Supporting Literature for Inclusion in the United State Fish and Wildlife Service 90-Day Finding on a Petition to List the Eastern or Southern Rocky Mountain Population of the Boreal Toad as an Endangered or Threatened Distinct Population Segment

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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, 10^6 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 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 primary 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).
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; Krieger and Hero, 2007). The effects of Bd infection can vary intraspecifically (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 surrounding Grand Teton National Park (GRTE) with boreal toad activity (Patla and Peterson, 2004). During repeated nocturnal visits from May 15 2006 to June 12, 206 (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 × 2 × 9), requiring 72 animals.
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 18°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 ± 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 ($F_{1,53} \leq 1.6$, $P \geq 0.21$).

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; 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 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).

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).

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<table>
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<tr>
<th>Site (abbreviation)</th>
<th>UTMs N, E (NAD 27, Zone 12)</th>
<th>Elevation (m)</th>
<th>Date sampled [No. of adults tested]</th>
<th>Avg. water temperature (°C)</th>
<th>Avg. pH</th>
<th>Avg. specific conductance (µS/cm)</th>
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<td>1908</td>
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<td>Blackrock Pond (BR)</td>
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<td>7.9</td>
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<td>5/16 [19]</td>
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<td>Snake River Quarry (QU)</td>
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<td>5/17 [22]</td>
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zoosporangia may have released more zoospores. Control broth was prepared similarly, except that we scraped sterile TGhL 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 $4.5 \times 10^4$ zoospores/ml, and a cumulative 4-day dose of $4.5 \times 10^6$ 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 200x 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 (±SE) relative humidity (63% ± 1) than with lids (93% ± 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 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 more dilute (0.1 ml of 9.8 × 10^6 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.

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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} ≤ 1.0 and P ≥ 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

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.
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 (χ² = 64, P < 0.001) and population (χ² = 3.0, P = 0.083), but not access to dry sites (χ² = 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 (χ² = 21.0, P < 0.001; χ² 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 reflex; Nichols et al., 2001) and dissection at death revealed no gross abnormalities. Control toadlets grew during the experiment (0.004 g/day, t(219) = 4.8, P < 0.001), but exposed toadlets did not (−0.002 g/day, t(231) = 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 (χ² = 2.9, P = 0.087; Figure 4a, grey line). The source population-by-time dry interaction was marginally significant (χ² = 2.7, P = 0.102) and greatly improved the model fit. We included the survival days-by-time dry interaction (χ² = 3.8, P = 0.052) to correct for nonpro-
portional hazards. All other interaction terms and body mass were not significant ($\chi^2 \leq 2.3, P \geq 0.131$) 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 nonlinear 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 ($0.36 \pm 0.02$) than in control toadlets ($0.19 \pm 0.02$). The wet-dry treatment did not affect this rate ($F_{1,54} = 1.1, P = 0.296$) but interacted with the exposure treatment ($F_{1,54} = 5.4, P = 0.024$). Hence, more shed skin was observed in wet control aquaria ($0.22 \pm 0.02$) than in dry ($0.16 \pm 0.02$), yet there was no difference between wet ($0.35 \pm 0.03$) and dry ($0.38 \pm 0.02$) 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 $\chi^2 \leq 1.3, P \geq 0.258$). Only four animals died during the 68-day
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 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 Blackrock 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.
(P ≥ 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 toads 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 $4.5 \times 10^6$ 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 $2.9 \times 10^6$ 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 shortsens 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.

ACKNOWLEDGMENTS

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Temperature, hydric environment, and prior pathogen exposure alter the experimental severity of chytridiomycosis in boreal toads

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ABSTRACT: Prevalence of the pathogen Batrachochytrium dendrobatidis (Bd), implicated in amphibian population declines worldwide, is associated with habitat moisture and temperature, but few studies have varied these factors and measured the response to infection in amphibian hosts. We evaluated how varying humidity, contact with water, and temperature affected the manifestation of chytridiomycosis in boreal toads Anaxyrus (Bufo) boreas boreas and how prior exposure to Bd affects the likelihood of survival after re-exposure, such as may occur seasonally in long-lived species. Humidity did not affect survival or the degree of Bd infection, but a longer time in contact with water increased the likelihood of mortality. After exposure to ~10⁶ Bd zoospores, all toads in continuous contact with water died within 30 d. Moreover, Bd-exposed toads that were disease-free after 64 d under dry conditions, developed lethal chytridiomycosis within 70 d of transfer to wet conditions. Toads in unheated aquaria (mean = 15°C) survived less than 48 d, while those in moderately heated aquaria (mean = 18°C) survived 115 d post-exposure and exhibited behavioral fever, selecting warmer sites across a temperature gradient. We also found benefits of prior Bd infection: previously exposed toads survived 3 times longer than Bd-naïve toads after re-exposure to 10⁶ zoospores (89 vs. 30 d), but only when dry microenvironments were available. This study illustrates how the outcome of Bd infection in boreal toads is environmentally dependent: when continuously wet, high reinfection rates may overwhelm defenses, but periodic drying, moderate warming, and previous infection may allow infected toads to extend their survival.

KEY WORDS: Boreal toads · Disease severity · Chytridiomycosis · Temperature · Moisture · Acquired immunity

INTRODUCTION

Chytridiomycosis, an emergent skin disease caused by the fungus Batrachochytrium dendrobatidis (Bd), has been documented in over 200 amphibian species worldwide and has caused local extinctions and widespread population declines (Skerratt et al. 2007). Surveys have demonstrated that the prevalence of Bd infection increases in species inhabiting permanent water (Kriger & Hero 2007, Brem & Lips 2008), in species overwintering aquatically versus terrestrially (Longcore et al. 2007), and seasonally in cool months, both across species and within single populations (McDonald et al. 2005, Woodhams & Alford 2005). Infection patterns in the wild are consistent with an aquatic infectious stage unable to survive desiccation (Longcore et al. 1999, Johnson et al. 2003). Likewise, Bd grows optimally at moderate temperatures (17 to 25°C), with arrested growth at 28°C, and death at 30°C (after 8 d; Piotrowski et al. 2004). The development of

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First, we tested and distinguished the effects of humidity on the severity of chytridiomycosis in boreal toads. Pilliod et al. (2010) have just begun to test whether the manifestation of disease with temperature and with prior infection status (Bustamante et al. 2007, Murray et al. 2009). Experimental studies, however, have not manipulated the hydric environment, providing either facultatively (Lamirande & Nichols 2002, Berger et al. 2004) or obligately (Carey et al. 2006) wet environments. Although some riparian cloudforest species may experience frequent-to-constant contact with water (Lips et al. 2003), the habitats of other adult amphibians exposed to Bd vary widely. In addition, pond breeders like boreal toads, may be in contact with water frequently during breeding but thereafter move away from standing water for extended periods (e.g. from weeks to months; Bartelt et al. 2004). Phenological changes in habitat may be linked to changes in individual infection status within a season and over years in long-lived species (Corn 2007, Murray et al. 2009). Experimental studies, however, have just begun to test whether the manifestation of disease with Bd infection changes with hydric environment and with prior infection status (Bustamante et al. 2010, Ramsey et al. 2010).

Boreal toads vary regionally in their manifestation of chytridiomycosis with Bd infection (Muths et al. 2003, Pilliod et al. 2010). In this study, we sought to explore the severity of chytridiomycosis in boreal toads. First, we tested and distinguished the effects of humidity and direct contact with water on disease progression, in order to clarify our earlier findings (Murphy et al. 2009), which were potentially confounded by body mass or population (toads were from distinct clades; Goebel et al. 2009), and to explore how husbandry influences the outcome of Bd infection. Second, we tested how a moderate temperature increase, within the optimal range for growth of Bd, affects toad survivorship after Bd infection, and examined thermal selection by toads for evidence of behavioral fever. Third, we tested whether previous exposure influenced subsequent survivability of exposure by comparing the trajectory of infection in previously exposed and Bd-naive toads.

**MATERIALS AND METHODS**

We obtained 52 yearling boreal toads from the Colorado Division of Wildlife Native Aquatic Species Restoration Facility, offspring from several crosses of adults raised from eggs collected in Pitkin County, Colorado in 2001. During the 35 d until the first exposure was conducted, toads were housed at the ISU Animal Care Facility in two 100 l aquaria. We provided fresh dechlorinated water and crickets dusted with calcium (ReptoCal) daily. Aquaria were held under full spectrum lighting (Reptisun 5.0) and a 23 to 17°C 12 h day: 12 h night cycle, within the range of temperatures experienced by toads in the field (Hossack et al. 2009).

**Exposure 1. Effect of humidity and temperature on disease severity.** We selected 48 toads for an 8 treatment design that crossed three 2-level factors, each with 6 replicates: pathogen exposure (Bd exposed vs. control), humidity (high vs. low), and temperature (heated vs. ambient). Individuals ranged in weight from 5 to 33 g (mean 17.9), with no differences by treatment ($F_{7,40} = 0.2$, $p = 0.973$). Toads were housed individually in 8 l glass aquaria. Dechlorinated tap water (75 ml) was provided in two 9 cm diameter glass petri dishes, one at each end of the aquaria (back and front). Aquarium floors were dry. The pH (~7.2), alkalinity, and total dissolved solids of the water used were within the range measured at boreal toad-breeding sites in NW Wyoming (Hawk 2000). Moreover, the tap water did not compromise Bd, as we have induced lethal chytridiomycosis in toads exposed in this water in prior experiments (Murphy et al. 2009).

Heated aquaria had a 6 cm wide thermal strip (CaloriQue) under one end, while ambient aquaria lacked this strip. Temperature treatments (Table 1A) reflected the low to middle range of what a toad in the field could experience during mid-summer daylight hours (09:00 to 18:00 h; Carey 1978), without being consistently too hot to halt Bd growth ($\geq 28^\circ$C for 2 d; Pi-
inoculate (±1 SE) was 5.8 ± 0.8.

cillin-streptomycin (Hyclone). The average daily
caused infection: 20 of 24 exposed toads had
(demonstrated that drip inoculation
toads over clean, re-filled dishes. Scans of shed skins
water around the aquaria. Each day we inoculated
the infective solution, although they tracked some
method (see ‘Exposure 2’). Here toads could escape
‘drip’ inoculation to distinguish it from the ‘bath’
ping into the water dishes in aquaria. We term this
ping 1 ml of broth on the venter, with the excess drip-
for 5 d by holding them with a gloved hand and drip-
quantified with a hemocytometer. We inoculated toads
(Bartelt et al. 2003). Immediately prior to exposure, we swabbed
each animal’s venter with a polyester swab and sent
swabs in 70% ethanol to Pisces Molecular (Boulder,
Colorado, USA) for
testing via PCR (protocol modi-
isolate JEL #275 was
grown in pure culture on sealed TGhL (tryptone,
hydrolysed gelatine, lactose) plates and scraped with a
spatula into tryptone broth. For control inoculate, we
scraped sterile TGhL plates into tryptone broth. The
broth in both cases was treated with 0.2 mg ml–1 peni-
cillin-streptomycin (Hyclone). The average daily Bd
inoculate (±1 SE) was 5.8 ± 0.8 × 105 zoospores ml–1,
quantified with a hemocytometer. We inoculated toads
for 5 d by holding them with a gloved hand and dripping
1 ml of broth on the venter, with the excess dripping into the water dishes in aquaria. We term this ‘drip’ inoculation to distinguish it from the ‘bath’ method (see ‘Exposure 2’). Here toads could escape the infective solution, although they tracked some water around the aquaria. Each day we inoculated toads over clean, re-filled dishes. Scans of shed skins (see ‘Monitoring’) demonstrated that drip inoculation caused infection: 20 of 24 exposed toads had ≥1 shed with Bd sporangia within 3 wk of exposure.

Monitoring: We recorded whether toads were in contact with water (in water dish) once or twice daily. Toad temperatures were taken daily with a non-contact infrared thermometer (TTI Instruments) held ~10 cm from the toad’s dorsum (Rowley & Alford 2007). We recorded water temperatures in dishes every 2 d in control aquaria using a thermistor from an Omega handheld thermometer. Subsequently, we replaced dirty water dishes with clean ones and refilled them, and fed 3 to 5 crickets to each toad. Humidity and air temperatures were logged every 10 min in control aquaria (2 Onset loggers per treatment combination, rotated weekly). We weighed toads weekly to the nearest 0.1 g, each on sterile KimWipe (Kimberly-Clark), tared prior to measurement.

Each day, we prepared wet mounts of any shed skin found in each cage. These samples were scanned for Bd sporangia for 3 min at 200× under a light microscope. Samples were scored, blind to treatment, as negative (0, no Bd sporangia), positive (1, sporangia diffuse), strong positive (1.1, several large clusters), or very strong positive (1.2, clusters large, dense, and widespread), with borderline cases confirmed by 2 observers (P.J.M. and S.S.H.). We have successfully used this method to monitor Bd-infection status in boreal toads (Murphy et al. 2009) as have Berger et al. (2004) and Padgett-Flohr (2008) in other species.

Statistical analysis: We examined temporal trends in Bd infection status in four 16 d periods using infection scores from shed skin. A total of 16 d was sufficient time to include at least 1 shed per toad per period, and we calculated an average score per period for each toad. We compared infection score by treatment and time period using factorial, repeated measures ANOVA (SAS version 9.1 for this and all subsequent analyses). We tested how toad weight (average change weekly), rate of skin shedding (days skin found/60 total days), time in contact with water (proportion of total observations), and toad temperature (average of total observations) depended on treatment using factorial ANOVA.

Exposure 2. Effects of contact with water, temperature, and prior Bd exposure on disease severity. After 64 d without mortality and what appeared from the microscopic evaluation of the shed skins to be recover-

Table 1. Mean relative humidity (RH) and temperature (air, water, and Anaxyrus b. boreas dorsum) by treatment during (A) Period 1 (Days 0 to 64) and (B) Period 2 (Days 67 to 179)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>RH (%) ± 1SE</th>
<th>Air temp. (°C) ± 1SE</th>
<th>Water temp. (°C) ± 1SE</th>
<th>Toad temp. (°C) ± 1SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(min., max.)</td>
<td>(min., max.)</td>
<td>(min., max.)</td>
<td>(min., max.)</td>
</tr>
<tr>
<td>(A) Period 1 (post-Exposure 1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low RH, ambient</td>
<td>44 ± 2 (33, 57)</td>
<td>19.5 ± 0.2 (16.4, 23.6)</td>
<td>16.3 ± 0.1 (12.7, 17.9)</td>
<td>15.5 ± 0.1 (11.3, 19.9)</td>
</tr>
<tr>
<td>Low RH, heated</td>
<td>41 ± 3 (27, 68)</td>
<td>21.0 ± 0.4 (14.9, 27.1)</td>
<td>17.4 ± 0.3 (14.0, 31.5)</td>
<td>17.0 ± 0.3 (11.2, 28.8)</td>
</tr>
<tr>
<td>High RH, ambient</td>
<td>83 ± 4 (50, 100)</td>
<td>19.8 ± 0.2 (16.4, 24.0)</td>
<td>17.4 ± 0.1 (12.5, 20.3)</td>
<td>17.7 ± 0.1 (13.3, 20.9)</td>
</tr>
<tr>
<td>High RH, heated</td>
<td>76 ± 4 (37, 92)</td>
<td>24.0 ± 0.7 (16.0, 27.1)</td>
<td>20.9 ± 0.2 (14.7, 33.2)</td>
<td>21.9 ± 0.2 (14.1, 30.9)</td>
</tr>
<tr>
<td>(B) Period 2 (post-Exposure 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambient</td>
<td>41 ± 3 (24, 88)</td>
<td>19.6 ± 0.2 (18.3, 20.6)</td>
<td>15.6 ± 0.3 (12.1, 18.4)</td>
<td>15.0 ± 0.1 (11.7, 17.8)</td>
</tr>
<tr>
<td>Heated</td>
<td>34 ± 4 (23, 83)</td>
<td>21.6 ± 1.0 (19.0, 24.8)</td>
<td>18.0 ± 1.1 (11.6, 27.3)</td>
<td>17.8 ± 0.7 (11.4, 27.6)</td>
</tr>
</tbody>
</table>
ing animals (see Fig. 1, grey line), we swabbed each toad’s venter for diagnostic PCR and re-inoculated a subset of them with *Bd* using the bath method of Carey et al. (2006). By following their inoculation and husbandry methods, we sought to determine whether bath inoculation, constant contact with water, or both, were required for *Bd* infection to cause mortality in boreal toads. A second exposure also allowed us to assess whether prior exposure to *Bd* affects the progression of disease in re-exposed toads.

Boreal toads in Exposure 2 were distributed into 9 treatment groups (Table 2), 8 of which were a combination of three 2-level treatments (2 × 2 × 2) that crossed an animal’s Exposure 1 history (*Bd*-naïve or *Bd*-experienced) with a second inoculation treatment (*Bd* bath or control) and a contact with water treatment (obligate vs. facultative). The ninth treatment group matched the fourth group (obligate vs. facultative contact with water) except that after inoculation toads were placed in aquarium with a heat strip at one end. By comparing Groups 9 and 4, we tested the effect of moderate heat on disease progression. Contact with water was manipulated by providing 2 platforms (inverted glass petri plates, 0.8 cm high, 9 cm diameter) in each aquarium designated ‘facultative’. Toads could thus select a drier environment in these aquarium, while toads in the ‘obligate’ treatment without platforms always had at least their hind feet in water. A total of 51 toads were involved: 47 from Exposure 1 (1 was excluded due to a husbandry error) plus 4 *Bd*-naïve animals that had been raised throughout in 1 large aquarium under ambient temperature, low humidity conditions (as in Table 1A, row 1). From within each pre-exposure category (naïve or previously exposed), toads were randomly drawn and assigned to treatment groups (Group 1 to 4, 9 or 5 to 8, respectively, Table 2). We stratified random assignments according to 2 conditions in order of priority: (1) such that initial toad mass did not differ among groups (*F*8,42 = 0.2, p = 0.993), and (2) such that toads from each heat and humidity combination in Exposure 1 were proportionately represented among Exposure 2 groups.

**Bath inoculation:** Toads were exposed to *Bd* using the protocol of Carey et al. (2006). As above, we swabbed toads’ venter pre-exposure and had swabs tested for *Bd* by diagnostic PCR. We inoculated toads using a ‘bath’ for 3 d (Days 64 to 66 after Exposure 1 began). *Bd* and control inoculates were prepared daily as in Exposure 1. *Bd* inoculate averaged 1.2 ± 0.2 × 10^6 zoospores ml⁻¹. We dripped 1.5 ml of inoculate on each toads’ venter over small, inoculation chambers (709 ml; Glad Products) containing 40 ml of 20% Holtfreter’s solution. Toads could not escape the inoculate bath, which was ~3 to 4 mm deep across the bottom (ensuring coverage for 24 h). The mean concentration of *Bd* inoculate within chambers was 4.4 ± 0.8 × 10^6 zoospores ml⁻¹, and the cumulative inoculate per toad was ~5.4 × 10^6 zoospores ml⁻¹. Each day before inoculation, we rinsed and re-filled chambers with fresh Holtfreter’s solution.

**Monitoring:** After the 3 d bath inoculation period, toads were transferred to clean 8 l glass aquaria identical to those in Exposure 1, but filled with 350 ml of 20% Holtfreter’s solution (ensuring complete floor coverage ~4 to 5 mm deep between water changes) and covered with mesh lids. Unlike in Murphy et al. (2009), the toads here were heavier (Day 64 mean: 18.0 ± 1.0 g) and could not climb walls to escape wet aquarium bottoms. Hence, contact with water was obligate in treatments without platforms (although toads would occasionally stand on their hind feet against walls), and facultative when they were available. Even with platforms, the default conditions were wetter in Period 2 (Days 67 to 179) than Period 1 (Days 0 to 64): during Period 2 toads had to climb onto platforms to escape water, while during Period 1 the floors of the aquaria were nearly dry.

Once or twice daily we noted the toad’s position in the aquarium (back = –1, center = 0, or front

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Sample size</th>
<th>Inoculation 1 (Days 0–4)</th>
<th>Inoculation 2 (Days 64–66)</th>
<th>Contact with water</th>
<th>Water temp.</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Control</td>
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<tr>
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<td>7</td>
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<td>Obligate</td>
<td>Ambient</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td></td>
<td>Facultative</td>
<td></td>
<td></td>
</tr>
<tr>
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<tr>
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<td>5</td>
<td>(Bd-experienced)</td>
<td>Control</td>
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<td></td>
</tr>
<tr>
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<td>6</td>
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<td>Obligate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td></td>
<td>Facultative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>Control</td>
<td>Bd bath</td>
<td>Facultative</td>
<td></td>
</tr>
</tbody>
</table>

*In ‘Facultative’ aquaria, toads could climb on platforms to dry off; in ‘Obligate’ aquaria, no platforms were available. *See Table 1B
factorial ANOVA. We used ing position (average) depended on treatment using observations), body temperature (average), and rest-
total days), time dry on platforms (proportion of total change), rate of skin shedding (days skin found/112 platforms to that in comparable unheated aquaria.  
rank test to compare survival in heated aquaria with dependent covariates (Allison 1995). We used a log-
vival time (Carey et al. 2006) and any significant time-
mass as a covariate to help control for its effect on sur-
We changed the Holtfreter’s solution and platforms (where present) 3 times per week, at which time wet mounts of shed skin were scored for Bd as after Exposure 1. Prior to water changes on Days 84, 118, and 179, we filtered ~200 ml of liquid from 17 aquaria. Bd loads were quantified with quantitative PCR (qPCR) according to Kirstein et al. (2007). Sampling was limited by processing costs, although we selected aquaria randomly from treatment groups with surviving toads at sampling time without repeating aquaria. After water changes, toads were fed 3 to 5 crickets (dusted with ReptoCal).

Any toad that appeared severely lethargic or lacked a righting reflex was euthanized in MS-222 (2 to 3 g l⁻¹). Upon death, we dissected and scored ventral skin for Bd sporangia as above, and preserved skin and foot samples in 70% ethanol. We also conducted a gross necropsy on each toad, noting any abnormalities. The Eposure 2 study period ended on Day 179 (115 d after bath inoculation began), ~3 to 5 times the period necessary for 100% mortality at equivalent Bd dosage in Carey et al. (2006). We euthanized all survivors, examined skin wet mounts and internal organs, and collected tissues for Bd testing by diagnostic PCR.

Statistical analysis: We used proportional hazards regression to compare the effects of bath Bd exposure (Days 64 to 66), prior Bd exposure (drip, Days 0 to 4), and contact with water on toad survival. Models including the 100% surviving controls (all censored) would not converge. Hence, we compared toad survival based on 3 Bd-exposure groups (drip only, bath only, and drip + bath), contact with water (obligate vs. facultative), and their interaction. We included toad mass as a covariate to help control for its effect on survival time (Carey et al. 2006) and any significant time-dependent covariates (Allison 1995). We used a log-rank test to compare survival in heated aquaria with platforms to that in comparable unheated aquaria.

We tested how toad weight (average weekly change), rate of skin shedding (days skin found/112 total days), time dry on platforms (proportion of total observations), body temperature (average), and resting position (average) depended on treatment using factorial ANOVA. We used t-tests (heterogeneous variance) to compare these responses in Groups 9 and 4. We also used ANOVA to compare filtered Bd zoospore concentration in aquaria by date, treatment, and their interaction.

RESULTS

Exposure 1. Effects of humidity and temperature on disease severity

No toads died after the first exposure, but we detected differences in the degree of infection, growth, and skin shedding by treatment. Bd infection scores were lower in toads from heated than ambient aquaria, did not differ by aquarium humidity, and no interactive effects were evident (Table 3, Fig. 1). The mean score across treatments declined significantly during the last sampling period (Fig. 1, grey line). Based on diagnostic PCR of skin swabs, 0 toads were Bd positive at the start of Exposure 1, and 0 controls and 5 of 24 exposed were positive on Day 64 (4 ambient, 1 heated).

The Bd exposure and temperature treatments interacted in their effects on toad weight, although the main effect of neither was significant (Table 4). Warmer temperature decreased weight gain in control toads, but this effect was reversed in toads exposed to Bd (Fig. 2A). The humidity treatment did not affect weight, and no other interactions were observed. Skin shedding showed a similar interactive response to Bd exposure and temperature (Table 4), with the highest rate in exposed ambient aquaria, an intermediate rate in heated aquaria, and the lowest rate in control ambient aquaria (Fig. 2B). Overall, skin sheds were observed more frequently in toads exposed to Bd (mean d⁻¹ ± 1 SE: 0.14 ± 0.01) than in controls (0.08 ± 0.01) and less frequently in more humid (0.08 ± 0.01) than drier aquaria (0.14 ± 0.01).

Mean toad temperatures were 2 to 4°C lower than air temperatures but tracked the pattern in air tempe-ration by treatment (Table 1A). Both heating and humidity treatments altered toad temperature, and interacted significantly, but there were no effects of Bd exposure on toad temperature nor any other interac-

Table 3. Anaxyrus b. boreas. Repeated measures, factorial ANOVA of Bd infection scores over four 16 d periods following Exposure 1. For each period, a subject’s score was the average of the readings on skin shed during the previous 16 d. Factors were relative humidity (RH; low or high) and temperature (T; ambient or heated; Table 2). Analysis includes only exposed toads (n = 24; all scores were zero for the 24 controls).

<table>
<thead>
<tr>
<th>Factor</th>
<th>df</th>
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<th>p</th>
</tr>
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<tr>
<td>T</td>
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<tr>
<td>Period</td>
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</tr>
<tr>
<td>RH × Period</td>
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<td>0.124</td>
</tr>
<tr>
<td>T × Period</td>
<td>3, 37</td>
<td>1.3</td>
<td>0.300</td>
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<tr>
<td>RH × T × Period</td>
<td>3, 37</td>
<td>1.7</td>
<td>0.181</td>
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</table>
Exposure 2. Effects of contact with water, temperature, and prior Bd exposure on disease severity

Continuous contact with water accelerated the onset of lethal chytridiomycosis in boreal toads, but prior exposure to Bd and a moderate increase in aquarium temperature slowed the onset of lethal disease. All 10 unexposed toads survived (Fig. 3A, Groups 1 and 2), but only 7 of 41 toads exposed to Bd survived through to Day 179. Exposed survivors were all from aquaria with platforms (Fig. 3A, Groups 6, 8 and 9). By PCR, all exposed toads were strongly Bd positive at death or on Day 179, and 4 of 10 controls were weakly positive. The PCR-positive controls may have resulted from contamination during shipment (when several vial lids popped) or PCR analysis; scores of skin wet mounts showed no positive controls (Fig. 3B, Groups 1 and 2).

The proportional hazards regression showed that toad survival depended on Bd exposure treatment and contact with water (Table 5A). Although there was no main effect of contact with water, an interactive effect with Bd exposure arose because the availability of platforms had a stronger protective effect in twice-exposed toads (drip + bath, Fig. 3A, Group 7 vs. 8) than in toads exposed only by bath (Group 3 vs. Group 4) or drip (Group 5 vs. Group 6). When compared across exposure groups (Table 5B), the ability to dry off periodically increased mean survival time nearly 3-fold, from 18 to 48 d. Log-rank tests among exposure treatments also showed that bath inoculation reduced survival time compared to exposure via drip and drip + bath.

Toads usually began to die within a treatment group when their average Bd infection score was ≥0.8 for more than one 16 d period (Fig. 3B). In toads exposed by drip only, infection scores increased slowly and did not exceed this level until the fourth period (Day 128); at this time, scores peaked in aquaria without platforms (Group 5) and increased more slowly in aquaria with platforms, in which several toads survived longer (Group 6). In the double-exposed toads (drip + bath), scores quickly peaked in those in obligate contact with water (Group 7) but fluctuated at an intermediate level in those in facultative contact with water (Group 8).

A moderate increase in aquarium temperature (Table 1B) strongly protected toads against lethal chytridiomycosis (Fig. 3A, Table 5B). Toads in heated aquaria, however, retained high levels of Bd infection (Fig. 3B, Group 9).

Similar to after Exposure 1, toad weight and skin-shedding were affected by Bd exposure during Period 2.
Prior \textit{Bd} exposure by itself did not affect weight change, while bath exposure increased weight loss (Table 6). The exposure treatments had an interactive effect on weight change, such that toads exposed twice to \textit{Bd} (drip + bath) lost less weight than those exposed via bath alone (Fig. 4A). A similar pattern was observed with respect to the rate at which shed skin was detected, with no effect of drip exposure, an
increase with bath exposure, and an interactive effect between them (Table 6, Fig. 4B). The frequency of detecting skin sheds was higher in toads in obligate contact with water compared to facultative contact (Table 6). When Groups 9 and 4 were compared, toads in heated aquaria lost less weight ($t$-test, $t = 3.6$, df = 9, $p = 0.006$) and shed skin was detected less frequently ($t = 1.5$, $p = 0.162$).

The tendency to select dry sites differed by exposure treatment following Exposure 2. Prior Bd exposure did not affect the tendency to use platforms, but exposure via bath increased their use (Table 6). Again the treatments interacted such that twice-exposed toads used platforms less often than those exposed via bath alone (Fig. 4C). In unheated aquaria, the mean body temperature did not differ by treatment ($F_{1,37} = 2.2$, $p \geq 0.143$), nor did the mean resting position within aquaria ($F_{1,39} \leq 1.6$, $p \geq 0.215$). When resting sites in Groups 9 and 4 were compared, the mean in heated aquaria was shifted towards the heat strip ($t_9 = 2.2$, $p = 0.060$).

*Bd* zoospore concentrations in water (log-transformed) in a subset (n = 17) of *Bd*-exposed aquaria did not differ by exposure type, heat, or date ($F_{1,16} = 0.3$, $p \geq 0.331$). However, zoospore loads differed in the contact with water treatment (unheated aquaria only, $F_{1,13} = 12.3$, $p = 0.004$): zoospore loads (back-transformed means) were higher in aquaria without platforms (450 ml$^{-1}$) than in those with platforms (16 ml$^{-1}$).

**DISCUSSION**

**Periodic drying and moderate warming decrease the severity of infection**

We found that temperature and hydric environment significantly affected disease severity and the outcome of *Bd* infection in boreal toads, extending earlier experimental work on this species by Carey et al. (2006) that demonstrated the effects of *Bd* dose, duration of exposure, and body size on survival time. Toads that could warm themselves moderately, from 15 to 18°C, survived despite heavy *Bd* infections. Infected toads that could dry off periodically either survived (179 d), or survived longer than those that could not escape wet environments.

**Effects of temperature**

Exposure to high temperatures (37°C) can kill *Bd* in experimentally infected frogs (Woodhams et al. 2003),

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**Table 5. Anaxyrus b. boreas.** Survival analysis of boreal toads during Period 2 (Days 67 to 179). (A) Proportional hazards regression comparing survival by *Bd* exposure treatment (Exp), contact with water (CwH$_2$O), and their interaction (n = 37; excludes Groups 1, 2 [controls] and 9, Table 2). Average toad weight (Wt) and a time-dependent covariate of average weight (Wt TD) were included to improve fit and control for nonproportional hazards. (B) Log-rank tests comparing survival of specific treatment groups: (1) by *Bd* exposure treatment, (2) by contact with water, and (3) in moderately heated versus ambient aquaria. Significant effects in **bold**

<table>
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<tr>
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<tr>
<td>Wt</td>
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<td>Wt TD</td>
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<table>
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<th>p</th>
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<tr>
<td>Drip vs. Drip + Bath</td>
<td>5, 6 vs. 7, 8</td>
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<td>0.976</td>
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<tr>
<td>(2) CwH$_2$O</td>
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<tr>
<td>(3) Moderate heat</td>
<td>4 vs. 9</td>
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<td>8.8</td>
<td><strong>0.003</strong></td>
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**Table 6. Anaxyrus b. boreas.** Factorial ANOVA of 3 responses measured on 47 toads during Period 2 (Days 67 to 179): weight change, rate of skin shedding, and time dry (on platforms). All analyses exclude Group 9. Analysis of time dry only includes toads with platforms available. Factors were Exposure 1 (Exp1; *Bd* drip or control), Exposure 2 (Exp2; *Bd* bath or control), and contact with water (CwH$_2$O; obligate or facultative). Significant effects in **bold**

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
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<th>p</th>
<th>Skin shedding</th>
<th>p</th>
<th>Time dry</th>
<th>p</th>
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<td>15.3</td>
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<tr>
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<td><strong>0.002</strong></td>
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<td>3.9</td>
<td><strong>0.056</strong></td>
<td>5.8</td>
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<tr>
<td>CwH$_2$O</td>
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<tr>
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<td>0.308</td>
<td>1</td>
<td>1.5</td>
<td>0.234</td>
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<td>2.9</td>
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<tr>
<td>Exp1 $\times$ Exp2 $\times$ CwH$_2$O</td>
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<td>0.976</td>
<td>1</td>
<td>0.1</td>
<td>0.752</td>
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</tr>
<tr>
<td>Error</td>
<td>39</td>
<td>39</td>
<td>19</td>
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but we found that a moderate increase in average toad temperature decreased infection levels (Fig. 1) and increased survival following exposure (Fig. 3A, Group 9 vs. Group 4). Our finding is consistent with the survival benefit of moderate heating observed in 2 recent infection studies (Andre et al. 2008, Bustamante et al. 2010). Interestingly, after Exposure 2, heating did not decrease infection as measured by Bd sporangia in skin (Fig. 3B, Group 9 vs. Group 4), suggesting that the toads in heated aquaria did not substantially reduce the infection sustained during bath inoculation. The mean dorsal temperature of toads in heated aquaria (Table 1) was well within the growth optima of Bd in pure culture (Piotrowski et al. 2004), and only occasionally were temperatures achieved that may hinder Bd growth (maxima 27.6 to 30.6°C). At these temperatures, decreased toad mortality is more likely due to greater effectiveness of the amphibian immune response to Bd (sensu Matutte et al. 2000) than the reduced growth rate of Bd. In severely infected toads post-Exposure 2, this increase in resistance had a behavioral component as toads shifted their resting position towards heat strips, suggesting behavioral fever (Kluger 1978) to combat Bd infection.

Providing moderate heat also decreased secondary effects of Bd infection. Exposed toads in heated aquaria gained less weight than unexposed controls at ambient temperature following Exposure 1, suggesting an increase in metabolism without a corresponding increase in available food. But these toads did not lose weight as did the exposed toads in unheated aquaria (Fig. 2A). Likewise, the rate at which shed skin was observed among exposed toads was lower in heated than ambient aquaria (Fig. 2B). Trends were similar following Exposure 2. Andre et al. (2008) also noted persistent yet less severe effects of disease in infected Rana muscosa housed at 22°C compared to those housed at 17°C.

Effects of contact with water

Carey et al. (2006) reported on Bd infection trials with boreal toads in constant contact with water and suggested that infected toads would survive longer when given the chance to dry off periodically. We confirmed this hypothesis, finding that the availability of dry sites increased mean survival time from 18 to 48 d in toads exposed to 10^6 zoospores at 15°C. Our findings are consistent with Bustamante et al. (2010), who observed longer survival in infected golden frogs with access to dry sites than those without such access.

Contact with water increased the zoospore load and made the reinfection process more efficient, raising the chances that an infection reached a lethal threshold (sensu Carey et al. 2006). As measured by qPCR on aquariim water, continuously wet toads experienced ~30x higher Bd zoospore doses prior to water changes than did those with platforms (450 vs. 16 ml^-1, respectively). Infection subsided during Period 1 (Fig. 1) but increased during Period 2 even in toads that were not re-inoculated with Bd (Fig. 3, Groups 5 and 6). Infected skin was more likely to fall in water during Period 2 (wet aquarium floors) than Period 1 (water only in dishes). Also, we changed water daily during Period 1 but only every 2 to 3 d during Period 2 (as in Carey et al. 2006), allowing more time for zoospore production from the toad and shed skin.

As skin is compromised with Bd infection, toads in contact with water that is hypotonic to blood plasma may be less able to maintain electrolyte concentrations. Marcum et al. (2010) found marginally lower mean osmolalities in Bd-exposed boreal toads in continuous contact with water than in those in facultative contact. Reduced epidermal function and electrolyte loss, by hindering the conduction of action potentials and cardiac function, appears to be the proximate
cause of mortality in severe chytridiomycosis (Voyles et al. 2009). Toads in obligate contact with water may have also suffered more stress than those with platforms available, suppressing their immune response. Although we cannot rule out this possibility, the controls in obligate versus facultative contact with water showed no difference in survival or weight change. In both groups, we observed 100% survival and a mean weight increase of 0.1 g wk⁻¹. Moreover, platforms were not fully protective against lethal disease (Fig. 3A, Groups 4 and 6), indicating that the inoculation method, in addition to contact with water, influenced the outcome of infection. Bath inoculation likely resulted in an infection that covered a greater proportion of the skin than inoculation with dry refuges. A more severe infection may overwhelm any immune response, even if the animal can dry off post-inoculation.

Higher aquarium humidity did not significantly increase the severity of Bd infection (Fig. 1) unlike the 29% increase observed by Murphy et al. (2009). This earlier study used a more extreme humidity treatment (mean 93% vs. 76 to 83% here) and smaller toadlets (mean weight 0.5 g) that may have been more sensitive to the effect of humidity on infection. The lack of an effect of humidity could also be because the sheeting used to alter humidity slightly increased average temperature, perhaps boosting the immune response to Bd (see ‘Effects of temperature’ above).

**Prior exposure to Bd decreases the severity of infection**

Prior exposure to Bd halted or slowed the onset of severe chytridiomycosis, but only when toads could select dry sites. When exposed to ~10⁶ zoospores and platforms were available, toads previously exposed to Bd lived nearly 3 times longer and had lower infection scores than did Bd-naïve animals (Fig. 3, Group 4 vs. 8). Secondary effects of infection (weight loss and a tendency to select dry sites) were also smaller in Bd-experienced than Bd-naïve toads (Fig. 4A,C). Increased survival after prior exposure suggests the possibility of acquired immunity to this pathogen, although other responses, such as higher production of hydrophobic molecules combating infection (Woodhams et al. 2006, Rollins-Smith et al. 2009), cannot be excluded.

A recent infection study on Xenopus laevis found that both innate and acquired immunity are involved in the resistance to lethal Bd infection (Ramsey et al. 2010). Rollins-Smith et al. (2009) suggest that, mechanistically, the potential for adaptive immunity in Anaxyrus (Bufo) would be similar to that of Xenopus. Findings by Woodhams et al. (2007) also indicated changes in the components of the cellular immune system in response to Bd infection. However, in a highly Bd-susceptible tropical species, Rosenblum et al. (2009) found no increase in the expression of genes associated with immunity in infected compared to control animals. Unpublished work on boreal toads carried out in the laboratory of C. Carey had findings similar to our own, i.e. increased resistance to Bd with prior exposure, but only when dry microenvironments were available (Richmond et al. 2009). As noted above, contact with water likely enhances the effective dose of Bd, thereby reducing the effectiveness of any immune response. Increased time in water may also interfere with signaling pathways that mobilize acquired immunity (e.g. by Langerhans cells; Richmond et al. 2009) or drive a stress response that interferes with immune function.

**From the laboratory to the field: factors promoting endemic infection**

Our findings, and those of Andre et al. (2008) and Bustamante et al. (2010), suggest caution when making predictions about the effects of Bd on amphibians in the field based on its growth in vitro (i.e. the ‘chytrid thermal optimum hypothesis’ sensu Pounds et al. 2006). We found that a moderate increase in temperature within the optimal growth range of Bd did not eliminate infection but altered its outcome. Moderate temperature changes may affect the host response to Bd in a species-specific manner, which warrants tests with more species (per Carey et al. 1999).

Our study also suggests how boreal toads, a semi-aquatic species that moves between wet and dry microenvironments, feeding, basking, and resting (Hammerson 1999), may act behaviorally to reduce their level of infection. When available, we found that Bd-infected toads tended to select dryer sites (Fig. 4C) and warmer sites within aquaria, and these behaviors were associated with longer survival. Infected toads are likely to respond similarly in the field, as suggested by increases in mean temperature observed in golden frogs during a Bd epidemic compared to pre-epidemic means (Richards-Zawacki 2009). Survival of initial Bd infection also means individuals may clear infections and become reinfected (Corn 2007). If, as our findings suggest, previously exposed toads combat Bd infection more effectively, these individuals may serve as Bd reservoirs and vectors, reinfecting others at spring breeding aggregations. In the absence of overwintering tadpoles or a non-amphibian reservoir, Bd persistence is more likely if individuals carry low-level infections (Briggs et al. 2010). More-
over, the high fungal loads that led to lethal infections in our aquaria, and might drive density-dependent disease outbreaks in natural populations (sensu Briggs et al. 2010), may be highly unusual. In 4 active boreal toad-breeding sites with Bd-positive adults (2 in Wyoming and Colorado), 24 of 36 water samples were Bd-negative by qPCR, and the highest zoospore load observed during breeding was 0.2 mi⁻¹ (authors’ unpubl. data), ~80 to 2000 times below loads in our Bd-exposed aquaria. Hence, high prevalence (Murphy et al. 2009) and endemic persistence of Bd in boreal toads (Pilliod et al. 2010) is not surprising. In Panama, after epidemic decline of riparian amphibians, high Bd prevalence has been observed in pond-breeding species, and may also arise from their ability to act as Bd reservoirs and vectors (Brem & Lips 2008).

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Abstract: Chytridiomycosis is linked to the worldwide decline of amphibians, yet little is known about the demographic effects of the disease. We collected capture-recapture data on three populations of boreal toads (Bufo boreas [Bufo = Anaxyrus]) in the Rocky Mountains (U.S.A.). Two of the populations were infected with chytridiomycosis and one was not. We examined the effect of the presence of amphibian chytrid fungus (Batrachochytrium dendrobatidis [Bd]; the agent of chytridiomycosis) on survival probability and population growth rate. Toads that were infected with Bd had lower average annual survival probability than uninfected individuals at sites where Bd was detected, which suggests chytridiomycosis may reduce survival by 31–42% in wild boreal toads. Toads that were negative for Bd at infected sites had survival probabilities comparable to toads at the uninfected site. Evidence that environmental covariates (particularly cold temperatures during the breeding season) influenced toad survival was weak. The number of individuals in diseased populations declined by 5–7%/year over the 6 years of the study, whereas the uninfected population had comparatively stable population growth. Our data suggest that the presence of Bd in these toad populations is not causing rapid population declines. Rather, chytridiomycosis appears to be functioning as a low-level, chronic disease whereby some infected individuals survive but the overall population effects are still negative. Our results show that some amphibian populations may be coexisting with Bd and highlight the importance of quantitative assessments of survival in diseased animal populations.

Keywords: amphibian chytrid fungus, apparent survival, Batrachochytrium dendrobatidis, Bufo boreas, chytridiomycosis, Cormack-Jolly-Seber, mark-recapture, population decline

Efectos del Hongo Quitridio Anfibio sobre la Probabilidad de Supervivencia Individual en Sapos Boreales Silvestres

Resumen: La quitridiomicosis está ligada a la declinación mundial de anfibios, sin embargo se conoce poco sobre los efectos demográficos de la enfermedad. Colectamos datos de captura-recaptura de 3 poblaciones de sapos boreales (Bufo boreas [Bufo = Anaxyrus]) en las Montañas Rocalosas (E.U.A.). Dos de las poblaciones estaban infectadas con quitridiomicosis y una no. Examinamos el efecto de la presencia del hongo quitridio anfibio (Batrachochytrium dendrobatidis [Bd]; el agente de la quitridiomicosis) sobre la probabilidad de supervivencia y la tasa de crecimiento poblacional. Los sapos infectados con Bd tuvieron una menor
probabilidad de supervivencia promedio anual que los individuos no infectados en sitios en lo que se detectó Bd, lo que sugiere que la quitridiomycosis puede reducir la supervivencia en 31-42% en sapos boreales silvestres. Los sapos negativos a Bd en sitios infectados tuvieron probabilidades de supervivencia comparables a las de sapos que fueron negativos a Bd en sitios no infectados. La evidencia de que las covariables ambientales (particularmente las temperaturas frías durante la época de reproducción) influyeron en la supervivencia de sapos fue débil. El número de individuos en poblaciones enfermas declinó en 5-7%/año a lo largo de los 6 años del estudio, mientras que la población no infectada tuvo un crecimiento poblacional comparativamente estable. Nuestros datos sugieren que la presencia de Bd en estas poblaciones de sapos no está causando declinaciones poblacionales rápidas. Más bien, la quitridiomycosis parece estar funcionando como una enfermedad crónica, de bajo nivel, por lo cual algunos individuos infectados sobreviven pero los efectos sobre la población son negativos. Nuestros resultados muestran que algunas poblaciones de anfibios pueden estar coexistiendo con Bd y resaltan la importancia de evaluaciones cuantitativas de la supervivencia de poblaciones con animales enfermos.

Palabras Clave: batrachochytrium dendrobatidis, Bufo boreas, Cormack-Jolly-Seber, declinación poblacional, hongo quitridío anfibio, marca-recaptura, quitridiomycosis, supervivencia aparente

Introduction

The amphibian chytrid fungus (Batrachochytrium dendrobatidis [Bd]) is a widespread pathogen that is hypothesized to be the cause of mass mortality in some amphibian populations (Daszak et al. 2003). Chytridiomycosis results when Bd invades keratinized tissue of an amphibian and causes hyperkeratosis (Longcore et al. 1999; Pessier et al. 1999). Hyperkeratosis disrupts cutaneous function (Voyles et al. 2009) and may compromise the host's immune system (Rosenblum et al. 2008). Laboratory results confirm the pathogenicity of Bd in amphibians (e.g., Longcore et al. 1999), and field studies show chytridiomycosis has played a significant role in some declines of amphibian populations around the world (e.g., Berger et al. 1998; Bosch et al. 2001; Lips et al. 2006).

Despite the apparent lethality of Bd, the etiology of the disease is not completely understood. Information is still needed on factors that influence susceptibility of individuals and populations to chytridiomycosis, variability in pathogenicity of Bd, and the environmental conditions that may influence the host-pathogen dynamic. Innate defenses involved in resistance to chytridiomycosis occur in some amphibians (Woodhams et al. 2007), and sick animals may alter their behavior to raise their body temperature to combat the disease (Richards-Zawacki 2009). Consequently, some populations persist with Bd (Retallick et al. 2004; Briggs et al. 2005; Kriger & Hero 2006), whereas others are extirpated (Lips et al. 2006; Skerratt et al. 2007; Ryan et al. 2008).

Understanding the variability of the responses of populations to Bd requires information on demographic parameters, especially mortality rates of infected individuals in the wild. Despite the abundance of recent research on Bd, differences in demographic parameters, such as survival, between infected and uninfected individuals have been compared in only three studies. Retallick et al. (2004) were the first to examine the effects of Bd on anuran survival. They examined infected and uninfected individuals of Taudactylus eungellensis and Litoria wilcoxiilangguy over 4 years and found no evidence that survival differed between infected and uninfected frogs. Kriger and Hero (2006) also found no evidence that Bd infection affects survivorship. In both these studies, researchers used return rate (i.e., proportion of marked individuals captured in subsequent surveys) as the response variable. Return rate tends to be a biased estimator of survival because it assumes the probability of detection is constant over years, which is rarely the case (Mazerolle et al. 2007).

Using multistate, capture-recapture models of adult male Litoria pearsoniana, Murray et al. (2009) conducted the first robust study of the probability of surviving chytridiomycosis. They found that uninfected frogs had higher survival than infected frogs. Murray et al. (2009) studied a single population over a relatively short period of time (6 months), but their robust analysis provided much needed information on variation of population susceptibility and responses to chytridiomycosis in the wild. Similar studies conducted over longer periods of time that span multiple populations across the range of a species could greatly improve our understanding of this host-pathogen relationship.

We conducted a 6-year capture-recapture study on three populations of boreal toads (Bufo boreas [Bufo = Anaxyrus]) in the Rocky Mountains (U.S.A.) to examine the factors that influence survival and annual rate of population growth at two sites where Bd was detected (hereafter, infected sites) and one site where Bd was not detected (hereafter, uninfected site). The boreal toad is a good model for studies of chytridiomycosis effects because the species is susceptible to chytridiomycosis (Blaustein et al. 2005; Carey et al. 2006; Garcia et al. 2006), Bd has been found in boreal toad populations throughout the species’ range (Pearl et al. 2007; Muths et al. 2008), and populations of boreal toads have declined coincident with the detection of Bd in populations (Muths et al. 2003; Scherer et al. 2005).
Methods

Host Ecology

Boreal toads are the latest breeders among amphibians in the Rocky Mountains, and peak breeding activity for the species usually occurs during the latter stages of snowmelt (Vertucci & Corn 1996). Toads breed at night when temperatures are still cold, sometimes near freezing. Males congregate at the breeding site before females and compete for arriving females. This breeding behavior leads to much contact among individuals and may facilitate disease transmission in some amphibians (Rowley & Alford 2007). Additionally, the motile zoospores of Bd can move from infected to healthy toads through the water (Carey et al. 2006). Probably all females do not breed each year, but each female may lay over 5000 eggs in years she oviposits (Carey et al. 2005). The number of females at breeding sites is much lower than the number of males (e.g., at our sites approximately one female to 25 males). Survival to sexual maturity is low, but this has minimal effect on population growth rate, which is most sensitive to adult survival (Biek et al. 2002; Vonesh & De la Cruz 2002). Boreal toads may live ≥9 years (Carey et al. 2005), and adult survival can be high and stable among years (Scherer et al. 2008). Adult boreal toads are active for 5–6 months of the year and may have body temperatures of ≥30 °C during their active period (Carey 1978; Bartelt & Peterson 2005).

Selection and Characteristics of Sample Sites

We selected three sites along an 11° latitudinal gradient from Montana to Colorado (Fig. 1): a group of six ponds at Lost Trail National Wildlife Refuge, Montana (LT) (1090 m elevation), an oxbow pond near the Blackrock Ranger Station, Bridger-Teton National Forest, Wyoming (BR) (2082 m), and a group of three ponds at Denny Creek, San Isabel National Forest, Colorado (DC) (3360 m). We selected sites on the basis of existing information about toad populations (historical surveys indicated consistent annual reproduction) and logistical considerations. We first detected Bd at LT in 2005 and at BR in 2003. Bd has not been detected at DC. Other species of amphibians occur at these sites, but we did not test them for Bd.

We selected the environmental covariates in our demographic models a priori. We recorded maximum and minimum daily temperatures (SNOTEL 2009) and humidity (RAWS 2009) taken at weather stations that were 2–37 km away from the sites. We summarized weather data separately for the breeding season and active season at each site in each year. We defined the breeding season as 1 week prior to our first observation of a toad at each site (prior to egg laying) plus 28 days after the first observation. To examine the influence of stressors that occur during the breeding season (e.g., cold temperatures) on toad survival, we calculated the average daily minimum air temperature, counted the number of days of killing frost (temperatures ≤−4.4 °C [24 °F]; Natural Resources Conservation Service), and recorded the ordinal day of the last killing frost.

We defined the active season as the interval between the last day of killing frost in the spring and the first day of killing frost in autumn. Because warm temperatures during the active season may influence toad immune responses and Bd pathogenicity, we included two related covariates: average daily maximum air temperature during the hottest months (July and August) of the active season and a covariate representing the potential for toads to raise their body temperature above ambient air temperatures during the day. We calculated the latter covariate as the number of hours available to a toad during the active season when the animal could raise its body temperature above ambient air temperatures during the day. We calculated the latter covariate as the number of hours available to a toad during the active season when the animal could raise its body temperature above ambient air temperatures to ≥25 °C by basking. Basking hour estimates were calculated from a combination of known thermal associations of toads (Bartelt 2000) and results from a physiological modeling approach that uses first principles of environmental biophysics. Measures of air temperature, relative humidity, zenith angle of the sun, topographic features, and vegetative structure were used to estimate hourly
body temperatures of toads created by heat transfer and evaporative cooling at each study site over the active season (P.E.B., W.P. Porter, and R.W. Klaver, unpublished data).

Collection and Analyses of Samples
From 2003 through 2008 we sampled each population during the first 2–3 weeks of the breeding season (May at LT and BR, June at DC) in multiple capture sessions. During capture sessions, 1–6 workers used headlamps to search sites and adjacent wetlands and terrestrial areas for toads. Sampling began shortly after dark and continued for at least 30 min after the last toad was observed (2 h 30 min minimum search time per capture session). We captured toads individually with plastic bags. We injected a passive integrated transponder (PIT) tag into the dorsal subcutaneous tissue of newly captured individuals. We recorded, measured, and released all captured animals.

Before we measured or tagged toads, we tested a subset of them for Bd presence by swabbing the ventral skin of the body and hind feet with a sterile swab (Hyatt et al. 2007). We sealed each sample swab in a vial and placed each vial in an individual plastic bag. Sample swabs were then sent to Pisces Molecular (Boulder, Colorado) for analysis with polymerase chain reaction to detect Bd (Annis et al. 2004 as modified by J. Wood, personal communication). This technique reliably detects Bd in individuals over time (Hyatt et al. 2007).

Because we captured many fewer females than males, we used only males in this analysis. To avoid the potential bias of changes in Bd prevalence over time (e.g., seasons; Murray et al. 2009), we tested for Bd for 1–2 weeks near the beginning of the breeding season each year. To avoid potential contamination of the collected tissue and disease transmission among sites, we adhered scrupulously to clean procedures in the field (sensu Muths et al. 2008).

Statistical Analyses
We used MARK (White & Burnham 1999) to analyze the capture-recapture data. Because our focus was on survival probability and the effect of disease on that parameter, we used the Cormack-Jolly-Seber (CJS) model (Lebreton et al. 1992). The CJS model contains two parameters: apparent survival ($\Phi$) and capture probability ($p$). Apparent survival over interval $t$ ($\Phi_t$) is the probability that a marked individual in the population during the sampling period at time $t$ survives and remains in the population until the sampling period at time $t + 1$. This parameter is referred to as apparent survival, rather than true survival, because permanent emigration cannot be distinguished from death. Although permanent emigration can bias estimates of survival probability, we considered its effect negligible because earlier studies indicate it is near zero in male boreal toads (Muths et al. 2006). Hereafter, we refer to this parameter as survival or $\Phi$.

Whereas $\Phi$ and the covariates that affect $\Phi$ were the primary parameters of interest, we first evaluated models of $p$ to avoid unnecessary bias and imprecision in survival estimates (Lebreton et al. 1992). We identified the best model of $p$ by pairing the following five models with the global model for $\Phi$ (site $\times$ time): (1) $p$ was constant across years and sites (null model), (2) $p$ varied across years only, (3) $p$ varied among sites only, (4) $p$ varied across years and among sites, and (5) $p$ varied across individuals of different disease state.

We hypothesized $\Phi$ is lower in individuals that test positive for Bd than in individuals that test negative. We also hypothesized that the effects of environmental covariates on $\Phi$ differ between individuals of different disease states. To address these hypotheses, we evaluated 13 models (see Supporting Information). We assigned each captured individual to one of three possible disease states each year: tested and Bd positive, tested and Bd negative, and untested. We assigned a disease state for each marked individual in each year of the study, even if we did not recapture an individual in a given year. We assigned as untested the disease state of toads that were not captured, or were captured but not tested, in a particular year. We allowed each individual’s disease state to change between years; thus, disease state was modeled as a covariate that varied over time.

We then assessed five models of how environmental covariates interacted with disease to affect $\Phi$ in individuals. We designed each model so that the effect of the environmental covariate on $\Phi$ depended on the disease state of the toad (i.e., an interactive effect of the environmental covariate and disease state; see Supporting Information for details of CJS models) and assumed the effect of the environmental covariate would be similar among populations.

Finite Models of Population Growth
We used a reverse-time, capture-recapture model to estimate the annual rate of population growth (Pradel 1996). The annual rate of population growth ($\lambda$) can be defined as population size at time $t + 1$ divided by the population size at time $t$ ($N_{t+1}/N_t$). We modeled the average $\lambda$ directly with the $\lambda$ parameterization of the model. In addition to $\lambda$, the $\lambda$ parameterization included the same parameters in the CJS model, $p$ and $\Phi$. Because we had modeled these parameters already in our evaluation of models of survival probability, we used the structure for $p$ and $\Phi$ from the best model of that analysis (i.e., the model with the lowest AICc value). We evaluated three structures for $\lambda$: $\lambda$ is the same at the sites that were positive for Bd but different from $\lambda$ at DC; $\lambda$ is different at all three sites; and $\lambda$ is the same across sites.

Model Evaluation and Parameter Estimation
Using program RELEASE (Lebreton et al. 1992), we tested the fit of the capture data to the CJS model with the most
parameters \( \Phi(\text{site} \times \text{time}) \). We used \( \Delta Q_{\text{AICc}} \) (delta quasi-Akaike information criterion) values and Akaike weights \( (w_i) \) to determine which model(s) had the most support, given the data. The QAICc is a modification of AIC that accounts for small sample size and overdispersion (Burnham & Anderson 2002). The \( w_i \) quantifies the strength of evidence in support of a particular model, \( i \), and can be interpreted as the probability that the model is the best model of those in the candidate set (Burnham & Anderson 2002). We considered models within two QAICc units of the best model competitive models (Burnham & Anderson 2002). In some analyses, no single model was clearly better than other models that were evaluated, and inference from a single model was not robust. To address this issue, we used model averaging to derive estimates of parameters. To calculate these estimates, we extracted the estimate for a particular parameter from each model and computed a weighted average in which each estimate’s weight was the \( w_i \) of the model from which it came (Burnham & Anderson 2002).

**Results**

**Prevalence and Capture Probability**

From 2003 to 2008 we captured 2917 male toads from the three populations and tested 353 of them for Bd (Table 1). We did not detect Bd in the 140 samples taken at DC. The average annual naïve estimate of Bd prevalence was 62% at LT and 53% at BR.

The goodness-of-fit test indicated moderate overdispersion in the data \( \hat{c} = 1.75 \). Of the evaluated models of capture probability \( (p) \), results of model selection indicated strongly that \( p \) varied across years and among sites. Therefore, we used this \( p \) structure in all subsequent models that focused on factors influencing \( \Phi \). Capture probability in one of the infected sites (BR) (range 0.27–0.53) and the uninfected site (DC) (range 0.36–0.49) were similar. Capture probability at the other infected site (LT) was low (range 0.01–0.26).

**Effects of Bd on Survival**

Survival varied as a function of disease state of the individual (Table 2). The model-averaged estimate of the regression coefficients for the effect of being untested and the effect of being positive for Bd were both negative and 95% CIs did not include zero \( (\hat{p}_{\text{Bd-untested}} = -1.02 [95\% \text{ CI} -1.46 \text{ to } -0.57] \) and \( \hat{p}_{\text{Bd-positive}} = -1.47 [95\% \text{ CI} -2.57 \text{ to } -0.36] \) ). Toads that were infected with Bd had lower average annual \( \Phi \) (0.42, 0.53) than toads that were uninfected (0.73, 0.77) at the infected sites (Fig. 2). Average annual \( \Phi \) for toads that were negative for Bd at the uninfected site (DC) was comparable (0.76) to \( \Phi \) of uninfected toads at the infected sites (BR, LT). Untested toads at the infected sites had \( \Phi \) values intermediate (0.53, 0.61) to toads that were negative and positive for Bd, whereas \( \Phi \) values for untested toads (0.77) at the uninfected site were indistinguishable from \( \Phi \) values for toads that were negative at that site (Fig. 2).

**Table 1. Number of male toads captured, swabbed, and tested for Batrachochytrium dendrobatidis (Bd) and the naïve estimate of Bd prevalence at each site from 2003 to 2008.**

<table>
<thead>
<tr>
<th>Site, year</th>
<th>Number of toads caught</th>
<th>Number of toads recaptured</th>
<th>Number of toads tested</th>
<th>Number of toads positive for Bd</th>
<th>Naïve prevalenceb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>306</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>62.0</td>
</tr>
<tr>
<td>2004</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>2005</td>
<td>218</td>
<td>21</td>
<td>22</td>
<td>2</td>
<td>9.1</td>
</tr>
<tr>
<td>2006</td>
<td>371</td>
<td>41</td>
<td>31</td>
<td>29</td>
<td>93.5</td>
</tr>
<tr>
<td>2007</td>
<td>50</td>
<td>19</td>
<td>20</td>
<td>17</td>
<td>85.0</td>
</tr>
<tr>
<td>2008</td>
<td>42</td>
<td>10</td>
<td>28</td>
<td>17</td>
<td>60.7</td>
</tr>
<tr>
<td>BR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>259</td>
<td>0</td>
<td>28</td>
<td>12</td>
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</tr>
<tr>
<td>2004</td>
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<td>42</td>
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<td>65</td>
<td>5</td>
<td>85.3</td>
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<tr>
<td>2006</td>
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<td>76</td>
<td>28</td>
<td>16</td>
<td>57.1</td>
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<tr>
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<td>170</td>
<td>24</td>
<td>22</td>
<td>10</td>
<td>45.5</td>
</tr>
<tr>
<td>DC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>145</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>2004</td>
<td>80</td>
<td>59</td>
<td>24</td>
<td>0</td>
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<tr>
<td>2005</td>
<td>71</td>
<td>29</td>
<td>25</td>
<td>0</td>
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<tr>
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<td>88</td>
<td>26</td>
<td>37</td>
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<td>86</td>
<td>30</td>
<td>13</td>
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</tr>
<tr>
<td>2008</td>
<td>55</td>
<td>24</td>
<td>10</td>
<td>0</td>
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</tr>
</tbody>
</table>

aAbbreviations: LT, Lost Trail National Wildlife Refuge, Montana; BR, Blackrock, Wyoming; DC, Denny Creek, Colorado.
bThe number of individuals that tested positive for Bd divided by the total number of individuals tested. Population at LT was first tested in 2005.
Table 2. Set of models used to estimate the survival probability (Φ) of male boreal toads of different disease states captured at 3 study sites between 2003 and 2008.a

<table>
<thead>
<tr>
<th>Model rank</th>
<th>Model nameb</th>
<th>QAICc</th>
<th>ΔQAICc</th>
<th>w</th>
<th>Model likelihood</th>
<th>K</th>
<th>Qdeviance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Φ (disease state)</td>
<td>2114.78</td>
<td>0.00</td>
<td>0.20</td>
<td>1.00</td>
<td>18</td>
<td>2078.52</td>
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<tr>
<td>2</td>
<td>Φ (LT, BR + disease state)</td>
<td>2115.05</td>
<td>0.27</td>
<td>0.18</td>
<td>0.88</td>
<td>20</td>
<td>2074.73</td>
</tr>
<tr>
<td>3</td>
<td>Φ (LT, BR × disease state)</td>
<td>2115.46</td>
<td>0.68</td>
<td>0.14</td>
<td>0.71</td>
<td>20</td>
<td>2075.14</td>
</tr>
<tr>
<td>4</td>
<td>Φ (LT/BR + disease state)</td>
<td>2116.45</td>
<td>1.66</td>
<td>0.09</td>
<td>0.44</td>
<td>19</td>
<td>2078.16</td>
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<tr>
<td>5</td>
<td>Φ (disease state × KFdays)</td>
<td>2116.67</td>
<td>1.89</td>
<td>0.08</td>
<td>0.39</td>
<td>21</td>
<td>2074.52</td>
</tr>
<tr>
<td>6</td>
<td>Φ (Bd(0) BR, LT, Bd(+); Bd(0) at DC, Bd(−))</td>
<td>2116.68</td>
<td>1.89</td>
<td>0.08</td>
<td>0.39</td>
<td>21</td>
<td>2074.39</td>
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<tr>
<td>7</td>
<td>Φ (disease state × TMINbrd; DC)</td>
<td>2117.39</td>
<td>2.61</td>
<td>0.05</td>
<td>0.27</td>
<td>21</td>
<td>2075.04</td>
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<tr>
<td>8</td>
<td>Φ (LT, BR × Bd(0), Bd(+); Bd(0), DC)</td>
<td>2117.89</td>
<td>3.11</td>
<td>0.04</td>
<td>0.21</td>
<td>21</td>
<td>2075.54</td>
</tr>
<tr>
<td>9</td>
<td>Φ (disease state × KFlast; DC)</td>
<td>2118.96</td>
<td>4.18</td>
<td>0.02</td>
<td>0.12</td>
<td>21</td>
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<tr>
<td>10</td>
<td>Φ (disease state × TMAXact; DC)</td>
<td>2119.06</td>
<td>4.79</td>
<td>0.02</td>
<td>0.09</td>
<td>21</td>
<td>2077.22</td>
</tr>
<tr>
<td>11</td>
<td>Φ (LT, BR × Bd(O), Bd(+); DC)</td>
<td>2119.58</td>
<td>4.79</td>
<td>0.02</td>
<td>0.09</td>
<td>21</td>
<td>2077.22</td>
</tr>
<tr>
<td>12</td>
<td>Φ (disease state × BASKhr; DC)</td>
<td>2135.28</td>
<td>20.50</td>
<td>0.00</td>
<td>0.00</td>
<td>16</td>
<td>2103.08</td>
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</tbody>
</table>

The best five models showed no evidence that Φ varied between negative and untested individuals at DC, which lends validity to our supposition that the population was uninfected. The highest ranked model (i.e., lowest QAICc value) with different survival probabilities between disease states (untested vs negative for Bd) at DC (model rank 6, Table 2) was approximately 1.9 QAICc units higher than the best model of those examined (model rank 1, Table 2) and the associated regression coefficient, although positive, was centered approximately on zero (βBd−untestedat DC = 0.17; 95% CI -0.73 to 1.07).

Two models that included environmental covariates had strong support from the data (ΔQAICc ≤ 2). Estimates of regression coefficients from these models indicated that individuals that tested negative for Bd were influenced differently by the number of killing frost days and average daily minimum temperature during the breeding season than positive and untested individuals. For example, the estimate from the fifth-ranked model that included the number of killing frost days indicated no relationship between Φ and number of killing frost days for individuals that were negative for Bd (β = 0.13; 95% CI −15.72 to 15.98). The estimated regression coefficients, however, suggested a positive relationship between Φ and number of killing frost days for untested individuals (β = 5.60; 95% CI −17.00 to 28.21) and individuals that were positive for Bd (β = 3.35; 95% CI −26.69 to 33.39).

Models of Finite Population Growth

The best model indicated λ was the same for the three sites (Table 3). Due to uncertainty in model selection (ΔQAICc < 2), however, we derived model-averaged estimates of λ for each population. Over the 6 years of the study, the average population decline at infected sites was 5–7%/year (LT: λ = 0.93, 95% CI 0.49–0.99; BR: λ = 0.95, 95% CI 0.78–0.99), whereas at the uninfected site (DC) the population growth rate was mostly stable (λ = 1.00, 95% CI 0.91–1.09).

Discussion

The pathology of chytridiomycosis in amphibians is complex and variable because host resistance, disease pathogenicity, and environmental conditions vary. Our
results show that in the wild Bd reduces survival of infected adult boreal toads, a species that has declined in parts of its range and is considered "near threatened" by the International Union for Conservation of Nature (IUCN 2009). Nevertheless, contrary to patterns of rapid decline observed elsewhere following outbreaks of chytridiomycosis in amphibian populations (Skerratt et al. 2007; Ryan et al. 2008), we found that infected populations were declining relatively slowly even when over 40% of tested individuals in a population were infected with Bd. Our findings contribute to a growing body of evidence that some amphibian species and populations may coexist with Bd or that Bd is not an invariably lethal pathogen for all amphibians (Carey et al. 2006; Rachowicz et al. 2006). Given the widespread distribution of Bd and evidence that Bd has been present in many regions for ≥50 years (Ouellet et al. 2005), amphibian coexistence with Bd may now be the prevailing situation in some regions, particularly North America (Briggs et al. 2005; Longcore et al. 2007; Murray et al. 2009) and the United States (Corn 2007).

Understanding the effects of disease on population persistence requires estimates of survival, information that rarely is available for wild host populations (Jolles et al. 2005). We found that infected toads were 31% (LT) and 42% (BR) less likely to survive compared with uninfected toads at the infected sites. Furthermore, survival probabilities for uninfected toads at both infected and uninfected sites were high and similar. These findings are consistent with those of the only other analysis of survival probability of infected anurans in the wild. In a population of *L. pearsoniana* in Australia, monthly survival was consistently 38% lower in infected (Φ = 0.1–0.6) than in uninfected male frogs (Φ = 0.35–0.95; Murray et al. 2009). These findings contrast with two other Australian studies in which there were no statistical differences between return rates of infected and uninfected frogs, even in populations where 32% of tested frogs were infected with Bd (Retallick et al. 2004; Kriger & Hero 2006).

Our study provides additional evidence that some amphibian species and populations around the world are able to persist in the presence of Bd. The annual survival probabilities for infected toads were lower than for uninfected toads, but survival probability was still often >0.50 for those that were infected. This suggests that infected toads are more likely to die than uninfected toads, but the infected toads still have a fairly high probability of surviving to the next year. Two of our toads that tested positive for Bd in 1 year, tested negative in the next year (D.S.P. and E.M., unpublished data), which indicates individuals can rid themselves of the fungus over time. Clearing Bd infection has been reported in amphibians in Australia (Kriger & Hero 2006; Murray et al. 2009) and the United States (Corn 2007).

The influence of the environment on the dynamics of chytridiomycosis has not been well studied in the field because of the complexity of natural environments (e.g., microhabitat conditions), microhabitat selection by hosts, and the physiological responses of host and pathogen to the environment. Our results suggested that the direct effect of Bd on survival of adult boreal toads was larger than the indirect effect of the environmental covariates (i.e., temperature) on disease and toad survival. Results of model selection from among the models that included environmental covariates suggested that colder temperatures during the breeding season (i.e., more killing frost days) may have created less favorable conditions for Bd and thus increased survival of infected toads. These findings are consistent with Piotrowski et al. (2004), who suggest that infections at temperatures below 10 °C may not be fatal because growth of Bd is not favored. Nevertheless, the CIs around the regression coefficients for the number of killing frost days were approximately centered on zero; therefore, the potential for cold temperatures to suppress Bd activity needs further exploration.

Over the 6 years of our study, infected populations declined by about 5–7%/year, whereas the uninfected population remained relatively stable. Assuming mortality related to Bd is additive relative to other sources of mortality, it is possible that chytridiomycosis is removing some individuals from the population each year, but not causing mass mortality. This suggests that chytridiomycosis can function as an enzootic disease in which host
and pathogen coexist. Although a 5-7% annual decline could lead to extinction within a few years, models of amphibian extinction risk suggest that population persistence is possible if some infected individuals survive (Briggs et al. 2005), which appears to be the case in our infected populations. Rates of population decline, however, may also be influenced by population size; smaller populations may be affected disproportionately by demographic stochasticity or loss of alleles that afford disease resistance.

A complete understanding of this host-pathogen dynamic at the sites we examined depends on information on first arrival of Bd at infected sites and the time that elapsed to first infection. We have, however, only a general understanding of the history of Bd in the region. From museum records, it appears Bd first appeared in the western United States in the 1960s (Ouellet et al. 2005; Padgett-Flohr & Hopkins 2009). It is now widespread (Pearl et al. 2007; Muths et al. 2008; Padgett-Flohr & Hopkins 2009), and sufficient time has passed for some populations to have developed resistance. Therefore, we speculate our toad populations have experienced one of the following scenarios: (1) Bd arrived years or decades prior to initial surveys of these populations as an epizootic pathogen, and its presence resulted in low survivorship and a declining population. Initial population declines were followed by recovery of the population by resistant individuals, but the disease continues to reduce survival of some infected toads; (2) Bd arrived (possibly even recently) as a novel pathogen and has had low-level effects on survival since its arrival; (3) Bd arrived (possibly even recently) as a novel pathogen that caused no mortality until its interaction with other stressors associated with changing climatic conditions (e.g., warmer or drier winters, earlier springs).

The future of these populations is uncertain. Our data indicate chytridiomycosis is decreasing survival of adult male toads and causing slow population declines. But given the short duration of our study relative to the long lifespan of boreal toads, we are not yet convinced that these infected populations are threatened with extirpation.

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Supporting Information

Details of the hypotheses and descriptions of the Cormack-Jolly-Seber models used to evaluate the effect of disease state and environmental variables on survival are available as part of the online article (Appendix S1). The authors are responsible for the content and functionality of these materials. Queries (other than absence of the material) should be directed to the corresponding author.

Literature Cited


Pilliod et al. 2010