

Temperature alone does not explain patterns of *Batrachochytrium dendrobatidis* infections in the green frog *Lithobates clamitans*

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ABSTRACT: *Batrachochytrium dendrobatidis* (*Bd*) is an invasive, waterborne fungal pathogen that has caused significant declines and extinctions of amphibian species worldwide. Temperature is a major factor impacting the growth and spread of *Bd*, but little is known regarding the associated patterns in natural habitats. This study analyzed the temperature-associated trends, as correlated with season and microhabitat, of *Bd* prevalence and infection intensity in green frogs *Lithobates clamitans* in a temperate environment (central Ohio, USA). *Bd* was widely distributed at the study sites and found in more than half of the frogs sampled. *Bd* prevalence was significantly higher in the spring and in forested stream habitats compared to emergent wetland habitats. In contrast, *Bd* infection intensities tended to be higher in summer. Given the known temperature sensitivity of *Bd* as demonstrated in laboratory studies, these findings suggest that temperature may be an important factor determining *Bd* prevalence in green frogs at our study sites, but that factors other than temperature are more important in determining infection intensity. Our findings suggest that future monitoring of *Bd* among vulnerable species in regions experiencing seasonal temperature variation should study a range of environmental variables to better understand the dynamic relationship between *Bd* and its amphibian hosts.

KEY WORDS: Season · Microhabitat · Chytrid

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INTRODUCTION

The fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) has been implicated in the declines and extirpations of many amphibian populations worldwide (Mendelson et al. 2006, Wake & Vredenburg 2008, Blaustein & Johnson 2010). *Bd* kills amphibians by interfering with external water exchange, thereby disrupting ion balance and leading to heart failure (Voyles et al. 2007, 2009). Temperature appears to be a major factor affecting the interaction of *Bd* with amphibian hosts (Woodhams et al. 2003, Berger et al. 2004, Piotrowski et al. 2004, Puschendorf et al. 2009, Daskin et al. 2011). *Bd* has an optimal temperature range of 17 to 23°C (Piotrowski et al. 2004, Voyles et al. 2011) and an operative temperature range of 4 to

28°C (Woodhams et al. 2003). *Bd* dies at temperatures exceeding 30°C (Woodhams et al. 2003). Reproduction slows at 7 to 10°C (Voyles et al. 2011) and stops around 4°C (Woodhams et al. 2003), but the fungus can overwinter at temperatures approaching freezing on frogs in temperate regions (Garner et al. 2011, Savage et al. 2011). Laboratory studies on *Bd* conducted at 10 and 23°C have demonstrated that the fungus reproduces faster (i.e. produces more zoospores per unit time), is colonial, and is more infective at 23°C, whereas it produces more zoospores per sporangium at 10°C (Woodhams et al. 2008). The pathogenicity of *Bd* is more pronounced at 17°C than at 27°C (Berger et al. 2004). However, these laboratory results need to be extended to the field to understand how the fungus interacts with its amphibian hosts in natural environments.

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Large-scale *Bd*-related amphibian declines correspond to ecological 'hot spots' for amphibian diversity (Lips et al. 2006, Pounds et al. 2006). The climate of these regions generally matches the optimal temperature range of the pathogen, as indicated by laboratory studies (Bustamante et al. 2005). Impacts of *Bd* appear less strong in lowland tropical areas, where temperatures often exceed the thermal tolerance of *Bd* (Blaustein & Dobson 2006, Brem et al. 2007). However, it is unclear how both seasonal and daily variation in temperatures that are characteristic of a temperate environment may influence *Bd* pathogenicity and virulence.

Seasonal and daily temperature variation may also affect the host immune system. For example, there is evidence that *Bd* infection intensity may increase in amphibians over-wintering in hibernacula at low temperatures (Maniero & Carey 1997, Garner et al. 2011, Savage et al. 2011, and reviewed by Rollins-Smith et al. 2011), and this may facilitate spread of the fungus during the subsequent spring mating behavior, increasing the number of infected amphibians in the spring (Belden & Harris 2007). Therefore, temperate regions present interesting challenges to both host immunity and pathogen physiology that are not found in tropical regions. The dynamics which occur between host and pathogen in a temperate region may provide important insights for global amphibian conservation.

The strong seasonal variation in the climate of many temperate environments may have significant effects on the pathogenicity and reproduction rate of *Bd*. As discussed above, laboratory studies have found that temperature affects many aspects of the biology of *Bd*. Among wild populations, Kinney et al. (2011) found that 27% of sampled gopher frogs *Lithobates areolatus* in Indiana, USA, were *Bd* positive when they entered breeding pools, whereas 46% were *Bd* positive when they left the pools. It is likely that summer temperatures in temperate climates often exceed the lethal temperature for *Bd*, which may explain why cricket frogs *Acris crepitans* in Texas, USA, that tested positive in spring had cleared infections when temperatures increased in early summer (Gaertner et al. 2009). Other studies also indicate that as temperatures rise seasonally, *Bd* prevalence among amphibians declines (Kriger & Hero 2007a, Duncan Pullen et al. 2010).

Microhabitats utilized by amphibians experience different temperature profiles and different seasonal temperature variations that are likely to influence *Bd* population dynamics. Kriger & Hero (2007b) tested amphibians with different breeding strategies (e.g.

terrestrial, stream, pond, etc.) to determine differences in *Bd* prevalence by habitat, and found low *Bd* prevalence in species breeding in ephemeral ponds, increased prevalence in permanent pond breeders, and the highest prevalence in permanent stream breeders. Padgett-Flohr & Hopkins (2010) found that *Bd* persisted throughout the year in a Mediterranean climate despite monthly mean maximum temperatures only overlapping the optimal *Bd* temperature range 4 mo each year. Kinney et al. (2011) encountered *Bd* more commonly among amphibians living in deeper, cooler, permanent pools than in shallow, warmer, semi-permanent systems, and Conradie et al. (2011) found *Bd* to be more prevalent in gently flowing streams than in non-moving bodies of water or fast-moving streams. Foothill yellow-legged frogs *L. boylei* in central California had higher *Bd* prevalence in pool habitats with lower water temperatures (Lowe 2009). In a study of headwater streams in mountainous regions of the United States, *Bd* was detected in only 3% of 1322 individuals of 21 different species, suggesting that *Bd* is not common in headwater streams (Hossack et al. 2010.)

The overall aim of this study was to examine the dynamics of *Bd* in amphibian populations in a temperate environment and to gain insight into the potential impact of the fungus on the amphibian communities in such areas. Based on the thermal tolerance of *Bd*, the influence of temperature on amphibian immune response, and the temperature profile of a temperate region, we hypothesized that infection rates and intensities would be higher when temperatures were lower and more constant than when temperatures were higher and more variable. Here, we examined the correlation between temperature (in the context of season and microhabitat) and *Bd* prevalence and infection intensity in a temperate deciduous forest region.

MATERIALS AND METHODS

Study species

Our study focused on analyzing the effect of season and habitat on *Bd* infections in green frogs *Lithobates clamitans*. *L. clamitans* is a common, diurnal, generalist species that tolerates a broad temperature gradient, inhabits a wide variety of aquatic environments, and spends considerable time both in water and on land (Martof 1952). Forzán et al. (2010) reported that *Bd* was consistently detected among *L. clamitans* in the Canadian province Prince Edward

Island, and preliminary sampling for *Bd* in amphibian communities in central Ohio, USA, found that this species had the highest percent of infected individuals (Korfel 2012). *L. clamitans* are fairly tolerant of *Bd* (James et al. 2009, Schloegel et al. 2009, Kiesecker 2011 Gahl et al. 2012). *L. clamitans* therefore provided both an excellent estimate of *Bd* presence in the environment and the opportunity to test hypotheses regarding the impact of temperature as influenced by both habitat and season in our study area. *Bd* infection rates and intensities were studied in frogs during their entire annual period of activity in central Ohio.

Sampling sites and schedule

Sampling was conducted in protected park areas in the metropolitan area of Columbus, Ohio (40° N, 83° W, elevation range: 200 to 345 m above sea level). Sampling was performed for approximately 4 h weekly at each site (Sharon Woods, Battelle Darby, Blacklick, and Highbanks Metroparks) from spring through autumn in both 2010 (March 13 to November 11) and 2011 (April 14 to October 24) during the period of surface activity of green frogs. Two distinct habitats were sampled: emergent wetlands (n = 2) and closed canopy streams (n = 2).

Frog collection and swab sampling

Juvenile and adult green frogs were captured individually in plastic bags. Sampling for *Bd* was performed on frogs while they remained confined in the plastic bags. Each frog was swabbed following the techniques of Brem et al. (2007) and Briggs & Burgin (2003). The ventral drink patch was swabbed 10 times, and each hind thigh and foot was swabbed 5 times each (total of 30 strokes ind.⁻¹). Swabs were air dried, labeled, and stored in the freezer until they were processed by quantitative PCR analysis. Frogs were also marked by toe clipping (Heyer et al. 1994) so that *Bd* infections could be followed through time. A total of 273 green frogs were sampled during the 2010 (n = 33) and 2011 (n = 240) seasons.

PCR analysis and zoospore genomic equivalents

Taqman real-time PCR analyses of swab samples were conducted at the San Diego Zoo Amphibian

Disease Laboratory and followed a modified protocol by Boyle et al. (2004). Samples were run in triplicate, and the total sample dilution of zoospores was 100. If 2 of 3 wells were positive, then the sample was determined to be positive and indicative of a low level of infection. Zoospore genomic equivalents (GEs) were calculated by averaging the results for the 3 wells and multiplying by 100 to account for the dilution of the sample. GEs were log-transformed prior to statistical analysis.

Temperature data

In 2011, temperature data loggers (HOBO pendant temperature/light data logger 64k-UA-002-64; Onset Computer Corporation, <http://www.onsetcomp.com/products/data-loggers/ua-002-64>) were deployed at several of the sites we sampled in both 2010 and 2011. Two loggers were placed at each site to record ground surface and water temperatures, and each was set to collect 1 reading each hour. Terrestrial loggers were placed within 2 m of the aquatic habitat and covered with a thin layer of leaf litter. Aquatic data loggers were placed on the bottom of the aquatic habitat against the substrate to ensure aquatic readings regardless of changes in water level.

RESULTS

Prevalence and infection intensity

Bd was widely distributed in *Lithobates clamitans* at our field sites. *Bd* prevalence from spring through fall was statistically similar in 2010 and 2011: 51.5% infected (n = 33) and 49.2% infected (n = 240) respectively (p = 0.854, Fisher's exact test, 2-tailed). Infection intensities in individual frogs ranged dramatically from <1 to 190 755 zoospore GEs. Mean infection intensity among infected frogs was 22 250.8 ± 46945.1 GEs in 2010 and 678.8 ± 6698.5 GEs in 2011, but this difference was not statistically significant (1-way ANOVA, p < 0.001).

Seasonal and habitat temperature data

Ground surface and aquatic temperatures differed significantly among the different seasons and between different habitats (Table 1, Fig. 1). Mean

Table 1. Aquatic temperatures by season for forested stream and emergent wetland habitats in 2011. Data are based on hourly readings collected during the specified season of amphibian activity: spring (April 9 – May 31); summer (June 1 – August 31); autumn (September 1 – November 30); winter (December 1–30). SD: average standard deviation of daily temperatures calculated to determine the variation among temperatures recorded over a 24 h period

Season	n	Aquatic temp. (°C)			
		Mean	Max.	Min.	SD
Forested stream habitat					
Spring	1284	15.14	23.77	7.48	1.21
Summer	2140	20.35	27.57	16.33	0.63
Autumn	2186	12.20	19.66	4.73	0.37
Winter	707	4.85	9.37	1.00	0.64
Annual	6317	14.74	27.57	1.00	0.66
Emergent wetland habitat					
Spring	1284	17.76	33.95	6.57	2.48
Summer	2208	24.72	33.12	18.33	0.95
Autumn	2185	13.83	28.85	5.96	0.56
Winter	707	5.70	7.38	4.00	0.16
Annual	6317	17.49	33.95	4.00	1.03

emergent aquatic temperatures were significantly higher than mean closed canopy stream aquatic temperatures (1-way ANOVA, $p < 0.001$) (Fig. 1a), and emergent aquatic temperatures were more variable than closed canopy stream temperatures (Fig. 1b). Water temperatures were most variable at emergent sites in the spring (average standard deviation of daily temperature, 2.48°C) and least variable in closed canopy streams in the autumn (average standard deviation of daily temperature, 0.37°C) (Fig. 1). The water temperature range in emergent wetlands was 28°C during the entire monitoring period of amphibian activity, whereas the temperature range in forested streams was 22°C for the same period. The amount of daily temperature variation increased from spring to summer. Variations in temperature in both 2010 and 2011 were slightly higher than averages from the past 10 yr (NOAA 2012) during the months of amphibian activity (March to October), and 2011 received approximately 140% the normal precipitation.

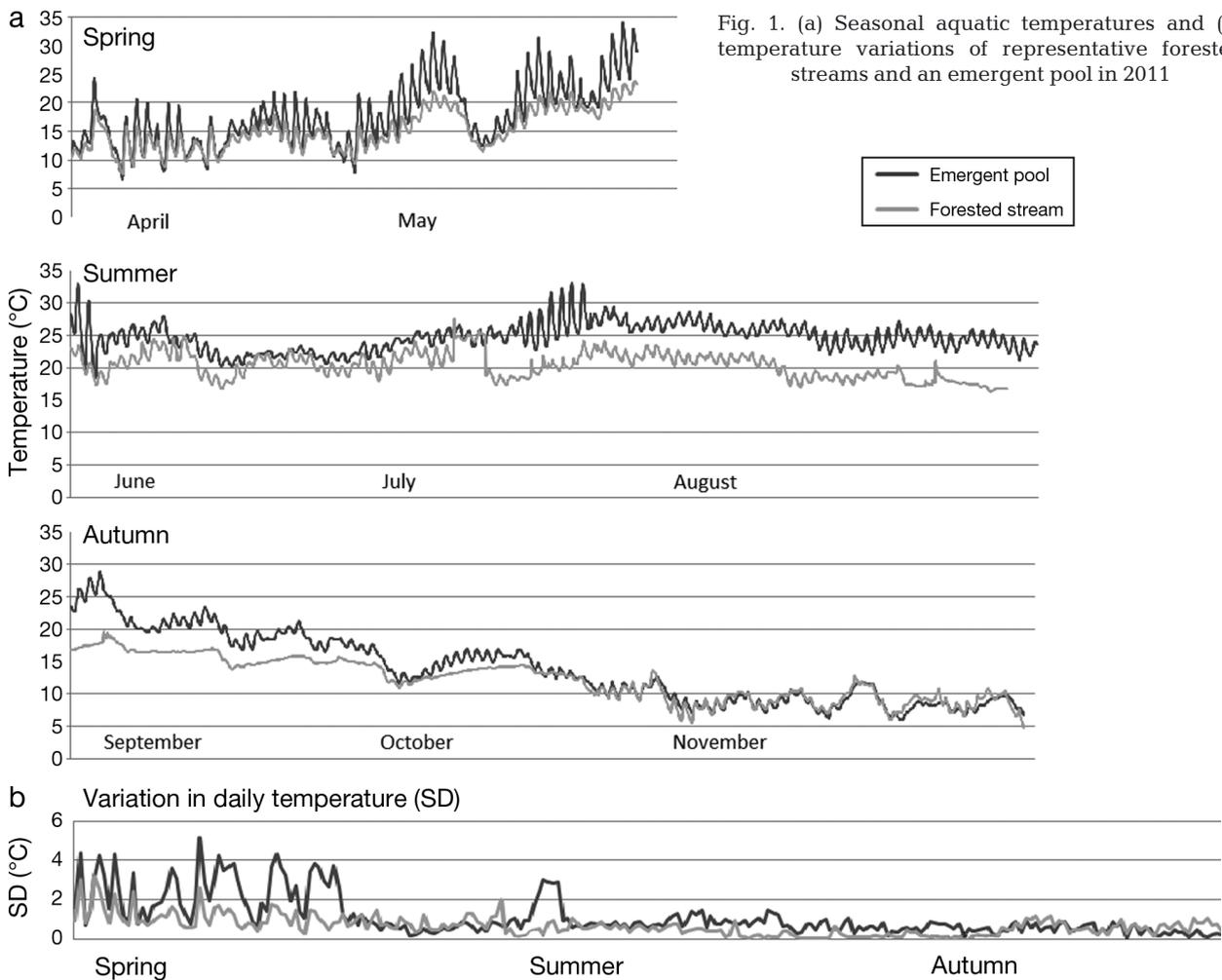


Fig. 1. (a) Seasonal aquatic temperatures and (b) temperature variations of representative forested streams and an emergent pool in 2011

Habitat differences in *Bd* prevalence and intensity

Bd prevalence in *L. clamitans* was significantly higher in closed canopy streams (52.6% positive, $n = 247$) than in emergent wetlands (15.4%, $n = 26$) (Fisher exact test, $p < 0.001$). In contrast, infection intensities tended to be higher in emergent wetlands ($18\,164.0 \pm 36\,206.9$ GEs, $n = 4$) than in closed canopy streams ($2961.8 \pm 18\,160.4$ GEs, $n = 130$).

Seasonal differences in *Bd* prevalence and intensity

Sampling data were grouped according to the following seasons: spring (March 13 – May 19), summer (June 8 – August 29), and autumn (September 12 – November 11). Because of limited samples collected from emergent wetlands, the following analyses are limited to samples collected from forested streams (Fig. 2). *Bd* prevalence at these sites was significantly different by season (Fisher's exact test, $p < 0.001$). *Bd* prevalence in *L. clamitans* peaked in spring (89% infected, $n = 36$), was lowest in the summer (38% infected, $n = 104$), and increased in autumn (55% infected, $n = 107$). Infection intensities demonstrated a different seasonal pattern. GE values were highest among summer samples ($8239.5 \pm 31\,852.5$ GEs, $n = 40$), lowest in spring (72.2 ± 230.2 GEs, $n = 33$), and decreased from summer to autumn (1040.3 ± 6455.5 GEs, $n = 61$). However, none of the differences in *Bd* infection intensity between seasonal samples were sta-

tistically significant (MANOVA pairwise comparison: autumn–spring $p = 0.24$, autumn–summer $p = 0.06$, spring–summer $p = 0.78$).

Infection intensity and ambient temperature

When data from all infected frogs in all seasons and from both habitats were combined, mean daily temperatures, maximum daily temperatures, and daily standard deviations of both aquatic and terrestrial temperatures were not correlated with *Bd* infection intensity (Fig. 1).

Data from recaptured frogs

Seven *L. clamitans* were recaptured multiple times during this study. All of the recaptured frogs carried infections in either spring or autumn, but 4 of them were infection-free in the summer. Infection intensities were low (<115 GEs, mean: 22.7 GEs) for all of the recaptured frogs.

Results from dead and/or frogs displaying clinical signs of disease

During the course of the study 5 *L. clamitans* were sampled that displayed signs that can be associated with chytridiomycosis. Two of these frogs tested positive for *Bd*, but infection intensities were low (<1.0 GE). One of 7 frogs that likely had been dead from 1 to several days tested positive for *Bd* (<1.0 GE). We cannot say conclusively that any of these frogs were sick or died due to *Bd* infection.

DISCUSSION

Prevalence and intensity of *Bd* infections in *Lithobates clamitans*

Our study documented widespread *Bd* prevalence in *L. clamitans* populations in central Ohio. Half of the *L. clamitans* sampled in this study tested positive for *Bd*. The high percentage of *Bd*-positive *L. clamitans* suggests that *Bd* is present in most aquatic environments in the region, and, although *L. clamitans* populations appear to be robust, most individuals likely are exposed to the pathogen. High infection intensities of *Bd* zoospores, as high as 2×10^6 GEs, were observed in asymptomatic *L. clamitans*. Some

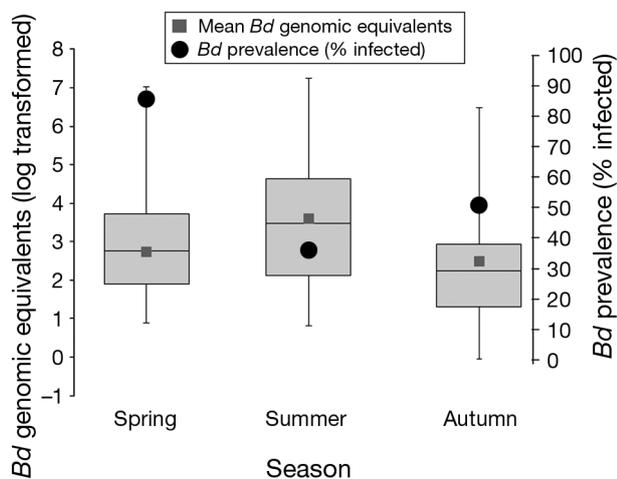


Fig. 2. Seasonal trends in *Batrachochytrium dendrobatidis* (*Bd*) prevalence (percent infected) and infection intensities measured in genomic equivalents of *Bd* among *Lithobates clamitans* in forested stream habitat. Box plots display maximum, minimum, interquartile range, and mean value

studies have proposed that the lethal GE threshold of 10^4 observed in mountain yellow-legged frogs *L. muscosa* by Vredenburg et al. (2010) may be generalizable to other frog species (Kinney et al. 2011). Our findings raise questions about the general applicability of a 10^4 lethal GE threshold for anuran amphibians.

Seasonal and habitat patterns of *Bd* prevalence and infection intensity

This study documented both seasonal and habitat differences in *Bd* prevalence and infection intensities in *L. clamitans* populations in central Ohio. Statistically significant differences in seasonal patterns of *Bd* prevalence were observed in closed canopy stream habitats that afforded a large sample size. In these forested streams the lowest prevalence occurred in the summer and higher prevalence rates were found in the spring and autumn. There were no significant differences observed in seasonal patterns of infection intensities, although there was a trend of increased infection intensities in summer. *Bd* prevalence and infection intensity therefore showed different seasonal patterns. The 2 habitat types sampled in this study (closed canopy streams and emergent wetlands) also showed different patterns of *Bd* prevalence and infection intensity. *Bd* prevalence was significantly higher in frogs collected from closed canopy streams. In contrast, infection intensities tended to be higher among frogs in emergent wetlands, although the difference was not statistically significant.

The different seasonal and habitat patterns observed for *Bd* prevalence and *Bd* infection intensity were unexpected. Based on the known temperature sensitivity of *Bd*, we expected both prevalence and infection intensity to be strongly tied to seasonal and habitat temperature trends and to be higher in lower temperature habitats and at lower temperature times of the year. The higher prevalence of *Bd* during the lower temperature spring and in the lower temperature closed canopy stream habitat supported this hypothesis. Forrest & Schlaepfer (2011) found 75 to 100% *Bd* prevalence among amphibians in Arizona, USA, in water at temperatures $<15^{\circ}\text{C}$ (similar to findings in spring and autumn in our study) and much lower prevalence ($<10\%$) in water at temperatures $>30^{\circ}\text{C}$ (similar to summer and emergent habitat findings in our study). Other studies have found that *Bd* is more prevalent in more shaded, lower temperature habitat in the temperate USA (Raffel 2010) and in

gently flowing streams compared to non-flowing water in African grasslands (Conradie et al. 2011). It is likely that shaded habitats and flowing water maintain lower temperatures more suitable for *Bd* survival (Raffel 2010).

In contrast to *Bd* prevalence, the seasonal and habitat patterns of *Bd* infection intensity were unexpected. Although no statistically significant relationship was found, infection intensities tended to be higher (rather than lower) in the summer months and in emergent wetland sites where temperatures were higher. Combined analysis of the entire sample of infected frogs from all seasons and both habitat types showed no relationship between infection intensity and daily mean, maximum, or standard deviation of sampling site temperatures. *Bd* infection intensity therefore did not show the negative relationship with ambient temperature associated with rates of *Bd* prevalence. Certain studies suggest that temperature should influence *Bd* infection intensity. For example, Maniero & Carey (1997) suggest that *Bd* infections are likely to increase when amphibian immune function is reduced at lower and more variable temperatures. Longo et al. (2010) report season as a strong predictor of both *Bd* prevalence and infection intensity among direct-developing *Eleutherodactylus coqui* and *E. portoricensis* in a tropical system. However, seasonal variation in tropical systems typically emphasizes differences in precipitation rather than temperature, so ambient temperature may not have been the dominant factor affecting *Bd*-host dynamics in this study. One possible explanation for our findings among green frogs in Ohio is that host immunity increases as temperature increases, so frogs tolerate higher *Bd* loads before succumbing to infection. Therefore, less *Bd*-associated mortality at higher rates of infection may lead to a higher mean infection burden in summer months. It is also possible that different environmental and/or organismal factors may contribute to seasonal and habitat patterns of *Bd* prevalence and *Bd* infection intensity in green frog populations in temperate central Ohio.

Potential factors behind seasonal and habitat patterns of *Bd* prevalence and infection intensity

Temperature is thought to be a major factor determining *Bd* pathogenicity and amphibian host susceptibility to *Bd* (Woodhams et al. 2003, Berger et al. 2004, Piotrowski et al. 2004, Puschendorf et al. 2009, Daskin et al. 2011). However, in our study, only *Bd* prevalence, and not *Bd* infection intensity, was corre-

lated with temperature. It is possible to speculate on the reasons for the different relationships of prevalence and infection intensity to temperature in our study. As mentioned above, Maniero & Carey (1997) suggest that *Bd* infections are likely to increase when amphibian immune function is reduced at lower and more variable temperatures. *Bd* prevalence is also likely to increase at relatively low temperatures. Woodhams et al. (2008) demonstrated that *Bd* zoospores live longer in lower temperature water and that *Bd* responds to cold shocks by temporarily increasing zoospore production. *Bd* reproduces faster at higher temperatures, zoospores were faster to encyst at higher temperatures (ultimately resulting in increased infectivity at 23°C compared to 10°C), and zoospores died at temperatures >30°C (Woodhams et al. 2008). Voyles et al. (2012) found that despite lower population growth rates at 4°C compared to 23°C, *Bd* activity rates were increased, which may ultimately result in higher zoospore encounter rates for amphibians at lower temperatures. According to these findings, we would expect *Bd* infections to be severe at lower temperatures when amphibian host immune systems are suppressed, the likelihood of encountering zoospores is elevated, and when *Bd* biology favors pathogenicity. We would expect *Bd* infections to be mild when temperatures are >30°C and overall *Bd* prevalence is low. However, results from our fieldwork indicate that infection intensity was not related to temperature and, in fact, tended to be highest during higher temperatures when *Bd* prevalence was lowest.

It is possible that high temperatures characteristic of emergent pools and summer months in central Ohio may favor re-encystment (increasing infection intensity on a single frog) but reduce the chance of *Bd* zoospores infecting a new host (decreasing prevalence within the population). The higher infection intensities observed in summer months may be explained by direct temperature effects on the fungus (i.e. facilitated re-encystment) but most likely are related to differential susceptibility among frogs in a population. Other stressors in higher temperature environments (e.g. parasites, etc.) may make certain individuals less capable of suppressing *Bd* infections. We suggest that future studies of *Bd* pathogenicity consider stress at the individual level.

CONSERVATION IMPLICATION

Although *L. clamitans* appears to be highly resistant to *Bd*, the seasonal and habitat patterns of the *Bd*

dynamics observed in this study may have implications for other more susceptible amphibian species. High infection prevalence in spring and high infection intensities in summer are of particular concern. Infection intensity likely involves a complex synergy of factors that include environmental variables, host immune system capabilities, and the timing of exposure. Frogs with high-intensity infections did not exhibit any symptoms of disease; however, none of these frogs were recaptured to determine survival. A small number of frogs with low-intensity infections were recaptured throughout the seasons and provide evidence that this species can gain and clear low-intensity infections. Our results suggest high-intensity infections may be of greater concern than widespread prevalence because it is high-intensity infections that likely result in mortality. Species more susceptible to *Bd* than *L. clamitans* that experience similar seasonal patterns of infection may display higher mortality.

Based on our findings, future monitoring for *Bd* presence in temperate areas such as Ohio should focus on springtime when *Bd* is most common. Amphibians emerging from overwintering sites may have reduced immune function in lower and fluctuating temperatures (Garner et al. 2011), as well as skin-to-skin contact during breeding events combined with optimal temperatures for *Bd* reproduction and decreased mortality, may exacerbate *Bd* spread. In contrast, monitoring for *Bd*-induced mortality, which likely occurs when *Bd* infection intensities reach a species-specific threshold, is dependent on factors other than temperature alone.

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