Bd*.

Bd and seasonality

Bd prevalence did not differ significantly among sites. However, *Bd* prevalence did differ significantly between seasons. *Bd* prevalence peaked at 45% in May when temperatures were between 18 and 19°C. The data from this survey support Kriger & Hero (2006), who found that in Queensland, Australia, *Bd* prevalence in the stony creek frog *Litoria wilcoxii* peaked at 58% when temperatures were between 12.3 and 19.4°C. These findings are also consistent with laboratory studies, showing that the fungus prefers temperatures between 17 and 25°C (Piotrowski et al. 2004). This variation with season might have an effect on the lack of chytridiomycosis within the studied area, given that the spores of the *Bd* fungus are not able to thrive at high temperatures (above 25°C) or in dry climates (Johnson et al. 2003). During the summer and autumn in Virginia, when *Bd* prevalence is at its lowest, the average temperatures are above 25°C and many areas can be very dry. As a result, the climate in Virginia and surrounding areas may be contributing to the lack of infection within the amphibian populations studied.

CONCLUSION

The present study of *Bd* is novel in that we took a systematic approach to surveying a wide range of species to not only understand the spatial extent of this fungus, but to evaluate possible associations between *Bd*, urbanization, and seasonal changes. This is important in understanding the characteristics and life cycle of *Bd* in its natural setting. It is also necessary in producing predictive and preventative approaches against this global emerging disease. This was the first extensive analysis of *Bd* in Virginia, supporting the hypothesis that *Bd* is endemic to central Virginia. *Bd* prevalence peaked in the spring when temperatures were between 14 and 25°C and dropped to below 2% when above or below this temperature range. Despite *Bd* prevalence reaching as high as 45% in the spring, no individuals were diagnosed with the clinical disease. Weather patterns may be contributing to the lack of chytridiomycosis infections in certain areas, such as in central Virginia, as the present study shows.

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