

Original Contribution

Distribution and Pathogenicity of *Batrachochytrium dendrobatidis* in Boreal Toads from the Grand Teton Area of Western Wyoming

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Abstract: The pathogen *Batrachochytrium dendrobatidis* (Bd), which causes the skin disease chytridiomycosis, has been linked to amphibian population declines and extinctions worldwide. Bd has been implicated in recent declines of boreal toads, *Bufo boreas boreas*, in Colorado but populations of boreal toads in western Wyoming have high prevalence of Bd without suffering catastrophic mortality. In a field and laboratory study, we investigated the prevalence of Bd in boreal toads from the Grand Teton ecosystem (GRTE) in Wyoming and tested the pathogenicity of Bd to these toads in several environments. The pathogen was present in breeding adults at all 10 sites sampled, with a mean prevalence of 67%. In an experiment with juvenile toadlets housed individually in wet environments, 10⁶ zoospores of Bd isolated from GRTE caused lethal disease in all Wyoming and Colorado animals within 35 days. Survival time was longer in toadlets from Wyoming than Colorado and in toadlets spending more time in dry sites. In a second trial involving Colorado toadlets exposed to 35% fewer Bd zoospores, infection peaked and subsided over 68 days with no lethal chytridiomycosis in any treatment. However, compared with drier aquaria with dry refuges, Bd infection intensity was 41% higher in more humid aquaria and 81% higher without dry refuges available. Our findings suggest that although widely infected in nature, Wyoming toads may escape chytridiomycosis due to a slight advantage in innate resistance or because their native habitat hinders Bd growth or provides more opportunities to reduce pathogen loads behaviorally than in Colorado.

Keywords: *Batrachochytrium dendrobatidis*, chytridiomycosis, boreal toads, Wyoming

INTRODUCTION

Since its discovery by Berger et al. (1998), the chytridiomycete *Batrachochytrium dendrobatidis* (Bd) has been detected in 257 species of amphibians (Olson and Ronnenberg, 2008). This pathogen, the cause of the skin disease chytridiomycosis, has been implicated as the pri-

mary cause of many amphibian population declines and several probable extinctions worldwide (Lips et al., 2006; Skerratt et al., 2007). Spread by aquatic zoospores, Bd infects the keratinized tissue of amphibians, which includes the mouthparts of tadpoles and the skin of juveniles and adults (Longcore et al., 1999). Recent evidence suggests that chytridiomycosis causes death by disrupting osmotic balance through electrolyte loss (Voyles et al., 2007).

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Amphibian species vary in their susceptibility to Bd. Responses range from death after exposure to as few as 100 zoospores (Berger et al., 1999), to the apparent ability to tolerate infection without manifesting chytridiomycosis (Daszak et al., 2004). Innate defenses against Bd, such as antimicrobial skin peptides, vary among species (Woodhams et al., 2007). In the tropics, occurrence of Bd seems to be habitat-related; species associated with permanent water experience higher pathogen prevalence and more disease (Lips et al., 2006; Kriger and Hero, 2007). The effects of Bd infection can vary intra-specifically (McDonald and Alford, 1999).

We have been puzzled by the divergent effects of Bd on the boreal toad, *Bufo boreas boreas*—a species of management concern in the Rocky Mountain region. Bd is widely distributed in toads from Colorado to Montana (Young et al., 2007; Muths et al., 2008). Populations of boreal toads in the southern Rocky Mountains of New Mexico, Colorado, and southeastern Wyoming have declined sharply (Corn, 2003), apparently from chytridiomycosis (Muths et al., 2003). Populations in the northern Rocky Mountains, however, are not known to have declined similarly (Corn, 2003), and a large population outside of Grand Teton National Park appears robust despite annual Bd prevalences of up to 50% in adults (Corn, 2007).

The difference in the response to Bd exposure between boreal toads in Wyoming and Colorado may be the result of regional differences in several factors, including the pathogenicity of Bd, the susceptibility of the host to Bd, and environmental conditions that determine the manifestation of advanced chytridiomycosis. In this study, we explored how Bd interacts with boreal toads from the Grand Teton ecosystem of northwestern Wyoming. We sought to quantify the prevalence of Bd at all known breeding sites, determine whether locally isolated Bd was pathogenic, determine whether toads from this region were susceptible to locally isolated Bd, and determine whether the pathogenicity of Bd in these toads varied with environmental humidity and contact with water. To achieve these objectives, we used a combination of field surveys and laboratory experiments involving juvenile toads raised from larvae.

MATERIALS AND METHODS

Quantifying Bd Prevalence at Boreal Toad Breeding Sites

From surveys during 2000–2003, we identified 16 sites between 1,908 and 2,082 m elevation within and sur-

rounding Grand Teton National Park (GRTE) with boreal toad activity (Patla and Peterson, 2004). During repeated nocturnal visits from May 15 2006 to June 12, 2006 (9 p.m. to 1 a.m.), we found breeding aggregations at 10 of these sites, and sampled ≥ 12 adults at each for Bd (Table 1; Figure 1). During visits, we used a YSI-63 multimeter to record water temperature, pH, and conductivity at 8–10 cm depth at three locations per site. We also sampled boreal toad eggs and larvae and adults of Columbia spotted frogs, *Rana luteiventris*, during these and other visits.

Sampling adults and juveniles for Bd involved capture with a gloved hand, noting the sex of adults, and swabbing the venter, legs, and feet thoroughly (~ 5 – 8 s) with a sterile “smartSpatula” (Investigen). Spatula tips were cut and stored in 70% ethanol, with gloves changed and scissors flamed between samples. We collected small sections of each egg mass found (~ 20 embryos or $< 0.5\%$ of typical clutch size). For larvae, we examined mouthparts for keratin loss (Fellers et al., 2001) and subsequently killed up to 36 larvae per site in MS-222. We sent all samples in 70% ethanol to Pisces Molecular (Boulder, CO) for Bd testing using a specific PCR assay (see Annis et al., 2004). Larval mouthparts were pooled three per vial to reduce costs.

For each site or lifestage, we calculated prevalence by dividing the number of PCR-positives by the number tested. Differences among sites were inferred by comparing binomial 95% confidence intervals (denoted {}). We used regression to explore relationships between physical factors and Bd prevalence and report AIC in addition to significance tests because our small sample size ($n = 10$ sites) limited statistical power.

Experiment 1: Susceptibility of Grand Teton Boreal Toads to Native Bd

In the laboratory, we tested whether Bd isolated from GRTE causes severe chytridiomycosis and mortality, and whether GRTE *B. b. boreas* are susceptible to this local isolate. We also took an initial look at the role that contact with water plays in disease progression. The design crossed three treatments: pathogen exposure (exposed vs. control), source population (Colorado vs. Wyoming), and access to dry sites (“wet”: platforms absent, vs. “dry”: platforms present). We replicated each combination 9 times ($2 \times 2 \times 2 \times 9$), requiring 72 animals.

Table 1. Locations, dates, and physical characteristics of ten boreal toad breeding sites sampled for *B. dendrobatidis* in 2006

Site (abbreviation)	UTMs N, E (NAD 27, Zone 12)	Elevation (m)	Date sampled [No. of adults tested]	Avg. water temperature (°C)	Avg. pH	Avg. specific conductance (μs/cm)
Nowlin Pond (NP)	4818872, 522372	1908	5/5 [20]	13.0	8.6	301
Romney Pond (RP)	4826689, 525301	1981	5/17 [20]	25.0	8.0	410
Schwabacher's Landing (SL)	4839952, 526473	1996	5/16 [20]	12.1	8.5	236
Blackrock Pond (BR)	4853370, 552480	2085	5/21 [20]	14.3	7.9	294
Willow Flats 1 (WF1)	4856731, 532356	2064	5/16 [19]	21.6	8.1	271
Willow Flats 2 (WF2)	4857067, 531752	2069	5/16 [15]	27.5	8.1	316
Colter Bay (CB)	4860685, 528170	2070	5/21 [12]	12.0	8.3	175
Steamboat Pass (ST)	4876505, 522173	2073	5/30 [24]	14.2	7.6	289
Snake River Quarry (QU)	4881781, 525403	2073	5/17 [22]	16.0	7.9	166
Flagg Ranch (FR)	4883291, 526008	2082	5/30 [17]	12.8	7.6	305
Ranges		1908–2082	5/5–5/30 [189]	12.0–27.5	7.6–8.6	166–410

Sites are listed from south to north (see Figure 1).

Toadlet Rearing

We reared approximately 200 boreal toad larvae from each of two sources. Larvae from the Colorado source population, known to be susceptible to Bd (Carey et al., 2006), were supplied by the Colorado Division of Wildlife Native Aquatic Species Restoration Facility (NASRF) in Alamosa, Colorado. The second source population, of unknown susceptibility to Bd, was Blackrock pond, Wyoming (Figure 1, BR; Corn, 2007). Larvae were raised at the ISU Animal Care Facility according to methods developed by the NASRF (Scherff-Norris et al., 2002) in separate 70-l tanks, 3–4 tanks per population. Air temperatures followed a 23 to 17°C day (15 h)–night (9 h) cycle. We removed moribund tadpoles and changed 80% of the dechlorinated water daily, using separate nets and siphons for each tank. Upon metamorphosis, we provided dry space and fed wingless fruit flies to the toadlets daily.

We selected 72 toadlets in November 2006 for the experiment; 36 from each population. Because the Colorado population was developmentally more advanced, randomly selecting animals from among those that appeared active and healthy led to a disparity in mass (mean $\pm$ SE), with Colorado (0.62 ± 0.02 g) heavier than Wyoming (0.46 ± 0.02 g) toadlets ($t_{57(2)} = 6.4$, $P < 0.001$). Randomization ensured no other treatment differences in mass ($F_{1,53} \leq 1.6$, $P \geq 0.21$).

Four days before inoculation, we placed each toadlet in a separate 709 ml plastic aquaria (Glad Products) with 10 ml of dechlorinated water, an inverted petri dish as a platform (6 cm diameter, 0.8 cm high), and a perforated lid. Aquaria were held in an incubator under a 23 to 18°C day (12 h)–night (12 h) cycle, with full-spectrum lighting (Reptisun 5.0). Each day we rinsed aquaria, provided fresh dechlorinated water, and fed each toad ~ 5 –10 wingless fruit flies (dusted with ReptoCal biweekly).

Pathogen Exposure

Toadlets were exposed to Bd using a protocol similar to that used in the study by Carey et al., (2006). Immediately before exposure, we swabbed each toadlet with a polyester swab to assess initial infection status. Swabs were sent in 70% ethanol for Bd testing to Pisces Molecular. Bd was isolated from a Blackrock, Wyoming, juvenile boreal toadlet (Figure 1, BR) following standard methods (Longcore and Berger, 2000), and grown in pure culture on sealed TGH plates. We harvested Bd from plates with a spatula into sterile tryptone broth (treated with penicillin-streptomycin, 0.2 mg/ml, HyClone) to achieve a solution averaging 1.1×10^6 Bd zoospores/ml, quantified with a hemocytometer. Zoosporangia were not counted nor filtered from the inoculate (as in Carey et al., 2006); hence doses should be considered minimum estimates because

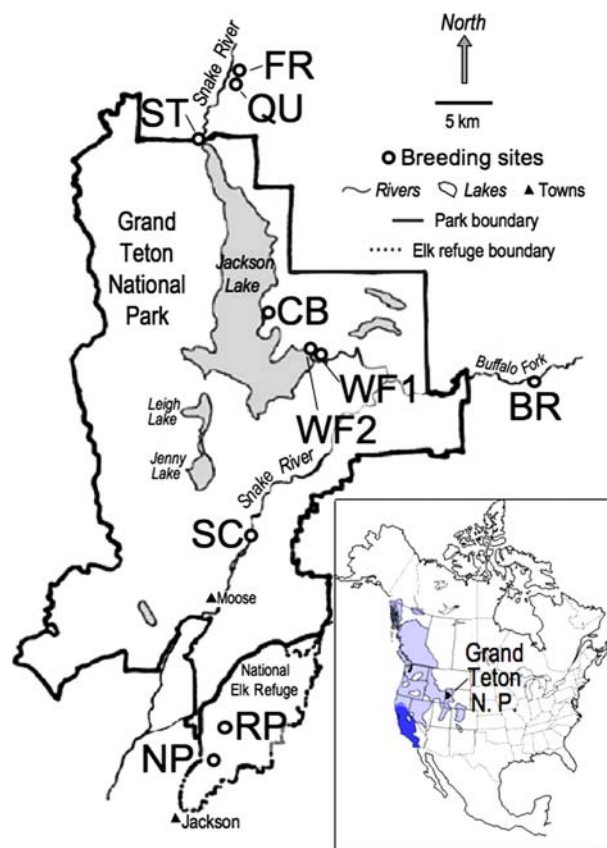


Figure 1. Grand Teton National Park ecosystem where boreal toad adults (*Bufo b. boreas*) from 10 breeding sites (2 letters) were sampled for Bd in May 2006. Sites in close proximity (NP & RP, WF1 & WF2, and FR & QU) were separated by ≥ 400 m and were hydrologically distinct. Inset gives location within western toad range (light shading = boreal toad; dark shading = California toad, *B. b. halophilus*). Site abbreviations defined in Table 1.

zoospores may have released more zoospores. Control broth was prepared similarly, except that we scraped sterile TGH plates into broth. We inoculated toadlets for 4 consecutive days by holding them with a gloved hand and dripping 1 ml of broth on the venter, with the excess dripping off toadlets into the aquaria. With 25 ml of water in aquaria (~ 3 mm deep across the bottom, ensuring full coverage for 24 h), exposed toadlets experienced Bd concentrations of at least 4.5×10^4 zoospores/ml, and a cumulative 4-day dose of 4.5×10^6 Bd zoospores. Each day we treated toadlets over refilled, clean aquaria and provided wingless fruit flies ad libitum.

Monitoring

We added a platform to each aquarium in the dry treatment after the exposure period. Aquaria were cleaned and

refilled daily with 25 ml of dechlorinated water. Toadlets were fed wingless fruit flies ad libitum every other day and weighed biweekly.

Our daily observations included noting resting site and collecting shed skin. We noted each toadlet's location once or twice per day to estimate "time dry," i.e., the proportion of time not in contact with water. A wet mount of each observed skin shed was examined at $200\times$ for Bd sporangia and designated as negative or positive. Any toadlet that appeared moribund (severely lethargic) or lacked a righting reflex was killed in MS-222. Upon death, dissected ventral skin was screened for Bd sporangia, and a skin and foot sample were preserved in 70% ethanol. We also examined each dead toadlet internally for gross abnormalities. The experiment ended 36 days after exposure, 1 day after the last "exposed" toadlet died. We killed all survivors, examined skin and internal organs, and collected skin swabs for Bd testing by Pisces Molecular.

Statistical Analysis

We compared the effects of three treatments—exposure to Bd, source population, and access to dry sites—on the survival of boreal toadlets using survival analysis (SAS 9.1). First, we tested treatment effects on survival separately with Kaplan–Meier tests. Treatments for which $P < 0.3$ were included in a Cox regression. We included two covariates in the Cox model: (a) toadlet body mass, to help control for mass differences in the source populations, and (b) time dry, to control for the fact that climbing aquaria walls diluted the "wet–dry" treatment. We report on interactions when they improved model fit (AIC). To account for nonproportional hazards, we included any significant time-dependent covariates (Allison, 1995).

We used analysis of covariance to interpret the interaction, observed in survival analysis, between exposed toadlets by source population and time dry. The response for each toadlet was the proportion of time alive. If the population-by-time dry interaction was judged important ($P \leq 0.20$, increased R^2_{adj}), we tested the effect of time dry on survival for each population separately.

We also assessed how exposure to Bd, source population, and access to dry sites affected time dry and the tendency to find shed skin in aquaria (days skin found/total days). For both responses (arcsine square root transformed), we report results from reduced models (without 3-way or 2-way interactions) if they had higher R^2_{adj} and interaction terms were insignificant.

Experiment 2: Effect of Access to Dry Sites and Relative Humidity on Susceptibility to Bd

Based on our results from Experiment 1, we sought to clarify the role that contact with water and relative humidity play in the pathogenicity of Bd to boreal toadlets. Given that using dry sites prolonged life but did not reduce mortality, we sought to test whether using dry sites, drier air, or both might reduce mortality at a lower dose of Bd. Secondly, by imposing treatments during inoculation, we tested how they might affect initial susceptibility to Bd infection.

Using protocols described in Experiment 1, we raised tadpoles from the Colorado NASRF to metamorphosis. We then exposed them to Bd as before, with four differences. First, we began environmental treatments during exposure based on relative humidity (high rh, plastic lid vs. low rh, mesh lid) and access to dry sites (wet, ramps absent vs. dry, ramps present). Covering aquaria with no-see-um mesh (Ace Hardware) created lower average ($\pm$ SE) relative humidity ($63\% \pm 1$) than with lids ($93\% \pm 2$), verified using Hobo loggers (Onset Computer) in four aquaria per treatment. For ramps, we used 4-cm transverse sections of PVC conduit, rather than low platforms as before, because toadlets preferred more vertical resting sites. Second, we used larger aquaria (1,920 ml) in an effort to increase the environmental differences between the treatments as experienced by toadlets. Third, animals received a lower dose of Bd than in the first experiment: 1 ml of 9.8×10^5 zoospores/ml for 3 consecutive days, a cumulative dose of 2.9×10^6 zoospores. The inoculate dripping off toadlets was more dilute (1.4×10^4 zoospores/ml) as 71 ml of dechlorinated water covered the bottom of the larger aquaria (enough to prevent full evaporation in the mesh treatment in 24 h). We used a lower dose with the goal of initiating infection across treatments, but not at such a severe level that environment played no role in the outcome of infection. Fourth, inoculate was given without antibiotics, because bacterial growth was not problematic with daily water changes. We randomly assigned six toadlets to each treatment combination. We also added a “recovery” treatment, in which six toadlets spent 14 days in high rh, wet aquaria and then were switched to low rh, dry aquaria. In all, the trial involved 30 ($2 \times 2 \times 6 + 6$) individually housed toadlets.

We maintained aquaria and monitored the same responses as in the first experiment. In daily 3-min scans of shed skin (wet mounts at $200\times$), we recorded more detail

than previously on the density of Bd sporangia: negative (0, no sporangia), positive (1, at least one cluster of sporangia), strong positive (1.1, several regions with large clusters), very strong positive (1.2, clusters dense and widespread). Unlike Experiment 1, we observed no mortality attributable to severe chytridiomycosis; hence, we ended the trial on day 68, more than twice the time necessary for 100% mortality at an equivalent Bd dose in the study by Carey et al., (2006). Because there were no survival effects, we analyzed infection intensity over time using data from shed skin. For each toadlet, we created seven responses, each their infection status based on shed skin during consecutive 10-day periods (9 days for periods 6–7). We tested how the two treatments, and their interaction, influenced infection intensity using repeated measures ANOVA, which produced normal residuals.

RESULTS

Quantifying Bd Prevalence at Boreal Toad Breeding Sites

We sampled 189 boreal toad adults at 10 breeding sites, yielding a prevalence of Bd across sites of 64.5% (Figure 2, right). From the estimates by site (Figure 2, left), we can infer from 95% CI that only a few differed significantly in Bd prevalence, with site ST having higher prevalence than BR, CB, WF2, and QU. Using simple regression, we found no significant relationships between Bd prevalence in breeding adults with average water temperature, H^+ ion concentration ($[H^+] = 10^{-pH}$), specific conductance, site elevation, or sampling date (for all 5 tests, $F_{1,8} \leq 1.0$ and $P \geq 0.346$). However, the positive relationship between $[H^+]$ and Bd prevalence produced a much better model fit (lower AIC) than the other factors. In multiple regression, the prevalence model with $[H^+]$ and elevation produced the best fit, and both factors were marginally significant, with Bd prevalence increasing with higher $[H^+]$ (lower pH, $F_{1,7} = 4.3$, $P = 0.078$) and decreasing with higher elevation ($F_{1,7} = 4.1$, $P = 0.081$).

We detected no difference in Bd prevalence by sex (males 60%, females 83%; Exact Test, $P = 0.406$), but few females were tested ($n = 6$ females, 183 males). Juvenile toads ($n = 24$, 2 sites) had approximately one-half the Bd prevalence of all adults (25% {12, 44}; Figure 2, right), and half that of adults at the same two sites: 62.5% {46.4, 76.7}. We found no Bd in tadpoles based on mouthpart scans or

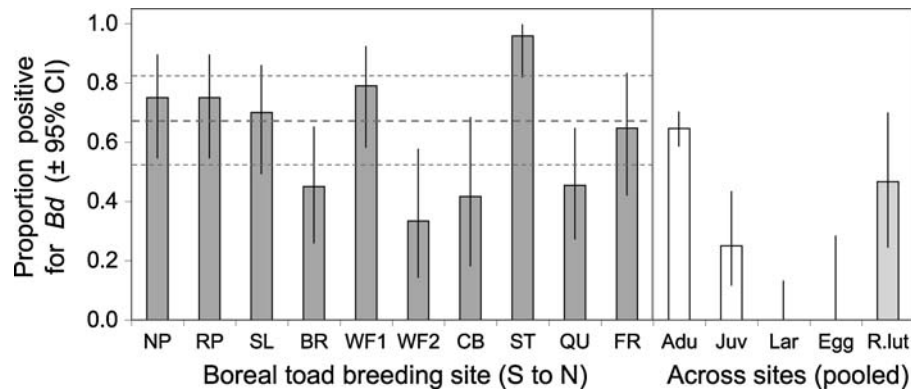


Figure 2. Prevalence ($\pm 95\%$ binomial CI) of the pathogen Bd within the Grand Teton ecosystem in boreal toad adults at 10 sites (dark bars; abbreviations and sample sizes in Table 1) and pooled across sites by life stage (white bars) and in co-occurring adults spotted frogs, *R.*

luteiventris (light bars). Prevalence was estimated from skin swabs or tissue samples with a PCR-based assay. The dashed line gives the grand mean prevalence in adult toads (67%, weighted by the number tested per site), with surrounding 95% CI (df = 9, light lines).

genetic tests, in which 0% were positive ($n = 26$ tests from 92 tadpoles, 4 sites). We also found no Bd in eggs ($n = 11$ tests, 3 sites). Adult Columbia spotted frogs had a similar prevalence (46.7%; $n = 15$; 3 sites) to adult boreal toads based on overlapping 95% CI.

Experiment 1: Susceptibility of Grand Teton Boreal Toads to Native Bd

We found that boreal toads were susceptible to, and died from, a Grand Teton isolate of Bd in the laboratory. Although the effect of Bd exposure on survival was clear (Figure 3), the effects of source population and access to dry sites were linked to toadlet mass and behavior (Figure 4a, b). Based on log-rank tests, we included exposure ($\chi^2_1 = 64, P < 0.001$) and population ($\chi^2_1 = 3.0, P = 0.083$), but not access to dry sites ($\chi^2_1 = 1.0, P = 0.317$), as factors in the Cox model.

The full Cox proportional hazards model that best fit the data showed a strong effect of exposure to Bd ($\chi^2_1 = 21.0, P < 0.001$; χ^2 all Wald tests). All exposed toadlets died within 35 days, whereas 93% of the controls survived. We based our analyses on 61 toadlets, excluding 11 from NASRF that were positive for Bd preexposure. Although we selected toadlets from tanks in which disease was not evident, these background Bd infections affected both populations, and may have originated in wild-caught Wyoming tadpoles (see *Toadlet rearing*). The toadlets that died (all exposed and 2 controls; Figure 3) were Bd-positive in terminal PCR tests and had heavy Bd infections in skin based on wet mounts. These animals exhibited advanced chytridiomycosis (excessive skin shedding, lack of righting

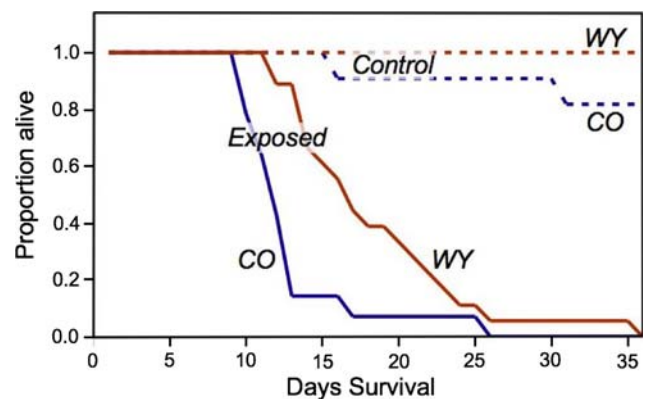


Figure 3. Survivorship of boreal toadlets in a 36-day experiment based on exposure to Bd (4-day dose $\approx 4.5 \times 10^6$ zoospores/toadlet vs. nonexposed controls) and source population (Colorado vs. Wyoming). In a proportional hazards analysis, exposure to Bd strongly reduced survival ($P < 0.001$) and survival of Wyoming toadlets was marginally higher than Colorado toadlets ($P = 0.052$).

reflex; Nichols et al., 2001) and dissection at death revealed no gross abnormalities. Control toadlets grew during the experiment (0.004 g/day, $t_{(2)19} = 4.8, P < 0.001$), but exposed toadlets did not (-0.002 g/day, $t_{(2)31} = 0.6, P = 0.543$).

There were weaker effects on survival of source population and time dry: Wyoming toadlets lived 5.2 days longer than Colorado toadlets ($\chi^2_1 = 3.8, P = 0.052$) and toadlets spending more time on aquaria platforms or walls lived longer ($\chi^2_1 = 2.9, P = 0.087$; Figure 4a, grey line). The source population-by-time dry interaction was marginally significant ($\chi^2_1 = 2.7, P = 0.102$) and greatly improved the model fit. We included the survival days-by-time dry interaction ($\chi^2_1 = 3.8, P = 0.052$) to correct for nonpro-

portional hazards. All other interaction terms and body mass were not significant ($\chi^2_1 \leq 2.3, P \geq 0.131$) and did not improve the model fit.

To clarify the population-by-time dry interaction from survival analysis, we compared survival time (percent of time survived, arcsine square root transformed) in the exposed toadlets by population and time dry using ANCOVA. We found a marginal effect of population ($F_{1,28} = 3.3, P = 0.082$), no effect of time dry ($F_{1,28} = 0.2, P = 0.653$), and a marginal interaction, i.e., a population-dependent, protective effect of remaining dry ($F_{1,28} = 2.1, P = 0.160$). When analyzed by population, spending more time dry increased survival time for Colorado but not for Wyoming toadlets (Figure 4a, thin solid vs. dashed line; all toadlets = grey line): i.e., no relationship with survival time was evident for toadlets spending $\geq 50\%$ of their time dry, which was true for nearly all Wyoming animals.

The proportion of time dry varied by toadlet mass and experimental treatment (Figures 4b and 5). Because mass and source population were not independent, we used sequential sums of squares in ANOVA to evaluate treatment effects on time dry, with mass as the first model term. We found strong effects on time dry of mass, exposure to Bd, population, and access to dry sites (all terms: $F_{1,55} \geq 16.2, P < 0.001$). Toadlets of greater mass spent less time dry, as did Colorado toadlets even when controlling for mass in part because a linear covariate could not remove all non-linear effects of mass on the ability to climb walls (Figure 4b; $>50\%$ of WY above line and $>50\%$ of CO below). Exposing toadlets to Bd and giving access to platforms also increased time dry (Figure 5). The only interaction that improved model fit, population-by-wet-dry, indicated that the wet treatment had a stronger effect on Colorado than Wyoming toadlets. Further analysis revealed that Bd exposure increased time spent on walls but not on platforms (Figure 5, trends in solid vs. dashed bars). Moreover, although Wyoming toadlets climbed aquaria walls more frequently ($F_{1,55} = 22.8, P < 0.001$), the tendency to climb walls in both populations was consistent across the wet-dry treatment ($F_{1,55} = 0.3, P = 0.566$).

We found shed skin more frequently with Bd exposure ($F_{1,54} = 44.5, P < 0.001$), with no differences by population ($F_{1,54} = 0.0, P = 0.976$). Shed skin was observed at nearly twice the rate (mean $\pm$ SE days with sheds/total days) in exposed toadlets (0.36 ± 0.02) than in control toadlets (0.19 ± 0.02). The wet-dry treatment did not affect this rate ($F_{1,54} = 1.1, P = 0.296$) but interacted with the exposure

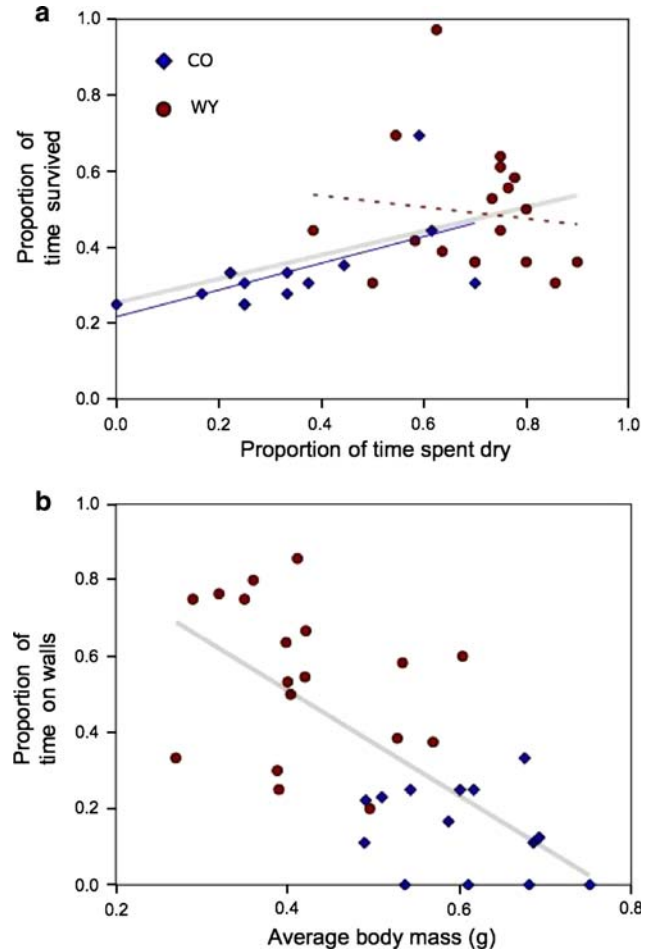


Figure 4. Boreal toadlets exposed to Bd ($n = 31$) survived longer when spending more time dry (a, grey line, $F_{1,30} = 6.9, P = 0.014, R^2 = 0.19$) and their body mass inversely predicted time dry on walls (b, grey line, $F_{1,30} = 26, P < 0.001, R^2 = 0.46$, slope = -1.38 ; for all toadlets, not shown, $F_{1,59} = 48, P < 0.001$, slope = -1.36). When population is considered in (a), the benefit of time dry weakens but the populations seem to have distinct slopes (ANCOVA, see text). Considered separately, more time dry increased survival in CO toadlets (thin line, $F_{1,12} = 6.4, P = 0.026, R^2 = 0.35$) but not WY toadlets (dashed line, $F_{1,16} = 0.3, P = 0.613, R^2 = 0.02$). In (b) there was no interaction by population ($F_{1,28} = 0.1, P = 0.825$).

treatment ($F_{1,54} = 5.4, P = 0.024$). Hence, more shed skin was observed in wet control aquaria (0.22 ± 0.02) than in dry (0.16 ± 0.02), yet there was no difference between wet (0.35 ± 0.03) and dry (0.38 ± 0.02) exposed aquaria.

Experiment 2: Effect of Access to Dry Sites and Relative Humidity on Susceptibility to Bd

The survival of toadlets did not differ based on access to dry sites or relative humidity (log-rank $\chi^2_1 \leq 1.3, P \geq 0.258$). Only four animals died during the 68-day

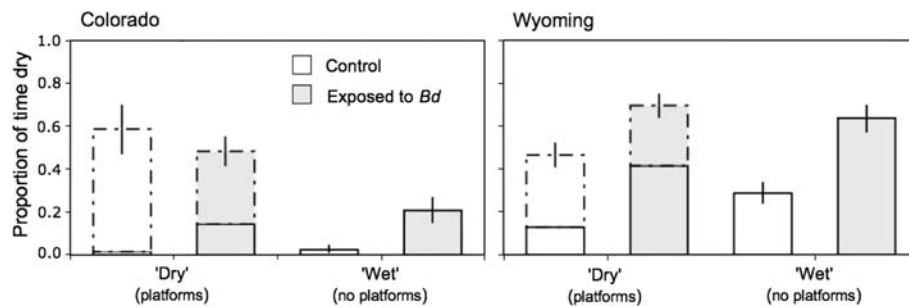


Figure 5. The proportion of time dry in Colorado and Wyoming toadlets was higher in those exposed to *Bd* than in nonexposed controls ($F_{1,55} = 25$, $P < 0.001$). The increase in time dry with *Bd* exposure was due to more time spent on walls (solid bars; $F_{1,55} = 19$, $P < 0.001$) not on platforms (dashed bars; $F_{1,55} = 1.2$, $P = 0.287$).

experiment (on days 24, 36, 53, and 58) and they had no detectable or light *Bd* infections in skin wet mounts and lacked other clinical signs of advanced chytridiomycosis (excessive shedding, lack of righting reflex). Despite lack of mortality, toadlets in high rh and wet (no ramp) aquaria had higher levels of *Bd* infection compared with those in low rh and dry aquaria (Figure 6). Decreasing contact with water through access to ramps reduced *Bd* infection more than experiencing lower relative humidity (45 vs. 29%). Ramps were more readily used than platforms in Experiment 1 (80 vs. 63% of time dry, respectively). The humidity

The wet treatment reduced time dry more in heavier CO than lighter WY toadlets ($F_{1,55} = 6.9$, $P = 0.011$). Values are least square means (± 1 SE) backtransformed from analyses of arcsine square root transformed data.

and wet-dry treatments did not interact ($F_{1,22} = 0.1$, $P = 0.752$) nor did time period with either treatment ($F_{6,117} \leq 0.7$, $P \geq 0.643$). Toadlets switched from wet, high rh to dry, low rh aquaria on day 15 experienced a non-significant recovery compared with those remaining in wet, high rh aquaria ($F_{1,10} = 2.9$, $P = 0.122$). Yet infection varied over time across all treatments, peaking day 40 and then declining (Figure 6, grey line).

DISCUSSION

Bd Prevalent in Grand Teton Boreal Toads Without Evident Disease

We found that *B. dendrobatidis* was widespread among boreal toad breeding sites in the Grand Teton ecosystem, and prevalent within sites (Figure 2). Adult toads at all ten of the sites sampled were positive for *Bd* and, on average, approximately two-thirds of them were positive for the pathogen. Yet we observed no signs of advanced chytridiomycosis (lethargy, anorexia, or excessive skin shedding, *per* Nichols et al., 2001) in the 189 adults sampled, nor have other surveys detected mortality attributable to *Bd* in Wyoming boreal toads since 6 of 13 found dead at Black-rock in 2001 were diagnosed with advanced chytridiomycosis using histology (Patla and Peterson, 2004). Similar observations, that *Bd* is prevalent without catastrophic mortality, are common in North American amphibians (Daszak et al., 2005; Ouellet et al., 2005; Adams et al., 2007; Longcore et al., 2007).

We found no simple relationships between *Bd* prevalence in adult boreal toads and water temperature, pH, specific conductance, site elevation, or sampling date

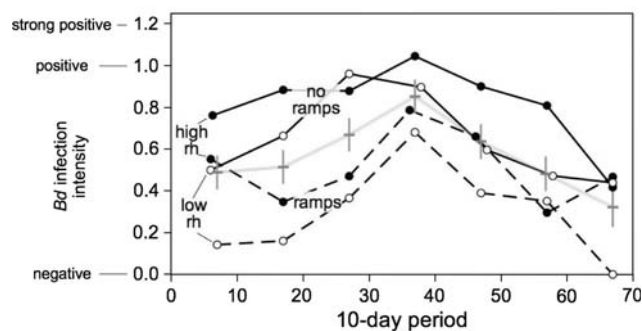


Figure 6. Boreal toadlets in aquaria with less access to dry sites (no ramps) and with higher relative humidity (rh) had consistently more intense *Bd* infection in shed skin ($F_{1,23} = 16.7$, $P < 0.001$ and $F_{1,23} = 5.7$, $P = 0.026$, respectively). Circles show least squares means by 10-day period for each treatment combination (SE's omitted for clarity): solid lines: wet, no ramps; dashed lines: dry, ramps present; filled circles: high rh; open circles: low rh. Average rh ($\pm$ SE) was $93\% \pm 2$ and $63\% \pm 1$ in high rh and low rh aquaria, respectively. The grey line connects mean infection intensity (± 1 SE) by period, which changed during the 68-day experiment ($F_{6,117} = 4.4$, $P < 0.001$). No treatment or temporal interactions were detected (see text).

($P \geq 0.346$). However, these negative findings must be interpreted cautiously because temperature and pH are known to fluctuate at lentic sites used by toads (Murphy, unpublished data; Hossack and Corn, 2008), and our sampling occurred on different nights at each site as aggregations were encountered. Given this limitation, multiple regression suggests that sites with higher pH at breeding time and at higher elevation tend to have lower Bd prevalences within GRTE. The finding for pH is consistent with reduced Bd growth observed in vitro at pH 8 vs. pH 7 (Piotrowski et al., 2004). A decrease in Bd prevalence in boreal toads with increasing elevation is consistent with a recent study that surveyed populations over a greater elevational range in three states (Muths et al., 2008). Overall, our results show that the range of elevation, water temperature, pH, and specific conductance at our GRTE breeding sites are insufficient to exclude Bd.

If Bd infection in GRTE boreal toads typically does not lead to disease or mortality, an alternative host is not required to maintain Bd. A decline in Bd prevalence during warm seasons has been observed in other amphibians (Kriger and Hero, 2006; Longcore et al., 2007), and four boreal toads telemetered in GRTE changed from Bd-positive to negative between July 12 and August 28, 2004 (site SL, Figure 1; Spear et al., 2005), suggesting that some individuals may clear Bd. However, if a few adults remain infected into winter burrows, they may expose others in the spring. Bd was not found in eggs or tadpoles in our survey (Figure 2). Eggs lack keratin thought necessary to sustain Bd. Toad larvae may carry Bd at prevalences we lacked the power to detect and, in any case, are unlikely reservoirs as they do not overwinter in pools. Columbia spotted frogs, found at three sites, had Bd prevalences similar to adult toads (47% {24, 70}). Unlike toads, these frogs may overwinter at breeding sites, providing an aquatic Bd reservoir. However, the Bd prevalence in toads at sites without frogs (65%) did not differ from sites with frogs (63%), suggesting that spotted frogs are not required to maintain high Bd prevalences within toads.

Bd Isolated from GRTE can Cause Chytridiomycosis in the Laboratory

Our experiments demonstrate that Bd isolated from juvenile toads from Wyoming (BR; Figure 1) can cause disease in boreal toadlets from both GRTE, Wyoming, and from the Native Aquatic Species Restoration Facility in Colorado. In the first experiment, when exposed to a 4-day dose

of 4.5×10^6 zoospores (45,000 Bd zoospores/ml within aquaria), 100% of Wyoming and Colorado toadlets died within 36 days (Figure 3). Consistent with previous studies (Nichols et al., 2001), advanced chytridiomycosis was evident in infected toads based on increased skin shedding compared with controls (skin found 1/3 vs. 1/5 of days) and lethargy and weight loss in infected toads before death (only control toads gained weight during the experiment). In Experiment 2, when Colorado toadlets were exposed to a lower 3-day dose of 2.9×10^6 zoospores in larger aquaria (14,000 zoospores/ml within aquaria), we observed an increase in infection to day 40 with a subsequent decline to day 68, as measured by the density of Bd sporangia in shed skin (Figure 6). Although both experiments confirm that Bd from Wyoming can cause disease (as measured by abundant sporangia in shed skin) in toadlets from both populations, their divergent outcomes indicate that chytridiomycosis may not always progress to advanced stages.

In Experiment 2, no toadlets died from chytridiomycosis when dosed at rates only slightly lower than in Experiment 1, whereas Carey et al., (2006) found 100% mortality within 40 days at doses as low as 10^4 zoospores (500 zoospores/ml in aquaria) and substantial mortality at even lower doses. This difference suggests that the lack of mortality we observed in Experiment 2 arose not from low dose per se but how the dose was experienced by toadlets. There were three differences between our two experiments. First, the aquaria were smaller in Experiment 1 (709 ml) than in Experiment 2 (1920 ml). Second, in Experiment 1 we attempted to restrict all toadlets to aquaria bottoms during exposure and did not provide dry sites in any aquaria until after this period. Conversely, in Experiment 2, we initiated environmental treatments during the exposure period (some aquaria received ramps and mesh lids). Third, Bd was administered with antibiotics in Experiment 1 (0.2 mg/ml) and without in Experiment 2.

The evidence is equivocal that environmental differences during exposure prevented the development of advanced chytridiomycosis and mortality in Experiment 2. We found no Bd-induced mortality even in the wet (no ramp) high rh treatment, suggesting that low humidity (mesh) or access to ramps were not the factors preventing mortality. However, evaporation of the floor water was faster in the larger aquaria of Experiment 2 than in the smaller aquaria of Experiment 1, leaving some dry space before refilling each day, regardless of the environmental treatment. Earlier trials in our laboratory also suggest that continuous direct contact with water is necessary for lethal

infection: boreal toadlets exposed in larger aquaria with ample dry refugia became infected, but did not develop advanced chytridiomycosis or die (Murphy and St-Hilaire, *unpublished data*). In addition, in both of our experiments the toadlets may have experienced lower effective doses of Bd than in Carey et al. (2006), because their small size allowed them to climb walls and temporarily escape the infective solution (mean mass 0.5 g in our study; 12 g in Carey et al., 2006). Lighter toadlets, better able to escape water by climbing walls, seemed to survive longer (Figure 4a, b). This effect of mass occurred over a small range, 0.3–1.0 g and does not contradict the positive effect of mass on survival time previously observed by Carey et al. (2006). In sum, in Experiment 2, the slightly lower dose combined with a greater opportunity for toadlets to escape wet surroundings, particularly before daily water changes, may have prevented mortality from disease. Indeed, Carey et al. (2006) suggest that enforced contact with water, as in all of their experiments, promotes reinfection and shortens survival time but does not reflect natural conditions. Our study supports this assertion and suggests that Bd infection may be chronic, and not always lethal, in *B. b. boreas* from both Wyoming and Colorado.

The lack of mortality we observed in Experiment 2 also may have been due to the fact that no antibiotics were given during exposure. Recent evidence suggests that skin microflora may be integral to an anuran's ability to combat Bd infection (Harris et al., 2006). Inoculating toadlets with an antibiotic solution, as in Experiment 1 or Carey et al. (2006), may reduce or eliminate these bacteria, and aid in the proliferation of Bd in the skin. Experiments comparing the response of boreal toadlets to Bd inoculation, with and without antibiotics, are necessary to ensure that laboratory trials are not reporting inflated levels of Bd virulence.

Hypotheses for Divergent Effects of Bd on Wyoming and Colorado Boreal Toads

Our findings show that Bd is widespread in Grand Teton boreal toads, with no evidence of recent disease or decline, yet a local Bd isolate can initiate lethal disease in these toads in the laboratory. What is protecting boreal toads from chytridiomycosis in Wyoming? Our results in the context of other studies suggest that both evolutionary and environmental factors may contribute to regional differences in the host-pathogen balance in this species.

From an evolutionary perspective, Colorado and northern Wyoming boreal toads are distinct lineages

(Goebel et al., 2009), and therefore may have different innate susceptibilities to Bd, perhaps arising from differences in skin peptides (Woodhams et al., 2007). Wyoming populations also may have developed greater Bd resistance if they suffered Bd-induced declines that went undetected before the advent of intensive monitoring during the late 1990s. Such a stable, endemic state for Bd has been observed in Australian amphibians post-decline (Retallick et al., 2004). Lower prevalences of Bd in boreal toads were noted in Colorado adults (13%, Muths et al., 2008) and in Wyoming juveniles (25%) compared with Wyoming adults (67%; Figure 2), which may be because fewer of both the former survive infection. When comparing juvenile survival experimentally, we found that infected Wyoming toadlets survived 5 days longer than those from Colorado (Figure 3). This difference at high Bd doses in the lab may translate to a greater disparity in juveniles and adults in the wild where animals are likely to encounter lower doses (Carey et al., 2006). However, the two experimental populations also differed in mass, and by climbing aquaria walls, lighter Wyoming toadlets may have experienced lower doses of Bd (Figure 4a, b). In sum, clarifying the role that innate resistance plays in the divergent effects of Bd in Colorado and Wyoming requires further study.

Regional differences in the environment, including both abiotic and biotic aspects of boreal toad habitat, also may affect the host-pathogen balance and explain the divergent effects of Bd in Wyoming and Colorado. On the microhabitat scale, reducing contact with water should reduce infection or reinfection by aquatic Bd zoospores (Carey et al., 2006). We found evidence for this in both experiments: longer survival in toadlets spending more time dry (Figure 4a) and reduced Bd infection in toads housed with access to ramps and at lower humidity (Figure 6). At the range tested, reducing humidity had a smaller protective effect than reducing contact with water.

Climbing walls was a strong experimental response to infection (Figure 5) as in the study by Carey et al., (2006). However, climbing in aquaria did not enable toadlets to raise their body temperature as it might through basking in the wild. This may explain why climbing, although it increased survival time, did not reduce mortality in Experiment 1. Behavioral fever is a well-documented response to disease in poikilotherms (Kluger, 1979) and raising body temperature sufficiently has been shown to clear Bd infection (Woodhams et al., 2003). Regional differences in boreal toad habitat, such as elevation or canopy cover, may make basking less effective against Bd in Colorado. For

example, basking during the breeding season increased the average body temperature of boreal toads to 23°C at one 2810 m site in Colorado (Muths and Corn, 1997), insufficient to kill Bd. Other regional differences in toad habitat, e.g., in water temperature, pH, etc., also may affect the host-pathogen balance in Colorado and Wyoming. A comparative study of how individuals, and their disease status, change with habitat selection is necessary to better test the hypothesis that environmental factors are contributing to the divergent effects of Bd in Colorado and Wyoming boreal toads.

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