Interspecific Variation in Susceptibility of Frog Tadpoles to the Pathogenic Fungus *Batrachochytrium dendrobatidis*

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Abstract: *As part of an overall biodiversity crisis many amphibian populations are in decline throughout the world. Numerous causes have been invoked to explain these declines. These include habitat destruction, climate change, increasing levels of ultraviolet radiation, environmental contamination, and the introduction of non-native species and diseases. Several types of pathogens have been implicated in contributing to amphibian population declines: viruses, bacteria, oomycetes, and fungi. One particular fungus, the chytridiomycete* Batrachochytrium dendrobatidis *may have caused amphibian population declines in several regions. This pathogen causes chytridiomycosis, which is fatal to newly metamorphic and adult amphibians of certain species. We present experimental evidence that larval stages may also be susceptible to exposure to* Batrachochytrium*. There was, however, differential sensitivity to* B. dendrobatidis *in larvae we examined. In laboratory experiments, larval western toads (*Bufo boreas*) exposed to* B. dendrobatidis *experienced increased mortality and behaviors that suggested they were affected by exposure compared with unexposed control tadpoles. Larvae of Cascades frogs (*Rana cascadae*), bullfrogs (*R. catesbeiana*), and Pacific treefrogs (*Hyla regilla*) did not die after exposure to* Batrachochytrium *and appeared to behave normally.* R. cascadae *larvae exposed to* B. dendrobatidis*, however, showed an increase incidence in mouthpart abnormalities, a characteristic effect of chytridiomycosis, compared with unexposed controls. These results show that* Batrachochytrium *can negatively affect some species of amphibians at the larval stage and not others. The implications of interspecific variation in susceptibility to fungal infection are broad.*

Key Words: amphibian population declines, chytridiomycosis, pathogens

Variación Interespecífica en la Susceptibilidad de Renacuajos al Hongo Patógeno *Batrachochytrium dendrobatidis*

Resumen: *Muchas poblaciones de anfibios están declinando en todo el mundo como parte de una crisis generalizada de biodiversidad. Numerosas causas han sido invocadas para explicar estas declinaciones. Estas incluyen la destrucción del hábitat, el cambio climático, el incremento en los niveles de radiación ultravioleta, la introducción de especies y enfermedades exóticas. Varios patógenos han sido implicados en la declinación de poblaciones de anfibios. Estos incluyen virus, bacterias oomicetos y hongos. Un hongo en particular, el quitridiomiceto,* Batrachochytrium dendrobatidis*, puede haber causado la declinación de poblaciones de anfibios en varias regiones. Este patógeno provoca quitridiomicosis, que es fatal para anfibios metamórficos y adultos de ciertas especies. Presentamos evidencia experimental de que las etapas larvarias también pueden ser susceptibles a la exposición de* Batrachochytrium*. Sin embargo, hubo sensibilidad diferencial a* B. dendrobatidis *en las*

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larvas que examinamos. En experimentos de laboratorio, las larvas de Bufo boreas *expuestas a* B. dendrobatidis *presentaron incremento en la mortalidad y comportamientos que sugirieron que estaban afectados por la exposición en comparación con renacuajos control no expuestos. Las larvas de* Rana cascadae, R. catesbeiana *e* Hyla regilla *no murieron después de ser expuestas a* Batrachochytrium *y parecieron comportarse normalmente. Sin embargo, en comparación con controles no expuestos, las larvas de R. cascadae expuestas a* B. dendrobatidis *mostraron incremento en la incidencia de anormalidades en la boca, un efecto característico de la quitridiomicosis. Estos resultados muestran que* Batrachochytrium *puede afectar negativamente a algunas especies de anfibios en la etapa larvaria y a otras no. Las implicaciones de la variación interespecífica de la susceptibilidad a la infección por hongos son amplias.*

Palabras Clave: declinación de poblaciones de anfibios, quitridiomicosis, patógenos

Introduction

Loss of global biodiversity is of major international concern. Although the exact number of species being lost is unknown, some estimate that the current rate of extinction is the greatest in the last 100,000 years (Eldridge 1998). Although habitat destruction appears to be the leading cause for species loss, numerous species are in jeopardy in regions where overt habitat destruction does not seem to be playing a role. Of course, it is difficult to find a habitat that is not at least subtly affected by some anthropogenically based contaminant. Nevertheless, it is challenging to find causes for loss of species in some regions because links to agents causing such losses are not always obvious. This has been especially true for amphibian populations. Numerous amphibian populations are declining throughout the world (Alford & Richards 1999; Houlahan et al. 2000). Like other groups of organisms, habitat destruction has drastically reduced many populations of amphibians. Other factors contributing to amphibian population declines are chemical contamination, global environmental changes (including global warming and increasing ultraviolet [UV] radiation from ozone depletion), introduced exotic species, and infectious diseases.

Recent evidence suggests that diseases may be an important factor in the declines of certain amphibian populations in some regions. Several pathogens appear to be important in this regard. These include viruses, bacteria, parasitic worms, protozoans, oomycetes, and fungi (e.g., Blaustein et al. 1994; Drury et al. 1995; Jancovich et al. 1997; Kiesecker & Blaustein 1997; Berger et al. 1998; Longcore et al. 1999; Pessier et al. 1999; Blaustein & Johnson 2003; Daszak et al. 2003). Pathogens may infect amphibians at various life stages and can be the proximate causes of mortality or can lead to sublethal damage such as severe developmental and physiological abnormalities.

Chytridiomycosis of amphibians is a newly discovered emerging infectious disease in which the pathogenic agent is the fungus *Batrachochytrium dendrobatidis* (hereafter, *Batrachochytrium*) (Berger et al. 1998; Daszak et al. 1999, 2003; Pessier et al. 1999; Longcore et al. 1999; Collins & Storfer 2003). *Batrachochytrium* infects the keratinized epidermis—the skin of metamorph and adult amphibians and the tooth rows and jaw sheaths of larvae (Berger et al. 1998; Longcore et al. 1999; Vredenburg & Summers 2001). In some regions dead and dying newly metamorphic individuals and adult anurans have been found infected with *Batrachochytrium* (Berger et al. 1998, 1999). Often, a single species whose metamorph and adult stages die with *Batrachochytrium* simultaneously have apparently healthy larvae (Berger et al. 1998; Bradley et al. 2002). These larvae often have mouthparts infected with *Batrachochytrium*, but the skin, which is not keratinized, is not infected (Berger et al. 1998; Bradley et al. 2002). Larvae with infected mouthparts also have been found in the absence of mass mortality events (Fellers et al. 2001). Although infection of mouthparts is often associated with damage and loss of tooth rows and jaw sheaths, mortality or other serious detrimental effects have not been observed in infected larvae (Berger et al. 1998, 1999).

Laboratory experiments have demonstrated that *Batrachochytrium* can infect and kill postmetamorphic stages (Berger et al. 1998; Nichols et al. 2001). Thus, *Batrachochytrium* infects both larval and postmetamorphic stages but is believed to kill only postmetamorphic stages (Berger et al. 1998, 1999). Observations of captive collections suggest that animals develop lethal infections after metamorphosis, when their skin becomes keratinized (Berger et al. 1998; Lamirande & Nichols 2002). It is also possible that animals that were not infected as larvae become infected after metamorphosis.

Daszak et al. (1999, 2003) suggest that larvae act as reservoir hosts for *Batrachochytrium*. Reservoir hosts have low susceptibility to a pathogen; thus, they can harbor nonlethal infections and serve as vectors that transmit the pathogen to other hosts which are more sensitive (Haydon et al. 2002).

In the absence of a reservoir host, if the density of susceptible hosts falls, because of either mortality from a disease outbreak or other factors, then the probability that an infectious individual will contact a susceptible individual is reduced. Below a critical threshold density of

susceptible hosts, this probability will be so low that the pathogen does not transmit and becomes locally extinct (Nokes 1992).

Reservoir hosts can exacerbate the severity of disease epidemics by allowing the pathogen to persist in an area even when the density of susceptible hosts is low. If amphibian larvae become infected with *Batrachochytrium* but do not suffer mortality, *Batrachochytrium* may persist even when metamorph and adult hosts are at low densities. Thus, *Batrachochytrium* could remain present in amphibian habitats after it has caused a die-off of metamorph and adult frogs and those susceptible life stages become rare. Surviving larvae that harbor *Batrachochytrium* infections may die when they metamorphose or may spread the fungus to the few metamorphs and adults that survived the initial outbreak (Daszak et al. 1999, 2003). If *Batrachochytrium* kills larvae, however, the epidemiology of the disease could be much different. Without a reservoir host, perhaps *Batrachochytrium* would have reduced potential to persist after it has reduced the density of susceptible hosts. The purpose of our study was to investigate the possibility that *Batrachochytrium* causes mortality at the larval stage, which would reduce the potential of larvae to act as reservoir hosts.

Methods

Experiment 1

In 2001 we collected larvae in early stages of development from natural breeding sites in Oregon in northwestern United States. Western toad (*Bufo boreas*; stage 25 [Gosner 1960]) larvae were collected on 1 July at Lost Lake (Linn County; elevation 1220 m) in the Oregon Cascade Mountains. Pacific treefrog (*Hyla regilla*; stages 29–33) and Cascades frog (*Rana cascadae*; stages 34–35) larvae were collected on 3 July from Parrish Pond (Linn County; elevation 1130 m) in the Oregon Cascades. Bullfrog (*R. catesbeiana*; stages 31–36) larvae were collected on 11 July from a pond in the E. E. Wilson Wildlife Refuge, 18 km north of Corvallis (Benton County; elevation 60 m), Oregon.

We separated and reared species in 9-L aquaria filled with 6 L of dechlorinated tap water. Ten larvae were reared per aquarium for all species except bullfrogs. Bullfrogs were reared five per aquarium. Rearing aquaria were on a natural photoperiod at 14±1° C. We fed larvae a 3:1 mixture of alfalfa pellets:Tetramin fish flakes (Tetra, Blacksburg, Virginia). Throughout the study, tanks with toads were aerated because toad larvae require highly oxygenated water.

Exposure to *Batrachochytrium dendrobatidis*

We grew *B. dendrobatidis* in pure culture on 10-cm-diameter petri dishes with standard TGhL nutrient agar medium, according to methods described in Longcore et al. (1999). We inoculated petri dishes containing nutrient agar with rapidly growing, liquid culture of isolate JEL215 of *B. dendrobatidis* (originally isolated from *R. muscosa* from California). *Batrachochytrium* culture dishes were incubated at 4° C for 15 days before use. Immediately after tadpoles were exposed to *Batrachochytrium*, we used a cytometer to count zoospores (the infective life stage of the pathogen) in random samples of unused *Batrachochytrium* dishes. The mean number of zoospores per dish was 2.78×10^6 (SE $\pm$ 7.52×10^5, $n = 3$). Zoospore densities in *Batrachochytrium* treatment tanks were approximately 1400 zoospores/mL for *Bufo boreas*, *H. regilla*, and *R. cascadae* and approximately 700 zoospores/mL for *R. catesbeiana.*

On 23 August (day 0), we exposed larvae to either a *Batrachochytrium* treatment or a control treatment for 48 hours. For each species, larvae were randomly assigned to a tank containing three petri dishes containing cultures of *Batrachochytrium* on nutrient agar (*Batrachochytrium* treatment) or a tank containing three sterile dishes with agar but no *Batrachochytrium* (control treatment). *Bufo boreas*, *H. regilla*, and *R. cascadae* were exposed in 9-L tanks filled with 6 L of dechlorinated tap water. For *Bufo boreas* and *H. regilla*, we used 30 larvae in each treatment (total $n = 60$ larvae). Eleven *R. cascadae* were used in each treatment (total $n = 22$). Because of their large size, *R. catesbeiana* were exposed in 38-L tanks filled with 12 L of water. Fifteen *R. catesbeiana* were used in each treatment (total $n = 30$ larvae). Immediately before exposure, we determined mass and developmental stage (Gosner 1960) for all *R. cascadae* and for a randomly selected sample in the other species (Table 1).

Postexposure Observations

After 48 hours (on day 2), larvae were removed from exposure tanks and randomly placed in 9-L postexposure tanks filled with 6 L of water. For *Bufo boreas*, *H. regilla*, *and R. catesbeiana*, larvae in each treatment were divided evenly between three tanks, for a total of six tanks per species. For *R. cascadae*, larvae exposed to *Batrachochytrium* were placed in one tank and control larvae (no *Batrachochytrium* exposure) were placed in another tank. Larvae in postexposure tanks were fed ad libitum a 3:1 mixture of alfalfa pellets:Tetramin fish flakes. Water

Table 1. Number of individual tadpoles measured, mean mass, and developmental stages per species before exposure to *Batrachochytrium dendrobatidis* (experiment 1).

Species	*Number measured*	*Mean mass ± SE (g)*	*Gosner stage*
Bufo boreas	18	0.35 ± 0.03	27–37
Hyla regilla	18	0.33 ± 0.03	30–38
Rana cascadae	22	0.72 ± 0.04	34–41
Rana catesbeiana	18	9.17 ± 0.52	31–37

was changed on day 6. At metamorphosis (tail 50% resorbed) we removed amphibians.

Daily we observed larvae for clinical signs of disease (i.e., behavior and activity), gross external lesions, and mortality. At the end of the study (30 days after the 2-day exposure), we humanely sacrificed surviving animals and preserved them in 70% ethanol. After the experiment, we examined preserved larvae with a hand lens for mouthpart abnormalities consistent with *Batrachochytrium* infection (Vredenburg & Summers 2001). Larvae were scored as normal if they possessed fully keratinized (black) jaw sheaths and tooth rows. Larvae that had keratin (black) missing on all or part of the jaw sheaths or tooth rows were scored as abnormal. Individuals that lost their mouthparts because of metamorphosis or scavenging by conspecifics could not be scored and were excluded from analyses assessing larval mouthpart abnormalities. We analyzed incidence of mouthpart abnormalities (percentage of tadpoles with abnormal mouthparts) for each species separately with Fisher exact tests. Analyses included all individuals with larval mouthparts, regardless of whether they died during the experiment. Including or excluding larvae that died during the experiment did not affect qualitative interpretations of results.

We measured *Batrachochytrium* infection of larvae with intact larval mouthparts. Dead larvae that lacked mouthparts because of conspecific scavenging were excluded from examination. All *Bufo boreas* with intact larval mouthparts were examined. For the remaining species, a subset of individuals with intact larval mouthparts was randomly selected from each treatment (Table 2). Ethanol-fixed larvae were sagitally sectioned and routinely processed for histology by embedding in paraffin, sectioning at 5–6 μm and staining with hematoxylin and eosin (HE). Individuals were scored as infected or uninfected based on the presence or absence of histologic lesions consistent with *Batrachochytrium* infection (characteristic spherical to flask-shaped chytrid thalli within the epithelial cells of the mouth parts) (Pessier et al.1999; Fellers et al. 2001). Individuals with autolyzed mouthparts were scored as undetermined. Individuals that failed to yield mouthparts adequate for investigation, despite repeated sectioning, were also scored as undetermined. Incidence of infection in *Batrachochytrium*-exposed versus control larvae was analyzed with Fisher exact tests. Individuals with undetermined infection status were excluded from analyses.

Individuals that lost larval mouthparts because of normal development (late-stage larvae and all fully metamorphosed individuals) were investigated for infection in a separate analysis. Epithelial cells of the outer layer of skin from both hind limbs were examined for spherical, thick-walled structures characteristic of *Batrachochytrium* infection. All examinations used to determine infection were blind with respect to treatment.

Experiment 2

In experiment 1, we treated all animals and aquaria in the same way. For example, all tanks were cleaned similarly and all tadpoles within exposure or control tanks were treated similarly. Moreover, for each species, tadpoles were exposed in one tank and compared with those that were in a control tank (not exposed). Therefore, differences between all tanks in experiment 1 were minimized. It is possible, however, that the results found for *Bufo boreas* in experiment 1 were due to some factor present in the aquarium in which *Bufo boreas* were exposed to *Batrachochytrium* other than factors pertaining to *Batrachochytrium* per se (e.g., a contaminant not found in any other tanks). Therefore, to further examine the effects of *Batrachochytrium* on toad larvae, we conducted an additional experiment with more replicates in which toad larvae were exposed individually to *Batrachochytrium*. On 7 August 2004 we collected toad tadpoles from Todd

Table 2. Number of individual tadpoles (percentage) infected and uninfected with *Batrachochytrium* in larvae that retained larval mouthparts (from experiment 1).

	Batrochochytriumtreatment			
Species	*infected*	*uninfected*	*undetermined*	*Total (two-sided p)**
Experimental treatment				
Bufo boreas	20 (67)	9 (30)	1 (3)	30 (<0.001)
Hyla regilla	13 (87)	0	2 (13)	15 (<0.001)
Rana cascadae	5 (83)	0	1 (17)	6 (= 0.018)
Rana catesbeiana	6 (86)	1 (14)	0	7 (= 1.0)
Control treatment				
Bufo boreas	0	25 (86)	4 (14)	29
Hyla regilla	0	13 (87)	2 (13)	15
Rana cascadae	0	3 (60)	2 (40)	5
Rana catesbeiana	6 (75)	2 (25)	0	8

**p values (Fisher's exact test) are comparisons within a species between the proportions of infected and uninfected larvae in* Batrachochytrium*-exposed versus control (unexposed) treatments.*

Lake (Deschutes County, Oregon; 1864 m elevation) and placed them in four 38-L rearing aquaria (approximately 50 per aquarium). Rearing aquaria were on a natural photoperiod at about 14±1° C. We fed larvae alfalfa pellets ad libitum.

Batrachochytrium strain and culturing techniques were identical to those described for experiment 1. On 24 August 2004 we placed 50 plastic cups (11-cm diameter) filled with 45 mL of dechlorinated tap water on a laboratory bench. We randomly assigned cups to contain petri dishes (5-cm diameter) with either *Batrachochytrium* ($n = 25$) or control petri dishes (agar, no *Batrachochytrium*) ($n = 25$). We placed one toad tadpole (stages 38–40, Gosner 1960) in each cup. The mean number of zoospores per dish was 4.04×10^7 (SE $\pm$ 7.12×10^6, $n = 3$). Zoospore densities in *Batrachochytrium* treatment cups were approximately 8.98×10^5 zoospores/mL. Based on the results from experiment 1, we decided, a priori, to run the experiment for 48 hour and then assess mortality.

Results

Experiment 1

We conducted separate statistical analyses (Fisher exact tests) for each species to determine whether survival differed between *Batrachochytrium* exposed and control groups. Within 24 hours of exposure, five *H. regilla* (two in the control and three in the *Batrachochytrium* treatment) and one *R. cascadae* in the control treatment died. We replaced these larvae with new individuals because death occurred within 24 hours after exposure. One *H. regilla* and one *R. cascadae* in control treatments died during the last 24 hours of the exposure period. Larvae that died during the exposure period were included in analyses but this did not affect interpretation of results. Three *H. regilla* in the control treatment metamorphosed before the end of the experiment. Three *R. cascadae* in the *Batrachochytrium* treatment and four in the controls metamorphosed. All metamorphs survived to the end of the study. No *Bufo boreas* or *R. catesbeiana* metamorphosed.

Bufo boreas survival was significantly reduced in *Batrachochytrium*-exposed versus control larvae (two-sided $p = 0.006$) (Fig. 1). Survival was not significantly different in *Batrachochytrium* exposed versus control larvae in the other three species ($p > 0.05$).

Mortality of *Bufo boreas* was first observed at the end of the exposure period (day 2; within 48 hours after exposure began). At this point, all *Bufo boreas* in the control exposure tank were actively swimming, but many *Bufo boreas* in the *Batrachochytrium* exposure tank were motionless at the bottom of the tank. Larvae were then transferred to postexposure tanks.

Once in the postexposure tanks, motionless larvae were prodded gently with a net. Three *Bufo boreas* in the

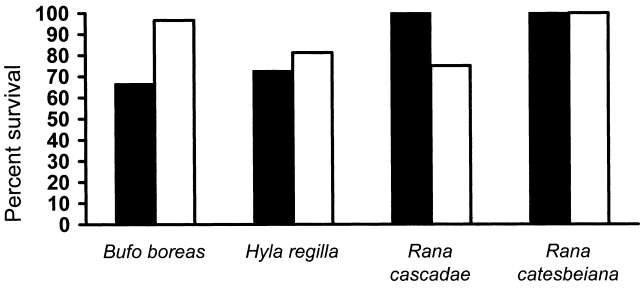

Figure 1. Percent survival of larvae in chytrid and control treatments for each of four species of anuran larvae (tested in experiment 1). Black bars represent Batrachochytrium *treatments, and white bars represent control treatments.*

Batrachochytrium treatment were unresponsive to prodding and determined to be dead. Twenty-six *Bufo boreas* larvae in the *Batrachochytrium* treatment were motionless at the bottom of tanks. When prodded, they moved slightly, and then became motionless. All 30 *Bfuo boreas* in the control treatment swam actively. Twenty-four hours after exposure ended (day 3; within 72 hours), fice more *Batrachochytrium*-exposed *Bfuo boreas* died, whereas no mortality occurred in the control treatment. At this point, surviving *Bfuo boreas* in the *Batrachochytrium* treatment "recovered" and exhibited behavior similar to control tadpoles. One *Batrachochytrium*-exposed *Bfuo boreas* larva died on day 8 and another died on day 15. One control *Bufo boreas* died on day 19. We saw no external lesions on any tadpoles.

Larvae of the other three species were not lethargic. For these species, there were no obvious differences in activity.

The incidence of mouthpart abnormalities per species differed significantly (Fig. 2). Abnormalities ranged from missing portions of the keratinized structures of tooth rows or jaw sheaths to complete absence of keratinized structures. There were no differences in the proportion of tadpoles with mouthpart deformities in the *Batrachochytrium* treatment compared with control treatment (no *Batrachochytrium* exposure) in *Bufo boreas* or *H. regilla* tadpoles. All bullfrog tadpoles in both regimes had deformed mouthparts. *Batrachochytrium*-exposed *R. cascadae* tadpoles, however, had a greater proportion of tadpoles with abnormal mouthparts than those in the control regime.

As determined by histology, incidence of infection in the *Batrachochytrium* treatment was high for *Bufo boreas, H. regilla,* and *R. cascadae* (Table 2). For these species, none of the control individuals displayed infection. Of the 10 *Bufo boreas* in the *Batrachochytrium* treatment that died during the experiment, none showed signs of infection (9 were scored as uninfected and 1 was scored as undetermined). In *R. catesbeiana*, incidence of infection was high in both *Batrachochytrium* and control

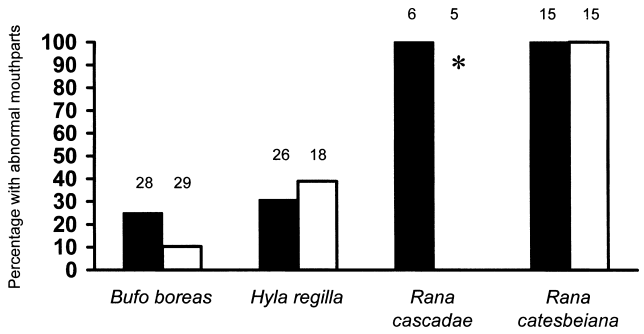

Figure 2. Percentage of larvae with abnormal mouthparts in individuals with mouthparts. Black bars represent Batrachochytrium *treatments. White bars represent control treatments. Numbers above bars are number of individuals that could be scored in each treatment group. *Significant difference between* Batrachochytrium *and control groups (two-sided* $p = 0.002$*).* Batrachochytrium *and control groups were not significantly different from one another (two-sided* $p > 0.05$*) in* Bufo boreas *and* Hyla regilla*.*

treatments but not significantly different between the two treatments. Infection in the control animals indicates that the wild-caught larvae were infected when brought into the laboratory.

Seven *H. regilla* (2 in the *Batrachochytrium* treatment and 5 in the control treatment) and 10 *R. cascadae* (2 in the *Batrachochytrium* treatment and 8 in the control treatment) lost larval mouthparts because of normal development. Infection status could not be determined for 1 control *H. regilla,* 1 control *R. cascadae*, and 1 *Batrachochytrium*-exposed *R. cascadae.* All other individuals lacking larval mouthparts were scored as uninfected.

Experiment 2

Within 48 hours after the experiment began, 16 of 25 larvae exposed to *Batrachochytrium* and 4 of 25 larvae from the control treatment died (Fisher exact test; two-sided $p = 0.001$). These results corroborate those we found in experiment 1 involving toad larvae. They suggest further that larvae of *Bufo boreas* are especially sensitive to *Batrachochytrium*.

Discussion

Our results suggest that (1) there are interspecific differences in larval susceptibility to *Batrachochytrium*, (2) larvae can be killed by exposure to *Batrachochytrium*, and (3) a toxic substance produced by the fungus, rather than chytridiomycosis per se, probably killed the tadpoles. In our study only *Bufo boreas* died from exposure to *Batrachochytrium*. *Bufo boreas* larvae from experiment 1 that died, however, did not have histologic evidence of infection. The mortality in *Bufo boreas* larvae after exposure to *Batrachochytrium* contrasts with the effects of exposure of other species similarly exposed to *Batrachochytrium*. In addition to mortality, *Batrachochytrium*-exposed *Bufo boreas* displayed signs that they were sick (disorientation, lethargy, weak response to prodding). The three species that did not show *Batrachochytrium*-induced mortality showed none of these behaviors. The signs of *Batrachochytrium*-exposed *Bufo boreas* were similar to those observed for naturally or experimentally infected postmetamorphic anurans: abnormal sitting posture, lethargy, slow response to tactile stimuli, loss of righting reflex, and anorexia (Berger et al. 1999; Nichols et al. 2001).

The mechanisms by which *Batrachochytrium* infections kill amphibians are unknown. One hypothesis suggests that the fungus produces lethal toxins, whereas another suggests that infection disrupts normal skin function affecting respiration and osmoregulation (Berger et al. 1998). These two mechanisms may work in combination (Daszak et al. 1999). *Batrachochytrium* has a 4-day generation time in culture (Longcore et al. 1999; Piotrowski et al. 2004), and *Bufo boreas* tadpoles began to die within 48 hours after exposure to *Batrachochytrium*. The results of experiment 1 suggested the possibility that a toxin was involved in causing larval mortality in *Bufo boreas*. The large sample size and individual exposure methods used in experiment 2 further support that hypothesis.

Our results are also consistent with reports that bullfrogs are susceptible to *Batrachochytrium* infection but do not die from it. Although metamorphic bullfrogs may carry *Batrachochytrium* infection, they appear to show no signs of being sick and do not die after exposure to *B. dendrobatidis* (Daszak et al. 2005). Our results suggest that larval bullfrogs and larvae of the other three species could potentially carry *Batrachochytrium* infection (Table 2).

The susceptibility of larvae to potential substances produced by *Batrachochytrium* has not been reported, and our results may have broad pathological and ecological implications. Of the four tested species, *Bufo boreas* was most susceptible to the *Batrachochytrium* regime. This is consistent with *Bufo boreas* susceptibility to other pathogens, including oomycetes, fungi, and parasites (Blaustein et al. 1994; Johnson et al. 2001; Kiesecker et al. 2001).

Different strains of *B. dendrobatidis* are genetically similar and are believed to be part of a recently emerged clone (Morehouse et al. 2003). Even though the strains are similar they may affect amphibians differently. Also, species that showed limited effects in our study may exhibit more severe effects from exposure to *Batrachochytrium* with longer exposure periods. Different populations of the same species may show different sensitivities to *Batrachochytrium*. Patterns of larval susceptibility, or resistance, may influence the natural history and

epidemiology of chytridiomycosis. These differences may help explain why some amphibian populations are more vulnerable to mass mortality events associated with *Batrachochytrium*, whereas other populations appear robust even though they contain infected individuals.

The ecological parameters influencing susceptibility of amphibians to *Batrachochytrium* in nature are largely unknown. It has been proposed that chytridiomycosis is widespread and found in amphibian populations in the tropics as well as in temperate regions (reviewed by Daszak et al. 2003). In some regions cofactors may enhance the susceptibility of amphibians to *Batrachochytrium* infection (discussed in Blaustein & Kiesecker 2002; Blaustein et al. 2003).

Cofactors are implicated in the emergence and transmission of various pathogens infecting amphibians (e.g., Kiesecker & Blaustein 1995; Cunningham et al. 1996; Taylor et al. 1999; Kiesecker et al. 2001; Blaustein & Johnson 2003), including *Batrachochytrium* (Bosch et al. 2001; Pounds 2001). Increasing ultraviolet (UV) radiation and contaminants leading to acid pollution from acid precipitation have been proposed as potential cofactors regarding outbreaks of chytridiomycosis (Blaustein & Kiesecker 2002). For example, changing water pH may influence *Batrachochytrium* outbreaks in Spain (Bosch et al. 2001). Both increasing UV and acid pollution may be involved in *Batrachochytrium* outbreaks in western North America. UV-B (280–320 nm) enhances the susceptibility of *Bufo boreas* eggs to oomycete infection (Kiesecker & Blaustein 1995; Kiesecker et al. 2001) and may do the same when *Batrachochytrium* is present. In Colorado, where amphibians are especially sensitive to low pH (Harte & Hoffman 1989; Kiesecker 1996), a small number of *Bufo boreas* have been found with *Batrachochytrium* (Muths et al. 2003). Thus, as in Spain, amphibians in Colorado may be more prone to infection in the presence of episodic acidification (Harte & Hoffman 1989).

Larval susceptibility to *Batrachochytrium* is significant for several reasons. Our results suggest that not only can larvae be reservoirs for disease transmission but they may also die directly from exposure. This could occur, for example, as ponds dry and concentrations of *Batrachochytrium* increase. This may be especially important in highly social tadpoles that form large schools, such as many species of toad, including *Bufo boreas* (Hoff et al. 1999). In large schools, which can be be hundreds of thousands of individuals (Blaustein 1988), there is great potential for disease transmission. For many species, schooling may be the only social behavior, besides mating, where individuals are in contact with conspecifics. Moreover, as larvae, many species are syntopic with other anurans and pathogens may be transmitted between species at the larval stage. In the Pacific Northwest, *Bufo boreas*, *R. cascadae*, and *H. regilla* are all syntopic at higher elevations. Moreover, *H. regilla* and *R. catesbeiana* are syntopic at lower elevations. There is frequent contact between these species in regions where they are syntopic.

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

Our results suggest that *Batrachochytrium* affected the mouthparts of *R. cascadae* larvae even though they did not appear to be sick and there was no mortality. Potentially, oral deformities may impair grazing ability and lead to reduced growth and slower development (e.g., Rowe et al. 1996). The body mass of larval *R. blairi* and *R. sphenocephala* and F1 hybrids infected with *Batrachochytrium* was less at metamorphosis than that of controls (Parris 2004).

Larvae experiencing sublethal effects such as reduced growth may suffer reduced competitive ability or increased predation. Furthermore, growth and development are crucial for amphibian larvae in ephemeral habitats that must metamorphose before the habitat dries up and for those in regions that must metamorphose before the onset of unfavorably cold temperatures (Blaustein et al. 2001). Perhaps most important, larvae with low energy reserves may be unable to meet the energy requirements necessary to respond to stressors such as UV radiation, contaminants, habitat alteration, unfavorable weather, chytridiomycosis, or other diseases. Indeed, disease resistance in amphibians may be highly context dependent (Kiesecker & Blaustein 1995; Taylor et al. 1999; Kiesecker 2002).

Poor condition at metamorphosis and postmetamorphosis, perhaps a consequence of exposure to *Batrachochytrium* during the larval stage, may increase mortality in postmetamorphic life stages. Amphibians are susceptible to carryover effects that operate on an early life stage but influence later life stages. For example, Belden and Blaustein (2002) found that *R. aurora* (red-legged frog) embryos exposed to ambient UV-B radiation in the field had reduced growth and impaired development at the larval stage. It is possible that exposure to harmful environmental factors in early life stages is influencing the susceptibility of subsequent life stages to *Batrachochytrium* and other pathogens and environmental stresses. Effects of *Batrachochytrium* that originate at the larval life stage deserve careful investigation.

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