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Survival of three species of anuran metamorphs exposed to UV-B radiation and the pathogenic fungus *Batrachochytrium dendrobatidis*

T. S. Garcia[1, 2,*], J. M. Romansic[1], A. R. Blaustein[1]

[1]**Department of Zoology, 3029 Cordley Hall, Oregon State University, Corvallis, Oregon 97331-2914, USA**

[2]***Present address:*** **Department of Fisheries and Wildlife, 104 Nash Hall, Oregon State University, Corvallis, Oregon 97331-3803, USA**

ABSTRACT: When exploring the possible factors contributing to population declines, it is necessary to consider multiple, interacting environmental stressors. Here, we investigate the impact of 2 factors, ultraviolet radiation and disease, on the survival of anuran amphibians. Exposure to ultraviolet-B (UV-B) radiation increases mortality and results in various sub-lethal effects for many amphibian species. Infectious diseases can also negatively impact amphibian populations. In this study, we exposed metamorphic individuals (metamorphs) to both UV-B and *Batrachochytrium dendrobatidis* (BD), a fungal pathogen and cause of the disease chytridiomycosis, and monitored survival for 3 wk. We tested for possible interactions between UV-B and BD in 3 species: the Cascades frog *Rana cascadae*; the Western toad *Bufo boreas*; and the Pacific treefrog *Hyla regilla*. We found strong interspecific differences in susceptibility to BD. For example, *R. cascadae* suffered a large increase in mortality when exposed to BD; *B. boreas* also experienced mortality, but this effect was small relative to the *R. cascadae* response. *H. regilla* did not show any decrease in survival when exposed to either factor. No synergistic interactions between UV-B and BD were found for any of the test species. A previous study investigating the impact of BD on larval amphibians showed different species responses (Blaustein et al. 2005a). Our results highlight the importance of studying multiple life history stages when determining the impact of environmental stressors. The contrast between these 2 studies emphasizes how vulnerability to a pathogen can vary between life history stages within a single species.

KEY WORDS: Emerging infectious diseases · *Batrachochytruim dendrobatadis* · Ultraviolet radiation · Multiple stressors · Amphibian metamorph

INTRODUCTION

The loss of biodiversity is a major international concern. The current rate of extinction may be greater than any known in the last 100 000 years (Eldridge1998). Despite the recognition that there are complex dynamics contributing to species loss, studies often focus on the direct effects of single factors. This is illustrated in attempts to understand the global decline of amphibian populations (Houlahan et al. 2000, Stuart et al. 2004). Recent studies directed at this problem suggest that global amphibian losses are the result of interactions between a number of highly context-dependent causal factors (Alford & Richards 1999, Blaustein & Kiesecker 2002, Collins & Storfer 2003), highlighting the need to investigate multiple stressors and synergistic impacts.

Major factors contributing to certain amphibian population declines include changes in atmospheric conditions and infectious diseases (Kiesecker & Blaustein 1995, Pounds et al. 1999, Blaustein et al. 2001, Kiesecker et al. 2001, Pounds & Puschendorf 2004, Pounds et al. 2006). Changes in temperature, cloud cover, precipitation patterns and changes in stratospheric ozone levels may all affect amphibians. In addition, several infectious diseases have been associated with amphibian population declines, such as oomycetes, fungi, bacteria and viruses (Drury et al. 1995, Jancovich et al. 1997, Haydon et al. 2002, Daszak et al. 2003).

*Email: tiffany.garcia@oregonstate.edu

Batrachochytrium dendrobatidis (BD) is one emerging infectious disease that appears to be affecting amphibian populations on a global scale (Berger et al. 1999, Daszak et al. 2003). Many aspects of how this fungus interacts with amphibians are unknown. For example, the means and rate of transmission under field conditions, the prevalence of infection amongst amphibian populations and the overall effect of the fungus on amphibians in the wild are poorly understood (Nichols et al. 2001, Vredenburg & Summers 2001, McCallum 2005). Furthermore, it is uncertain if BD is a novel pathogen or whether it has been chronically present with occasional disease outbreaks of chytridiomycosis, the disease caused by BD (Daszak et al. 2004, McCallum 2005, Ouellett et al. 2005, Rachowicz et al. 2005). Environmental co-factors could interact with BD to induce outbreaks, increase the virulence of BD directly, or hamper immune systems to increase the susceptibility of amphibian hosts.

Cofactors are implicated in the emergence and transmission of various pathogens infecting amphibians (Kiesecker & Blaustein 1995, Cunningham et al. 1996, Taylor et al. 1999, Kiesecker & Skelly 2001, Kiesecker et al. 2001, Kiesecker 2002, Blaustein & Johnson 2003, Christin et al. 2003, Gendron et al. 2003, Johnson & Chase 2004) including BD (Bosch et al. 2001, Pounds 2001, Parris & Beaudoin 2004, Parris & Cornelius 2004). Increasing UV radiation and contaminants from acid precipitation have been proposed as potential cofactors in outbreaks of BD (Blaustein & Kiesecker 2002) in western North America. Ultraviolet B (UV-B) enhances the susceptibility of *Bufo boreas* eggs to oomycete infection (Kiesecker & Blaustein 1995, Kiesecker et al. 2001) and may do the same when BD is present. In Colorado, where amphibians are especially sensitive to low pH (Harte & Hoffman 1989, Kiesecker 1996), a number of *B. boreas* have been found with BD (Muths et al. 2003). Thus, amphibians in Colorado may be more prone to BD infection in the presence of episodic acidification (Harte & Hoffman 1989).

In this paper, we investigated potential synergistic interactions between BD and UV-B (280 to 315 nm) radiation. UV-B radiation is an environmental co-factor that may enhance the effects of BD for a number of reasons. UV-B radiation is often harmful to amphibians and can be lethal to some species at certain developmental stages (Blaustein & Belden 2003). Moreover, UV-B may induce a number of sublethal effects in amphibians including physiological, developmental and behavioral anomalies (Blaustein et al. 2001). Stress from UV-B exposure may cause some amphibian species to become more susceptible to pathogenic infection. UV-B radiation interacts synergistically with at least one other pathogen, the oomycete *Saprolegnia ferax*, increasing mortality in amphibians when both UV-B and the pathogen are present (Kiesecker & Blaustein 1995, Kiesecker et al. 2001).

Climatic conditions influence UV-B exposure rates and the effects of water-borne pathogens. For example, UV-B exposure rates are, in part, determined by water depth, which depends on winter precipitation. In the Oregon Cascade Range, winter precipitation is modified by El Niño/Southern Oscillation events, resulting in a link between large-scale climatic patterns and mortality from *Saprolegnia ferax* in amphibian populations (Kiesecker et al. 2001). It is reasonable to assume that other pathogens, such as BD, would interact similarly with UV-B radiation. Recently, it has been hypothesized that harlequin frog populations in tropical America have declined due to a complex interaction of global warming, changes in moisture gradients and BD outbreaks (Pounds et al. 2006).

SYSTEM

Recently metamorphosed individuals (metamorphs) of 3 amphibian species were used in this study: the Cascades frog *Rana cascadae*, the Western toad *Bufo boreas* and the Pacific treefrog *Hyla regilla*. These 3 species were chosen because they vary in their susceptibility to UV-B radiation (Blaustein et al. 1998). Testing amphibian species with differential sensitivity to UV-B radiation allows us to quantify the range of possible synergisms with BD. All 3 species have aquatic larval stages and co-occur throughout much of the Oregon Cascade Range.

Blaustein et al. (2005a) examined larval mortality in these 3 amphibian species in response to BD exposure. *Bufo boreas* tadpoles were highly susceptible to BD, often dying immediately after BD exposure. *Rana cascadae* larvae, however, did not suffer significant mortality after exposure to BD, nor did *Hyla regilla* larvae. Sensitivity to environmental stressors, however, often varies between life history stages, and susceptibility to pathogens at one stage does not denote susceptibility at all stages. For example, *R. cascadae* suffers heavy mortality when exposed to UV-B radiation in the egg stage, while exposure at the larval stage may cause more sub-lethal damage than mortality (Blaustein et al. 1998). *B. boreas* is sensitive to UV-B exposure in its embryonic stage (Blaustein et al. 1994a) and after metamorphosis (Blaustein et al. 2005b) but this species is relatively resilient to UV damage as larvae (Little et al. 2003). *H. regilla*, however, is less susceptible to UV-B damage at the embryo stage (Blaustein et al. 1998) compared to the other species tested in this study, while long-term exposure to ambient UV-B can induce physiological and developmental abnormalities in me-

tamorphs and larvae (Hays et al. 1996). This study examined the effect of BD and UV-B exposure on the survival of metamorphs for all 3 species.

MATERIALS AND METHODS

Recently metamorphosed juvenile anurans were collected on September 2, 2005, from endemic breeding sites in the central Oregon Cascade Range: *Rana cascadae* and *Hyla regilla* at a sub-alpine meadow 2 km NW of Todd Lake, Deschutes County, Oregon, and *Bufo boreas* from Todd Lake, Deschutes County, Oregon. All animals were held at Oregon State University, Corvallis, Oregon, in a temperature-controlled room with natural photoperiod and fed crickets ad libitum during the 5 d holding period.

We used a $3 \times 2 \times 2$ factorial design; 3 species treatments (*Rana cascadae*, *Bufo boreas* and *Hyla regilla*), 2 UV-B treatments (present and absent) and 2 BD exposure treatments (present and absent). The experiment had 6 replicates per treatment combination (total n = 72), and metamorphs were randomly assigned to experimental units. Each experimental unit contained 5 metamorphs. Snout-vent lengths were measured on 14 metamorphs from each species (*R. cascadae* = 15.42 mm ± 0.36; *B. boreas* = 21.99 mm ± 0.31; *H. regilla* = 19.49 mm ± 0.40).

We first exposed metamorphs to UV-B treatments for 3 d. Metamorphs were housed in round plastic cups (500 ml) with a damp paper towel at the bottom. Filters were used to manipulate UV-B exposure: Mylar filters excluded 95% of UV-B radiation (UV absent), and high-density polyethylene filters removed 15% of UV-B radiation (UV present). All units were placed in a UV-B chamber with constant temperature and photoperiod (16°C, 12 h light:12 h dark) equipped with UV-B emitting light bulbs. Exposures were carefully designed so that amphibians received UV-B exposure relevant to their natural environment yet below lethal levels (Blaustein et al. 1998, Blaustein et al. 2005b). Metamorphs received exposure rates between 16.2 and 17.9 μW cm^{-2} in the UV present treatments and between 0.22 and 0.31 μW cm^{-2} in the UV absent treatments.

To simulate a natural UV-B environment, our lighting regime consisted of a 12 h day but only 5 h of UV-B exposure. In the morning (first 3 h of daylight) and evening (last 4 h of daylight), metamorphs were exposed to only full spectrum bulbs emitting negligible levels of UV-B (Vita-Life; Durotest). During peak UV periods (11:00 to 16:00 h) metamorphs were exposed to UV-B emitting bulbs (UV-313; Q-Panel). UV-B was measured with a UV-B probe (model PMA2100, Solar Light).

BD treatments were applied immediately following UV-B exposure. Metamorphs from each experimental unit were transferred to corresponding Petri agar dishes that were either cultured with BD or left sterile. Each dish was flooded with 1.0 ml of ultrapure H_2O immediately prior to the addition of metamorphs and an additional 1.0 ml was added 12 h later to allow for zoospore mobility. Dishes were covered with 2 mm^2 mesh window screen and secured with rubber bands to prevent metamorphs from escaping and to force contact between the metamorph and the BD-covered Petri plate surface. After 24 h of exposure to BD, all metamorphs were transferred to clean Petri dishes (14 cm diameter, 2 cm tall) and monitored for a 3 wk period. Paper towels were placed at the bottom of the dishes and constantly kept moist. Each individual was fed 3 crickets wk^{-1}. Mortality was noted throughout the experiments, and dead metamorphs were removed. At the end of the experiment, surviving metamorphs were sacrificed. All metamorphs were preserved in 70% ethanol.

BD culturing methods. We used *Batrachochytrium dendrobatidis* isolate JEL 215 grown in pure culture on Petri agar dishes, according to standard protocol (Longcore et al. 1999). Petri dishes were incubated at 22°C for 11 d before use. Immediately prior to the experiment, all Petri dishes were inspected visually, and those with BD growth that appeared substantially more or less than that present in the majority of the dishes were excluded. Zoospores (the infective stage) were counted in a random sample using a cytometer, and the mean number of zoospores per dish (±SE) were 2.08×10^7 ($\pm 2.86 \times 10^6$).

Statistical methods. Respective survival at 7, 14 and 21 d post exposure to UV-B and BD treatments were analyzed using repeated measures multivariate analysis of variance (MANOVA). Survival was calculated by determining the percentage of surviving metamorphs in each experimental unit for all 3 exposure time points. All data were angular transformed. During the 3 d of UV-B exposure and prior to the UV-B exposure, 3 *Bufo boreas* metamorphs died in 3 separate experimental units. As a result, proportional *B. boreas* data was not available for use with the full model analysis. Only *Hyla regilla* and *Rana cascadae* data were compared for species survival differences. Each species, however, was analyzed separately using repeated measures MANOVAs to identify species-specific response to UV-B and BD over time.

RESULTS

Exposure to BD had a significant negative effect ($p < 0.05$) on survival for *Rana cascadae* and *Bufo boreas* (Table 1, Fig. 1a,b). No UV-B effect or UV-B × BD inter-

Table 1. *Rana cascadae, Bufo boreas* and *Hyla regilla*. Repeated measures MANOVA by species on UV-B and disease (*Batrachochytrium dendrobatidis*, BD) effects on survival. Between-subjects shows differences in overall survival, and within-subjects indicates survival over 3 wk. Num: numerator; Den: denominator. *Statistically significant values ($p < 0.05$)

Source	df		MS	*F*	p
	Num	Den			
Rana cascadae					
Between-subjects					
UV-B	1	20	0.009	0.184	0.672
Disease	1	20	0.329	6.588	0.018*
UV-B × Disease	1	20	0.001	0.014	0.908
Within-subjects					
Time	2	19	0.325	3.085	0.069
Time × UV-B	2	19	0.016	0.152	0.859
Time × Disease	2	19	0.009	0.091	0.913
Time × UV-B × Disease	2	19	0.085	0.803	0.463
Bufo boreas					
Between-subjects					
UV-B	1	20	0.003	0.062	0.805
Disease	1	20	0.342	6.832	0.016*
UV-B × Disease	1	20	0.003	0.062	0.805
Within-subjects					
Time	2	19	0.001	0.009	0.990
Time × UV-B	2	19	0.003	0.029	0.971
Time × Disease	2	19	0.342	3.245	0.061
Time × UV-B × Disease	2	19	0.003	0.029	0.971
Hyla regilla					
Between-subjects					
UV-B	1	20	0.05	1.0	0.329
Disease	1	20	0.05	1.0	0.329
UV-B × Disease	1	20	0.05	1.0	0.329
Within-subjects					
Time	2	19	0.00	0.000	1.000
Time × UV-B	2	19	0.00	0.000	1.000
Time × Disease	2	19	0.00	0.000	1.000
Time × UV-B × Disease	2	19	0.00	0.000	1.000

action was found for either *R. cascadae*, *B. boreas* or *H. regilla* (Table 1, Fig. 1). Species differed in their response to both disease and survival over time (Table 2). *H. regilla* responded to UV-B and BD with almost 100% survival, and *R. cascadae* experienced considerable mortality by the third week. Mortality in *R. cascadae* due to BD exposure was significant within the first week, but the impact of the disease effect decreased over time as control mortality increased. And while disease impacted *B. boreas*'s survival, this effect did not change significantly over time (Table 1).

DISCUSSION

This study shows clear species-specific differences in response to BD. *Rana cascadae* metamorphs were highly susceptible to BD and died in large numbers

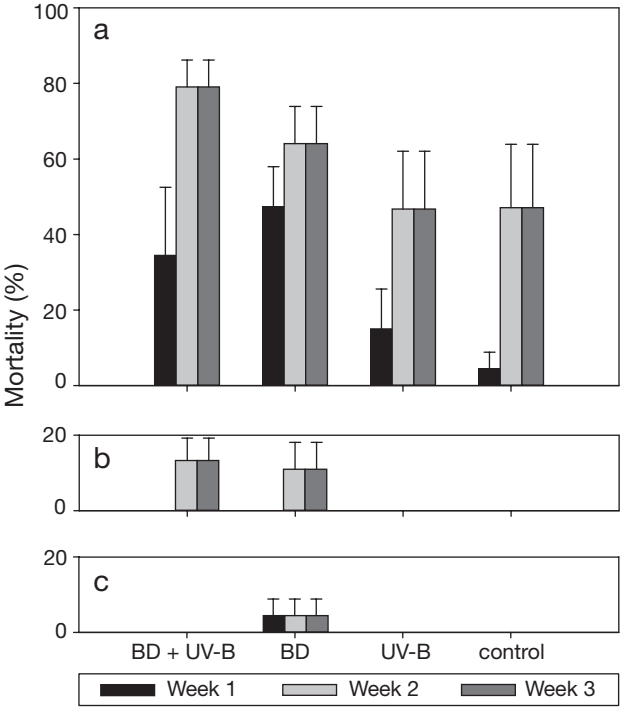

Fig. 1. *Rana cascadae, Bufo boreas and Hyla regilla.* Percent mortality of the metamorphic (a) Cascades frog, (b) Western toad and (c) Pacific treefrog exposed to *Batrachochytrium dendrobatidis* (BD) and UV-B radiation over a 3 wk observation period. Error bars are +SE, and data were angular transformed

within 1 wk of exposure regardless of the presence of UV-B. *Bufo boreas* showed a similar trend with increased mortality after exposure to BD, but overall the *B. boreas* mortality was relatively low compared to *R. cascadae*. Mortality did not increase when *H. regilla* metamorphs were exposed to BD or UV-B. Our results suggest that there are species-specific differences in susceptibility to BD and newly metamorphic anurans can be killed by exposure to BD. In addition, these results differ significantly from a previous study which tested the larval stage of these 3 species (Blaustein et al. 2005a). This can be interpreted to mean that within the same species, different life stages may show different sensitivities to BD.

Exposure to UV-B radiation did not significantly increase mortality in our study in any of the 3 species. Blaustein et al. (2005b) showed that ambient levels of UV-B killed newly metamorphosed *Bufo boreas*. In the present study, similar intensities of UV-B (16.1 to 17.9 µW cm^{-2}) did not kill *B. boreas* metamorphs. However, *B. boreas* in the previous study (Blaustein et al. 2005b) were exposed to UV-B for almost twice as long as those in our study, thus accounting for the differences in mortality. Our study was designed to limit the time of exposure, and sub-

Table 2. *Rana cascadae* and *Hyla regilla*. Repeated measures MANOVA for UV-B and disease (*Batrachochytrium dendrobatidis*, BD) effects on anuran metamorphs. Between-subjects shows differences in survival between species, and within-subjects indicates survival over 3 wk. Num: numerator; Den: denominator. *Statistically significant values ($p < 0.05$)

Source	df		MS	*F*	p
	Num	Den			
Between-subjects					
Species	1	41	1.918	78.651	<0.0001*
UV-B	1	41	0.001	0.046	0.831
Disease	1	41	0.184	7.555	0.008*
Species × UV-B	1	41	0.009	0.403	0.529
Species × Disease	1	41	0.132	5.425	0.025*
UV-B × Disease	1	41	0.0002	0.009	0.926
Within-subjects					
Time	2	40	0.169	3.394	0.043*
Time × Species	2	40	0.495	9.899	0.0003*
Time × UV-B	2	40	0.007	0.154	0.858
Time × Disease	2	40	0.005	0.092	0.912
Time × Species × UV-B	2	40	0.008	0.154	0.858
Time × Species × Disease	2	40	0.005	0.092	0.912
Time × UV-B × Disease	2	40	0.041	0.811	0.452

sequent mortality from UV-B, based on the results of previous work (Blaustein et al. 2005b).

We found no evidence for synergistic interaction between BD and UV-B on survival in the 3 test species. However, it is reasonable to assume that synergistic effects between UV-B and other factors may occur in these species. For example, previous work on *Hyla regilla* showed synergistic effects between contaminants and UV-B (Blaustein et al. 2003) and pathogens and UV-B in *Bufo boreas* (Kiesecker et al. 2001). However, *H. regilla* is more resistant to UV-B than the other species we examined (Blaustein et al. 1994a,1998). Because *H. regilla* is relatively resistant to UV-B, it is not surprising that *H. regilla* metamorphs had high survival in our study compared with the other species. *B. boreas* metamorphs did not suffer a significant increase in mortality in UV-B exposed treatments, but longer exposure to UV-B may be necessary to observe a detrimental synergistic interaction. Any interaction between UV-B and BD in *Rana cascadae* metamorphs, however, was difficult to detect due to the high mortality in treatments with BD. This result should not be interpreted to mean UV-B does not play a synergistic role with disease in *R. cascadae*; further investigation quantifying dose-dependent responses is needed. High background mortality in *R. cascadae* may have been caused by high stress levels due to capture and handling, which is characteristic of this species (Belden et al. 2003).

These results provide additional data on the effects of BD on different life history stages and different species of anuran amphibians. Blaustein et al. (2005a) showed that larval *Bufo boreas* were highly susceptible to BD exposure, and that a toxic substance produced from BD probably caused mortality in these toad larvae. However, in our study, *B. boreas* metamorphs, while showing an increase in mortality when exposed to BD, did not experience a massive die-off. *R. cascadae* metamorphs, however, were highly vulnerable to mortality from BD exposure in this study, but larval *R. cascadae* in our earlier study (Blaustein et al. 2005a) were not. Neither *H. regilla* larvae (Blaustein et al. 2005a) nor their metamorphs (our study) showed increased mortality as a result of BD exposure. This difference in susceptibility to BD and life stage illustrates the need to test all stages of anuran development when assessing effects of environmental stressors.

The mechanisms by which BD kills amphibians are poorly understood. One hypothesis suggests that the fungus produces lethal toxins, whereas another suggests that infection disrupts skin function, thus affecting respiration and osmoregulation (Berger et al. 1998). These 2 mechanisms may work together (Daszak et al. 1999). BD has a 4 d generation time in culture (Longcore et al. 1999, Piotrowski et al. 2004). Anuran metamorphs do not typically die, at least in laboratory tests, from chytridiomycosis until approximately 2 wk after initial BD exposure (Nichols et al. 2001, Carey et al. 2006). In the present study, *Rana cascadae* larvae and metamorphs began dying within 48 h after exposure to BD (Blaustein et al. 2005a, present study). These results and those reported previously suggest the possibility that a toxin, and not chytridiomycosis, was involved in causing mortality in metamorphic and larval stages.

The ecological parameters influencing susceptibility of amphibians to BD in nature are largely unknown. BD is widespread and found in amphibian populations in the tropics as well as in temperate regions (Daszak et al. 2003). Different strains of BD may be genetically similar (Morehouse et al. 2003). However, even though the strains are similar, they may affect various species and populations of amphibians differently. This could explain why some amphibian populations are more vulnerable to mass mortality events associated with BD, whereas other populations appear robust despite the presence of infected animals.

Susceptibility to BD after metamorphosis is significant for several reasons. Our results suggest that not only can metamorphs be reservoirs for disease transmission, but they may also die directly from exposure. This could occur under a variety of scenarios when anurans congregate (Blaustein & Walls 1995). For example, large numbers of frogs and toads may synchronously metamorphose (Neill 1957, Arnold & Wassersug 1978) with individuals having close contact with many others, such as *Bufo boreas* (Blaustein et

al. 2005b). Aggregations of frogs and toads may occur when they are under physiological stress (Johnson 1969). In many species, large aggregations of breeding adults may occur (Sullivan et al. 1995). Impacts of increased metamorph mortality from BD on overall population growth rates may also be significant. Metamorph survival has a strong influence on the population dynamics of many anuran species relative to larval survival (Wilbur 1980, Biek et al. 2002). The susceptibility of metamorphs to BD could have significant consequences on declining amphibian populations.

The possibility of non-amphibian substrates as a reservoir of BD must be considered (Johnson & Speare 2003). Some pathogens, such as the water mold *Saprolegnia ferax* (Blaustein et al. 1994b), may be present continually in the environment because they are saprobes. Persistence of BD as a saprobe would have essentially the same effect as a host reservoir. That is, BD could persist in amphibian habitats between outbreaks (Daszak et al. 2003).

We have now examined the effects of BD on 2 life history stages in several species of anuran amphibians. Our results suggest that the larval stage of one species, *Bufo boreas* (Blaustein et al. 2005a), is more susceptible to BD than the metamorph stage. In another species, *Rana cascadae*, post-metamorphic stages were more susceptible to BD than were the larvae. Other species were unaffected after exposure to BD at any stage. The questions arising from these differences in susceptibility would suggest that we should examine different species and different life stages to determine how BD affects amphibians.

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