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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 ~10^6 *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 10^6 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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lethal chytridiomycosis, however, depends not only on *Bd*, but on the host's immune response. Resistance to *Bd* has been linked to antimicrobial skin peptides and other hydrophobic molecules (Woodhams et al. 2007, Rollins-Smith et al. 2009) and cutaneous bacteria (Harris et al. 2006), both of which may change in effectiveness depending on body temperature and hydric environment (Matutte et al. 2000, Rollins-Smith et al. 2002).

Although the effects of varying temperature on chytridiomycosis have been studied experimentally, the outcomes *in vivo* have not always been consistent with the temperature optima of *Bd* in pure culture. For example, Carey et al. (2006) found no difference in survival time in infected boreal toads *Anaxyrus boreas boreas* housed at 12 and 23°C, although 12°C is well below the optimal growth range of *Bd*. Likewise, 2 studies have found that moderate changes in temperature within the optimal growth range of *Bd* (from 17 to 22–23°C) increased survival in exposed amphibians (Andre et al. 2008, Bustamante et al. 2010). Amphibians often modify their body temperature behaviorally, including in response to infection (Hutchison & Dupré 2002), and a recent field study suggests behavioral fever in frogs during *Bd* outbreaks (Richards-Zawacki 2009). Host-specific responses to *Bd* suggest that predictions of population declines that are based on average air temperatures and *Bd* growth *in vitro* are likely simplistic.

Most experiments challenging post-metamorphic amphibians with *Bd* 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 how microenvironment and prior exposure interact to affect 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*-naïve 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 (≥28°C for 2 d; Pi-

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)

Treatment	RH (% ± 1SE) (min., max.)	Air temp. (°C ± 1SE) (min., max.)	Water temp. (°C ± 1SE) (min., max.)		Toad temp. (°C ± 1SE) (min., max.)
(A) Period 1 (post-Exposure 1)			Back dish,	Front dish	
Low RH, ambient	44 ± 2 (33, 57)	19.5 ± 0.2 (16.4, 23.6)	16.3 ± 0.1,	16.8 ± 0.3 (12.7, 17.9)	15.5 ± 0.1 (11.3, 19.9)
Low RH, heated	41 ± 3 (27, 68)	21.0 ± 0.4 (14.9, 27.1)	17.4 ± 0.3,	25.1 ± 0.7 (14.0, 31.5)	17.0 ± 0.3 (11, 28.8)
High RH, ambient	83 ± 4 (50, 100)	19.8 ± 0.2 (16.4, 24.0)	17.4 ± 0.1,	17.5 ± 0.1 (12.5, 20.3)	17.7 ± 0.1 (13.3, 20.9)
High RH, heated	76 ± 4 (37, 92)	24.0 ± 0.7 (16.0, 27.1)	20.9 ± 0.2,	28.0 ± 0.4 (14.7, 33.2)	21.9 ± 0.2 (14.1, 30.9)
(B) Period 2 (post-Exposure 2)			Center of aquarium floor		
Ambient	41 ± 3 (24, 88)	19.6 ± 0.2 (18.3, 20.6)	15.6 ± 0.3 (12.1, 18.4)		15.0 ± 0.1 (11.7, 17.8)
Heated	34 ± 4 (23, 83)	21.6 ± 1.0 (19.0, 24.8)	18.0 ± 1.1 (11.6, 27.3)		17.8 ± 0.7 (11.4, 27.6)

otrowski et al. 2004). We manipulated humidity using different aquarium lids, using clear plastic sheeting for high humidity and no-see-um mesh (Balson Hercules Group) for low humidity. The humidity treatments (Table 1A) were within the observed range during daylight in montane habitat, with 'low' approximating the median of dry woodlands and 'high' approximating conditions in burrows or closed-canopy habitat near water (P. J. Murphy unpubl. telemetry data).

Drip inoculation: Toads were exposed to *Bd* using a protocol that simulated natural conditions in which toads enter water periodically to rehydrate (Bartelt et al. 2004). 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 *Bd* testing via PCR (protocol modified from Annis et al. 2004). *Bd* 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} penicillin-streptomycin (Hyclone). The average daily *Bd* inoculate (±1 SE) was $5.8 \pm 0.8 \times 10^5$ 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 thermister 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-

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 (*Bd*-naïve + *Bd* bath + facultative contact with water) except that after inoculation toads were placed in aquaria 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 aquaria, 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 × 10^4 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 aquaria (back = –1, center = 0, or front

Table 2. *Anaxyrus b. boreas.* Design of the Exposure 2 experiment involving a second Bd inoculation (Days 64 to 66) and 112 d of monitoring (Days 67 to 179). The experiment distributed 51 toads across 9 treatment groups. Five groups were *Bd*-naïve and 4 *Bd*-experienced from the Exposure 1 experiment. Among the *Bd*-naïve toads, more were allocated to the *Bd* bath treatments (Groups 3 and 4) than to the controls (Groups 1 and 2) in order to increase the power to detect differences in disease progression due to time in contact with water

Treatment group	Sample size	Inoculation 1 (Days 0–4)	Inoculation 2 (Days 64–66)	Contact with water[a] (Period 2: Days 67–179)	Water temp.[b]
1	5	Control	Control	Obligate	Ambient
2	5	(*Bd*-naïve)		Facultative	
3	7		*Bd* bath	Obligate	
4	7			Facultative	
5	6	*Bd* drip	Control	Obligate	Ambient
6	5	(*Bd*-experienced)		Facultative	
7	6		*Bd* bath	Obligate	
8	6			Facultative	
9	4	Control (*Bd*-naïve)	*Bd* bath	Facultative	Heated

[a]In 'Facultative' aquaria, toads could climb on platforms to dry off; in 'Obligate' aquaria, no platforms were available. [b]See Table 1B

[over heat strip where present] = 1) and whether it was in contact with water (on the floor) or not (on a platform, if available). We measured the dorsal surface temperature of each toad using a non-contact infrared thermometer 3 to 5 times per week at times ranging from mid-morning to early evening. Toads were weighed weekly as after Exposure 1.

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 Kirshtein 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 temperature 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). Significant effects in **bold**

Factor	df	*F*	p
RH	1, 19	0.1	0.833
T	1, 19	13.5	**0.002**
RH × T	1, 19	0.1	0.747
Period	3, 37	4.9	**0.006**
RH × Period	3, 37	2.1	0.124
T × Period	3, 37	1.3	0.300
RH × T × Period	3, 37	1.7	0.181

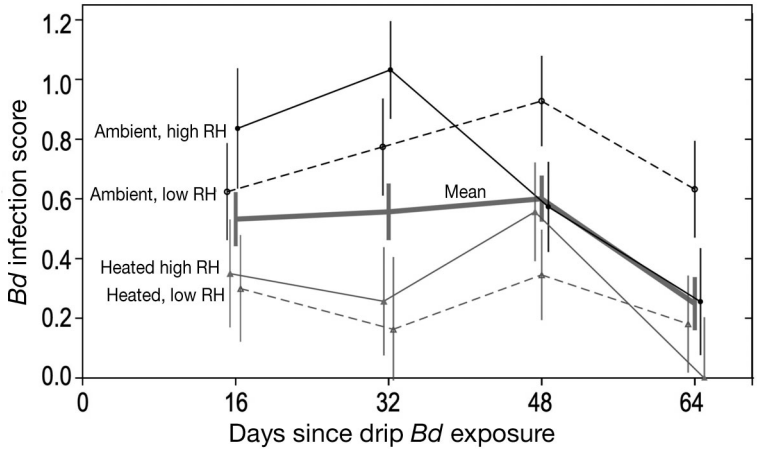

Fig. 1. *Anaxyrus b. boreas.* Infection scores based on shed skin in *Bd*-exposed toads (n = 24) by microenvironment treatment during Period 1 (Days 0 to 64). Each point represents the treatment mean (±1 SE) based on the average infection scores of 6 toads for the previous 16 d. Thick grey line connects the means across treatments (±1 SE) by time period. Mean scores differed by temperature and time but not humidity, and there were no interactions (Table 3). Table 1A gives the means and ranges of the temperature and humidity treatments. RH: relative humidity

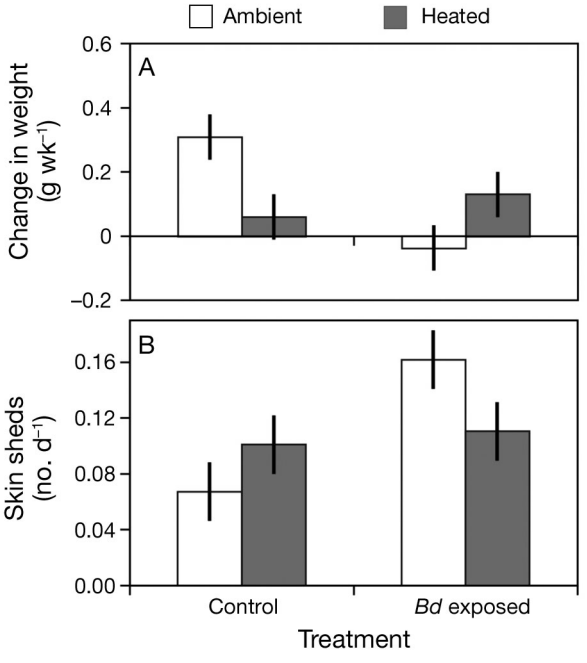

Fig. 2. *Anaxyrus b. boreas.* (A) Weight change and (B) rate of skin shedding in toads during Period 1 (Days 0 to 64) by pathogen and temperature treatments (mean ± 1SE, n = 12, humidity treatments pooled). Pathogen exposure and temperature interacted to affect growth and skin shedding (Table 4). Table 1A gives the means and ranges of the temperature treatments

tions (Table 4). *Bd* exposure and temperature did not change the tendency of toads to select wet sites, yet toads in high humidity aquaria spent less time in water dishes than those in drier aquaria (Table 4).

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.

Table 4. *Anaxyrus b. boreas.* Factorial ANOVA of 4 responses measured on 48 toads during Period 1 (Days 0 to 64): weight change, rate of skin shedding, body temperature, and time in water. Factors were *Bd* exposure (Exp; *Bd* or control), relative humidity (RH; low or high) and temperature (T; ambient or heated; Table 2). Significant effects in **bold**

Factor	df	Weight change		Skin shedding		Toad temperature		Time in water	
		F	p	*F*	p	*F*	p	*F*	p
Exp	1	1.3	0.268	6.1	**0.018**	0.3	0.581	0.7	0.423
RH	1	1.7	0.207	6.5	**0.015**	398.8	**<0.001**	5.2	**0.028**
T	1	1.9	0.178	0.2	0.677	254.1	**<0.001**	0.4	0.512
Exp × RH	1	0.1	0.830	0.6	0.428	0.0	0.967	0.3	0.576
Exp × T	1	9.3	**0.004**	4.1	**0.050**	1.0	0.328	0.0	0.865
RH × T	1	0.6	0.442	1.8	0.190	63.3	**<0.001**	0.4	0.512
Exp × RH × T	1	0.0	0.854	0.3	0.571	0.3	0.606	0.7	0.396
Error	40								

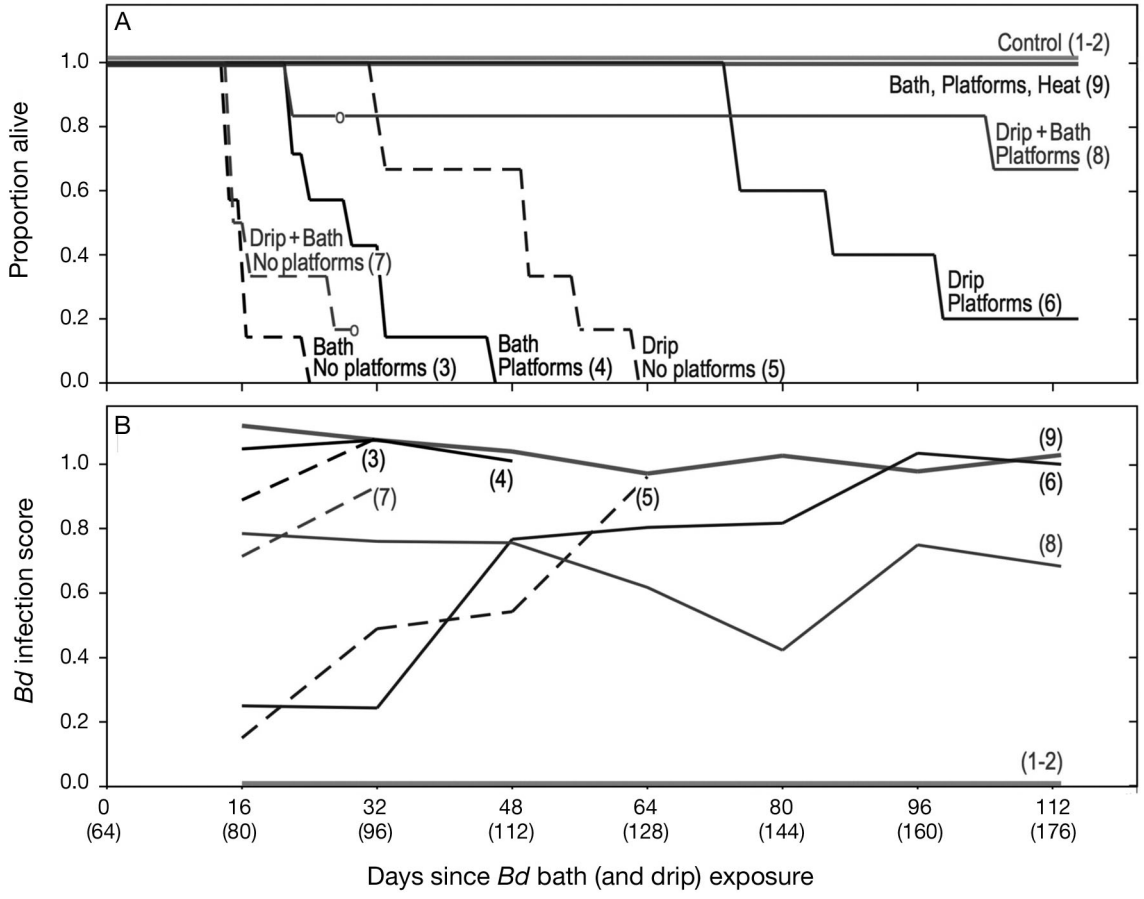

Fig. 3. *Anaxyrus b. boreas.* (A) Survivorship and (B) *Bd* infection score in toads during Period 2 (post-Exposure 2, Days 67 to 179). Numbers in parentheses correspond to treatment groups (Table 2). Toads were exposed to *Bd* by drip on Days 0 to 4, by bath on Days 64 to 66, by both methods at their respective times (drip + bath), or remained unexposed (control). Half of each exposure combination were in obligate contact with water (dashed lines), while the other half were in facultative contact (solid lines). In (B), lines link points that are averages based on living toads in that treatment during the previous 16 d (SEs omitted for clarity). Circles (o) in (A) indicate censored individuals (n = 3) due to a husbandry error on Day 93. Proportional hazards analysis showed effects on survivorship of exposure type, contact with water, and temperature (Table 5A)

Prior *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 *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

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_2O), 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**

(A) Proportional hazards regression

Variable	df	χ^2	p
Exp	1	9.1	**0.003**
CwH_2O	1	0.6	0.450
Exp × CwH_2O	1	3.9	**0.048**
Wt	1	1.5	0.223
Wt TD	1	3.4	0.067

(B) Log-rank tests

Contrast	Groups compared	df	χ^2	p	n
(1) Bath vs. Drip	3,4 vs. 5,6	1	20.4	**<0.001**	25
Bath vs. Drip + Bath	3,4 vs. 7,8	1	4.1	**0.044**	26
Drip vs. Drip + Bath	5,6 vs. 7,8	1	0.0	0.976	23
(2) CwH_2O	3,5,7 vs. 4,6,8	1	4.2	**0.040**	37
(3) Moderate heat	4 vs. 9	1	8.8	**0.003**	11

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_2O; obligate or facultative). Significant effects in **bold**

Source	Weight change			Skin shedding			Time dry		
	df	*F*	p	df	*F*	p	df	*F*	p
Exp1	1	0.1	0.737	1	2.8	0.103	1	1.6	0.219
Exp2	1	15.1	**0.000**	1	7.4	**0.010**	1	15.3	**0.001**
Exp1 × Exp2	1	10.5	**0.002**	1	3.9	**0.056**	1	5.8	**0.027**
CwH_2O	1	0.6	0.432	1	5.5	**0.024**			
Exp1 × CwH_2O	1	1.1	0.308	1	1.5	0.234			
Exp2 × CwH_2O	1	0.0	0.979	1	2.9	0.095			
Exp1 × Exp2 × CwH_2O	1	0.0	0.976	1	0.1	0.752			
Error	39			39			19		

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 ($F_{1,37} \leq 2.2$, $p \geq 0.143$), 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 ($F_{1,16} \leq 0.3$, $p \geq 0.331$). 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),

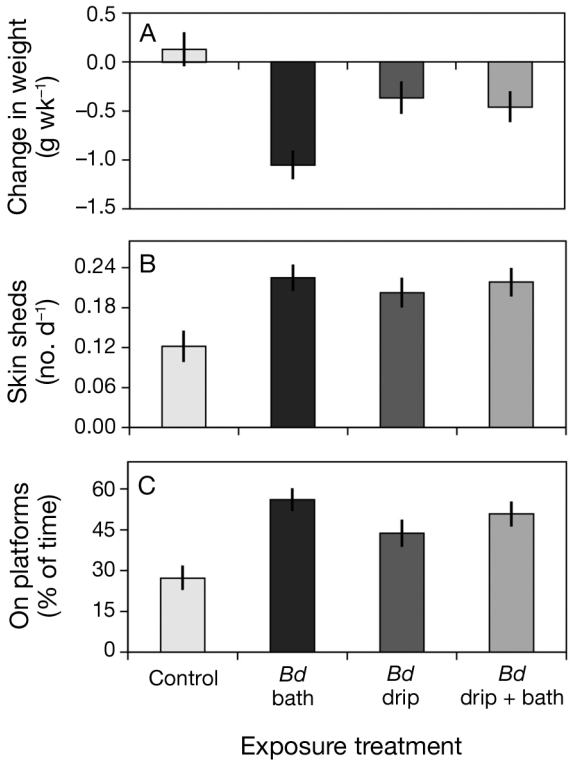

Fig. 4. *Anaxyrus b. boreas.* (A) Weight change, (B) skin shedding, and (C) use of platforms in toads in ambient aquaria by exposure treatment during Period 2 (Days 67 to 179). Means (±1 SE) in (A) and (B) pool data from aquaria with and without platforms (n = 10 to 14), while those in (C) are from aquaria with platforms only (n = 5 to 7). For all 3 responses, Exposures 1 and 2 interacted, meaning that the effects of drip + bath exposure on toads were non-additive (Table 6)

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 ~30× 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 ~10^6 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 ml^{-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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