Does Chytridiomycosis Disrupt Amphibian Skin Function?

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Chytridiomycosis, a disease caused by the fungus *Batrachochytrium dendrobatidis* (Bd), potentially disrupts osmoregulation or respiration across the skin of amphibians it infects, releases toxins into the host, or both. We investigated whether infection with Bd alters water balance or metabolic rate of the hylid frog *Litoria raniformis*. Frogs were held in laboratory conditions simulating those in which Bd epizootics had been observed in the field. We inoculated six frogs with infective Bd zoospores, held the subjects in individual containers, and compared their course of infection and associated physiological measures with those of six controls. Experimental subjects exhibited clinical signs of chytridiomycosis during the early period of infection, one week after they were inoculated, possibly due to invasion of Bd into the skin. These clinical signs were accompanied by significant inhibition of rehydration through the skin. However, we detected no changes in metabolic rate attributable to chytridiomycosis after one week. Five months after inoculation, all but one of the infected subjects had survived. Molecular testing confirmed that surviving frogs, although aclinical, still were infected. Control and infected subjects showed no difference in water balance or metabolism. These results provide evidence of inhibited rehydration in individuals exhibiting clinical signs of chytridiomycosis. However, aclinical chytridiomycosis does not severely affect amphibian skin function. Frogs that survive infection by Bd, even if they remain infected, may suffer no significant impairment in their physiological responses. The disease progression, with initial clinical signs of chytridiomycosis followed by apparent full recovery, is consistent with an adaptive immune response to Bd infection. Further research is needed to determine how Bd causes clinical chytridiomycosis and the immunological mechanisms by which hosts respond to Bd.

GLOBAL amphibian declines have been occurring since the 1960s (Houlahan et al., 2000; Alford et al., 2001; Stuart et al., 2008). Many amphibian population declines have occurred under stable environmental conditions in ecologically pristine habitats (Laurence et al., 1996; Lips, 1998, 1999; Bosch et al., 2001; Bell et al., 2004), and observations of sick frogs suggest that emerging infectious diseases may be responsible. The pathogenic amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Bd), first identified by Berger et al. (1998), appears to underlie mass mortality events. Over the past ten years, Bd has been found associated with frogs in declining (but also in apparently stable) populations in many areas around the world (Retallick et al., 2004; Lips et al., 2007; Longcore et al., 2007; Fisher et al., 2009; Kilpatrick et al., 2010). Available evidence suggests that Bd has been introduced into many localities (Morehouse et al., 2003). As no other pathogen is linked so closely to amphibian population declines, many believe that Bd is the proximate cause of global amphibian declines (Skerratt et al., 2007).

Despite the developing consensus that a subset of amphibian declines are attributable to the spread of Bd, mechanisms by which chytridiomycosis affects amphibians remained unstudied until recently. Susceptible species typically demonstrate progressive deterioration in condition, culminating in mortality in many (Bosch et al., 2001; Nichols et al., 2001; Raverty and Reynolds, 2001) but not all (Davidson et al., 2003) species. Infection might cause hyperkeratosis, thereby impeding respiration or water balance, or hosts might be poisoned by a fungal toxin (Berger et al., 1998; Pessier et al., 1999). Voyles et al. (2009) found that Bd infection limits cutaneous absorption of sodium and potassium in infected *Litoria caerulea*, and suggested that lower plasma concentrations of these electrolytes induce mortality by causing asystolic cardiac arrest. Direct measurements of the effect of Bd infection on amphibian respiration, dehydration, and osmotic water absorption have not been undertaken.

New Zealand's amphibian fauna includes four extant species of endemic frog (but see Holyoake et al., 2001) in the archaic endemic family Leiopelmatidae and three introduced Australian frogs from the family Hylidae (Bell, 1982; Bell et al., 1998; Vörös et al., 2008; King et al., 2009). One endemic, *Leiopelma archeyi*, and two of the Australian species, *Litoria raniformis* and *L. aurea*, have suffered reductions in distribution over the last decade (Bell, 1999; Pyke et al., 2002; Bell et al., 2004). Bd has been identified in populations of all these species, both those that have declined and those that are stable. The first recorded epizootic, in 1999, occurred in a *L. raniformis* population in which adults and metamorphs demonstrated clinical signs of chytridiomycosis leading to mortality (Waldman et al., 2001). An unusually high number of frogs present in the pond suggested that stressors associated with crowding may have compromised victims' immunological defenses (Glennemeir and Denver, 2002; Davis and Maerz, 2008) and enhanced rates of transmission, thereby increasing their vulnerability to chytridiomycosis. No loss of body condition was noted, and gross necropsy and histological investigations of carcasses failed to implicate causes of mortality other than Bd (Waldman et al., 2001).

Amphibian population declines may result from multiple interacting factors (Waldman and Tocher, 1998). Some amphibians have effective innate immune defenses to Bd (Rollins-Smith et al., 2002; Davidson et al., 2003), whose efficacy may be influenced by environmental factors

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(Davidson et al., 2007), including the presence of possibly symbiotic microorganisms (Woodhams et al., 2007). Adaptive immune responses to Bd also have been suggested (Richmond et al., 2009; Robert and Ohta, 2009) but have yet to be documented. To comprehend the ecological consequences of chytridiomycosis, a broad understanding of how host physiology changes in response to infection by Bd is needed.

Here we extend upon previous physiological investigations of Bd on amphibians by examining how infection with Bd affects respiration, dehydration, and osmotic water absorption in *L. raniformis*. We examine these effects on adult frogs held in laboratory conditions simulating those under which they show clinical signs of chytridiomycosis in the field. We compare metabolic and osmoregulatory (dehydration and rehydration) responses of frogs that we experimentally infected to those of control frogs, all known to be previously free of Bd infection. We expected that infection with Bd would impair rehydration in subjects demonstrating clinical signs of chytridiomycosis.

MATERIALS AND METHODS

Animals.—We collected adult *L. raniformis* in December 2001 from adjoining ponds in Alexandra, New Zealand (45°15′S, 169°23′E). The source population had been monitored carefully for more than 20 years, and no sick or dying frogs were ever observed. All individuals from this population that we had sampled for Bd infection by histology and PCR tested negative since the discovery of Bd in New Zealand. Further, subjects used in this experiment were tested for Bd infection by PCR assay and histological analyses (see below) prior to their use, and all tested negative. In contrast, other populations of *L. raniformis* that we monitored in surrounding regions experienced decline associated with the presence of Bd (Waldman et al., 2001). The frogs were housed at the University of Canterbury, Christchurch, in terraria with a constantly flowing water supply (23°C). Air temperature varied from 22 to 25°C, and photoperiod followed natural ambient conditions (12–15 hours light). Frogs were fed larval *Tenebrio* (mealworms) and nymph crickets on an *ad libitum* basis.

The *L. raniformis* ($n = 6$ control and 6 Bd-infected) used in these experiments comprised a high density, cold temperature treatment as part of a wider study of the susceptibility of frogs in New Zealand to Bd. Prior to the infection experiment, the frogs were held at 5°C, densities of 25 m^{-2}, in semi-darkness for six weeks. Temperature conditions were similar to those experienced by *L. raniformis* in New Zealand winter. We did not expect that these conditions would affect the frogs' respiratory capabilities, and we found that their water balance was unaffected (Carver, 2004). In addition to high density, low temperature and confinement in captivity can induce stress and immunosuppression in amphibians (Coddington and Cree, 1995; Maniero and Carey, 1997; Raffel et al., 2006). Indeed, even small temperature decrements can increase Bd infection rates and mortality in laboratory (Andre et al., 2008) and wild (Richards-Zawacki, 2010) frog populations.

Protocol for infecting frogs.—From April to September 2003 (late autumn to early spring), we inoculated experimental subjects with zoospores harvested from a culture of Bd that originally was isolated from a juvenile *Litoria lesueuri* in Australia in March 2000 by Lee Berger and colleagues. This culture was the most virulent of three Bd strains isolated from Australian frogs, and caused mortality in other infection studies that were run contemporaneously with ours (Berger et al., 2005). Zoosporangia were cultured on mTGhL agar (8 g tryptone, 2 g gelatin hydrolysate, 4 g lactose, 10 g agar and doubly distilled water with 200 mg penicillin-G and 300 mg streptomycin sulphate to inhibit bacterial growth). Sporangial agar cultures were incubated at 23°C for a minimum of four days before zoospores were harvested by flooding the plate with 2 ml of DS solution (artificial pond water: 1 mM KH_2PO_4, 0.1 mM $MgCl_2$, 0.02 mM $CaCl_2$). The plates were left to stand for 3 min, which induced zoosporangia to release fresh zoospores. The supernatant was pipetted off and the process repeated twice more. Concentrations of zoospores then were determined by hemocytometer.

Experimental subjects were inoculated over the ventral abdomen, hind legs, and digits with ca. 100,000 freshly harvested zoospores in 1 ml of DS solution. We had found, in preliminary trials, that this dose induced consistent clinical signs of chytridiomycosis. Controls were inoculated with 1 ml DS solution washed off clean TGhL agar plates (no Bd). Frogs then were incubated for 120 min in a glass jar with 20 ml DS solution (depth sufficient to immerse ventral abdominal region, 5 mm) at room temperature (19–22°C). During this period, jars were rotated gently to ensure complete exposure of subjects' skin to Bd zoospores and thereby maximize possibilities for infection. Each subject then was shifted to an individual plastic container holding moist paper towels and a reservoir (containing the incubation DS solution plus remaining zoospores) to allow hydration and reinfection for the remainder of the experiment. Controls were treated in exactly the same manner, but without exposure to Bd zoospores.

After incubation, frogs were held in a controlled temperature room (15 ± 0.5°C, 12:12 hour light:dark photoperiod) for 12 days without food and were fed once weekly thereafter. This temperature, typical of field conditions experienced by *L. raniformis* at this time of year, was suitable for growth and survival of Bd (Piotrowski et al., 2004). The experimental conditions allowed continual Bd reinfection of subjects, as presumably can occur in natural ponds. Respiratory measurements, followed by water balance measurements, commenced at seven days after inoculation and continued for four days. Sloughed skin of control and infected subjects was collected weekly from the containers in which they were housed and was tested for the presence of Bd. We were unable to precisely ascertain from what body regions all samples originated, though active sloughing of skin was predominantly observed over the ventral abdomen, hind limbs, and digits. We anticipated that infected subjects would suffer mortality shortly after infection, but as most frogs survived, we opportunistically took respiratory and water balance measurements during a second period, five months after inoculation.

Chytrid testing.—We tested skin samples for Bd by a PCR assay developed in our laboratory (Šadic and Waldman, unpubl.) supplemented by histological analyses of samples. We extracted total genomic DNA using xanthogenate (Tillet and Neilan, 2000). Skin was put into suspensions of 50 µl of TE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8.0; 100 µg/ml RNAase) in a 1.5 ml microfuge tube. Freshly made xanthogenate (750 µl: Fluka, Switzerland), 10 mM Tris-Cl,

pH 7.4; 20 mM EDTA, pH 8.0; 1% SDS; 800 mM ammonium acetate; and 10 μg/ml proteinase K were added to each tube and mixed by inversion. Samples were incubated at 70°C for 2 h. Supernatants were transferred to the new tubes, mixed with equal volumes of isopropanol, and incubated for 3 h at 25°C. The DNA was collected by centrifugation at 4,800 for 10 min; pellets were washed once using 70% ethanol, after which they were air-dried and suspended in ddH_20.

We amplified the extracted DNA using primers (for 5′–GACATGGTAGCCAGAGCAT–3′, rev 5′–GCCTTCGCAA-TAGTTTGTCC–3′) that target a 218 bp region of the Bd-18s small subunit (SSU) rRNA genomic sequence, based on the BLAST nucleotide database (National Center for Biotechnology and Information, Bethesda, MD). The 25 μl PCR reaction comprised 0.5 μM forward and reverse primers, 1× *Taq* PCR buffer, 2.0 mM $MgCl_2$, 200 μM dNTPs, and 1 unit of *Taq* DNA polymerase. Amplification (in an Eppendorf Mastercycler gradient thermocycler) consisted of an initial 5 min denaturation at 94°C, followed by 30 cycles with denaturation at 94°C for 45 s, annealing at 54°C for 30 s, and extension at 72°C for 45 s. After 30 cycles, the final extension was at 70°C for 7 min and analyzed by electrophoresis on 2% agarose gels. Positive and negative controls were run together with samples from each subject.

For histological confirmation, selected samples were dehydrated, cleared with terpineol, and embedded in paraffin. Sections were microtomed at 5 μm and stained with Mayer's double strength haemalum and eosin. Some sections were stained with Ayoub-Shklar stain to aid detection of Bd. Identification of Bd was based on the diagnostic characters described by Berger et al. (1999).

Metabolic measures.—Rates of resting oxygen (O_2) consumption, carbon dioxide (CO_2) production, and breathing were obtained at 15 ± 0.5°C, under conditions of normoxia (21% O_2, air). Gas exchange measurements were made on all subjects at one week and five months after inoculation. Each frog's mass was recorded prior to (W_a) and after (W_b) metabolic measurements ($\overline{X}$ mass, $((W_a+W_b)/2)$ ± SE: one week, control 29.29 ± 4.26 g, chytrid 29.71 ± 4.57 g: five months, control 31.53 ± 1.91 g, chytrid 32.87 ± 2.25 g). For measurements of metabolism, subjects were placed into a darkened 0.22 L acrylic plastic chamber through which air flowed. Frogs were acclimated to the respiration chambers for periods of 30–60 min before any metabolic recordings were made.

Rates of O_2 consumption ($\dot{V}O_2$) and CO_2 production ($\dot{V}CO_2$) were determined by flow-through respirometry (low-range respiration package, Qubit Systems, Kingston, Ontario). Dry air was supplied by positive flow (V_I) at a rate of 0.4 $L.min^{-1}$ and the excurrent gas was forced through a drying column and analyzed for fractional concentrations of O_2 (F_eO_2) and CO_2 (F_eCO_2) (galvanic cell O_2 sensor and S151 infrared CO_2 gas analyzer, Qubit Systems). Incurrent fractional concentrations of O_2 (F_iO_2) and CO_2 (F_iCO_2) were measured at the beginning ($_aF_iO_2$ and $_aF_iCO_2$) and end ($_bF_iO_2$ and $_bF_iCO_2$) of each frog's metabolic measurement. These measurements were used to adjust drift (equations 1 and 2) in excurrent fractional concentrations of O_2 ($_dF_eO_2$) and CO_2 ($_dF_eCO_2$) output gas recordings throughout the metabolic measurements (equations 3 and 4):

$$ {}_dF_iO_2 = \frac{-({}_aF_iO_2 - {}_bF_iO_2)}{T_t} $$

$$ {}_dF_iCO_2 = \frac{-({}_aF_iCO_2 - {}_bF_iCO_2)}{T_t} $$

where $_dF_iO_2$ and $_dF_iCO_2$ are drift in incurrent O_2 and CO_2, and T_t (min) is the total time excurrent O_2 and CO_2 output were recorded:

$$ {}_dF_eO_2 = F_eO_2 + T \times {}_dF_iO_2 $$

$$ {}_dF_eCO_2 = F_eCO_2 + T \times {}_dF_iCO_2 $$

where T is the time (min) at which the metabolic output was recorded.

The O_2 and CO_2 output were recorded with Logger Pro Version 2.1.1 (Vernier International, Sarasota, FL) over 20 to 30 min. In all cases, $\dot{V}O_2$ and $\dot{V}CO_2$ were determined with the frog quiet and resting within the chamber, as indicated by a steady value on the recorded trace (on average over 4 to 5 min). $\dot{V}O_2$ and $\dot{V}CO_2$ measurements are expressed as dry gas under standard temperature and pressure conditions and normalized for flow rate and body mass (equations 5 and 6):

$$ \dot{V}O_2 = \frac{V_I(F_iO_2 - F_eO_2)}{((W_a + W_b)/2)} $$

$$ \dot{V}CO_2 = \frac{V_I(F_eCO_2 - F_iCO_2)}{((W_a + W_b)/2)} $$

At the conclusion of the metabolic measurements, subjects were returned to their plastic containers and breath counts were made 15 min later. Breathing was observed as contractions of the flanks or nares over a 5 min period. Frogs that had not settled by 15 min after being placed into their container (erect posture, rapid movement of buccopharyngeal region, and movement within their container) were left for a further 10 min before breath counts were attempted a second time.

Water balance measurements.—Water balance also was measured at one week and five months after Bd inoculation. Both control and infected subjects were dehydrated using methods described by Cree (1984). The frogs were induced to urinate by applying gentle pressure on the abdomen and insertion of a cannula into the cloaca. Subjects then were blotted dry with a paper towel and their initial body mass (W_0) measured to the nearest mg. Following this, the frogs were placed onto a gauze platform (mesh diameter 1–2 mm) in a glass jar containing dehydrant (silica gel crystals) in the base. The lid of the jar was perforated to increase air circulation around the frog and consequently the rate of dehydration. Dehydration was conducted at 23 ± 0.5°C and 40–45% relative humidity. Rate of dehydration was determined gravimetrically (evaporative water loss, EWL) over 4 to 8 hours. Frogs' masses were taken every half hour until they had lost ca. 10% of their W_0 (range 6.8–12.6%). These frogs then were used in the rehydration experiment.

Rehydration of subjects followed methods described by Cree (1984, 1988). Bladder-emptied, dehydrated frogs were placed into shallow glass containers containing DS solution (depth sufficient to cover ventral abdominal region, 5 mm). The shallow depth of the containers ensured that all frogs were forced to maintain a posture similar to that observed during periods in which they conserve water (Pough et al., 1983).

Table 1. Metabolism of Control and Bd-infected *L. raniformis* ($\bar{X}$ ± SE) One Week and Five Months after Inoculation. $\dot{V}O_2$ and $\dot{V}CO_2$ ($\mu L g^{-1} \cdot min^{-1}$) and breath rate (contractions min^{-1}).

Experimental procedure	Time post-inoculation	Control	Infected	*t*	df	*P*
$\dot{V}O_2$	1 Week	2.70 ± 1.36	1.55 ± 1.30	0.85	10	0.425
	5 Months	4.66 ± 1.68	2.87 ± 1.71	0.93	9	0.375
$\dot{V}CO_2$	1 Week	2.26 ± 0.41	1.48 ± 0.40	1.71	10	0.119
	5 Months	1.85 ± 0.44	1.77 ± 0.36	0.14	9	0.896
Breath rate	1 Week	2.17 ± 1.82	2.37 ± 0.96	0.70	10	0.510
	5 Months	5.93 ± 1.64	5.36 ± 1.32	0.29	9	0.778

Time necessary to rehydrate varies before urination into the bathing medium occurs (Cree, 1985a, 1988). We thus used a relatively short period between mass measurements, as verified in other species with similar water balance responses, including the closely related *Litoria aurea* (Cree, 1985a, 1988), to reduce the chance of urination. Subjects' masses were obtained every 15 min for the first hour, then once at two hours and again at four hours. Upon removal from the rehydration containers, the frogs were blotted dry and their mass recorded to the nearest mg. Urine that had accumulated in the bladder during that time was removed and frogs' masses were retaken. This allowed the rate of rehydration to be divided into two components, water uptake (the difference between the two masses) and urine accumulation. This procedure assumes that subjects did not urinate into the bathing medium between measurements of mass (Cree, 1985a, 1988).

Statistical analysis.—We analyzed differences in measures of respiration and dehydration using Student's t-test (two-tailed), and differences in water balance measures (during rehydration) using univariate repeated-measures ANOVA. A linear polynomial was used to estimate the percentage of change across the repeated measures. Normality and equality of variances were assessed using Shapiro-Wilk's test of normality and Levene's homogeneity of equal variance tests. Non-normal data were first normalized by log transformation. For all measures during rehydration (rehydration, urine accumulation, and percentage of body mass stored in the bladder), rates and storage proportions did not significantly differ within groups, between 15 min intervals, during the first hour. Thus, in all these cases, mean individual rates and storage proportions were analyzed. All statistical tests were conducted with SPSS 11.0.

RESULTS

Clinical chytridiomycosis.—All infected frogs showed initial clinical signs two days after they were inoculated with Bd zoospores. Both control and infected subjects sloughed skin, but infected frogs sloughed visibly more copious amounts. Infected subjects sloughed skin predominantly from the ventral abdomen, hind limbs, and digits, but also to a much lesser extent from the flanks and dorsum. Frogs with chytridiomycosis were observed to be lethargic and to have a depressed posture with splayed legs and dilated pupils. Infected subjects also struggled less in response to handling and lacked coordination in jumping, compared to control frogs.

Sloughing of skin and changes in animal condition, as observed here, are consistent with other studies (Berger et al., 1998, 1999; Lips, 1999; Longcore et al., 1999; Pessier et al., 1999; Bosch et al., 2001; Nichols et al., 2001). In susceptible frogs, chytridiomycosis typically results in clinical signs just a few days before death (Bosch et al., 2001; Nichols et al., 2001; Raverty and Reynolds, 2001). Instead, we found that by six days after inoculation with Bd zoospores, infected subjects started to recover. Posture and coordination improved, skin sloughing reduced, and pupils were noticeably less dilated. However, struggling response to handling was noticeably less in the infected frogs up until day 10. By day 11, the infected frogs were indistinguishable in condition from the controls.

Following the completion of respiration and water balance measurements, all infected subjects appeared to fully recover, showing no further clinical signs of chytridiomycosis. Nevertheless, the infected frogs still showed evidence of infection with the pathogen, as determined by PCR tests and histological analyses of sloughed skin. In one case, however, apparent recovery from chytridiomycosis was followed by a sudden and severe onset of clinical signs and subsequent mortality two months after infection. Husbandry conditions were maintained unchanged during this period. Five months after infection, the remaining infected subjects showed no signs of ill health prior to or immediately after the water balance and respiratory measurements. No deterioration in condition, signs of disease, or other signs of poor health were observed in the control frogs at any point.

Metabolism.—No significant difference in metabolic or breathing rates were observed between control and Bd-infected subjects either one week or five months after infection (Table 1). A non-significant trend of decreased O_2 consumption and CO_2 production rates in infected frogs, relative to the controls, was observed at both one week and five months after infection (Table 1). This trend was most evident in CO_2 production for infected subjects one week after infection (Table 1).

Water balance.—All subjects adopted a water conserving posture (Pough et al., 1983) for most of the dehydration period (4–8 hours). In most cases, the frogs also pressed their bodies against the wall of the dehydration chambers, thereby reducing surface area exposed to evaporative water loss. Nevertheless, high rates of EWL were observed for both control and Bd-infected subjects, both one week (control 0.25 ± 0.02, chytrid 0.25 ± 0.02; $\bar{X}$ $mg.g^{-1}.min^{-1}$ ± SE) and five months (control 0.25 ± 0.02, chytrid 0.25 ± 0.03) after infection. Rates of EWL were not significantly different between control and infected frogs (one week $t = 0.01$, 10 df, $P = 0.991$; five months $t = 0.11$, 9 df, $P = 0.918$). All frogs (control and infected) also were observed to become more

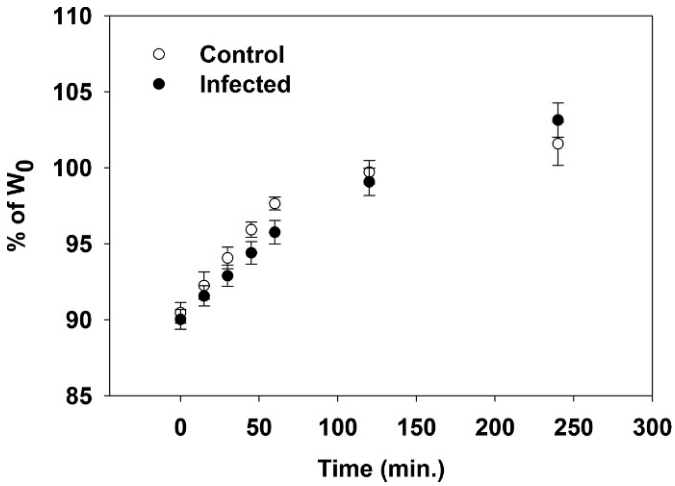

Fig. 1. Rehydration ($\bar{X}$, % of initial body mass [W_0] ± SE) for control and Bd-infected *L. raniformis* one week after inoculation with zoospores. Subjects initially were dehydrated to approx. 90% of their initial body mass and were allowed to rehydrate with their ventral surface submerged in water. % of W_0 denotes percent of initial body mass after dehydration and prior to urine removal.

lethargic over the dehydration period—slower, weaker, and less likely to struggle during handling.

Rehydration within each treatment was rapid in the first hour and declined in the subsequent three hours (Fig. 1, Table 2) at both one week and five months after inoculation. During this time, all subjects resumed struggling behavior to levels similar to those observed before the experiment was initiated. Two hours after the commencement of rehydration, most subjects had rehydrated to equivalent levels of W_0 (Fig. 1).

The rate of rehydration, one week after inoculation, differed between the control and infected frogs (Fig. 1, Tables 2, 3). During the first hour, the infected subjects demonstrated reduced rates of rehydration. However, rehydration rates reversed during the remainder of the period (Table 2). At five months after inoculation, the rehydration rates of control and infected frogs did not significantly differ (Table 3).

The rate of urine accumulation within treatments reveals a pattern similar to that observed for rehydration (Table 2). Within each treatment, urine accumulated more quickly during the first hour than during the subsequent three hours (Tables 2, 3). Urine accumulation did not differ between the treatments at either one week or five months post-infection (Table 3). There was, however, a non-significant indication that urine accumulation rate was reduced in infected subjects one week after inoculation (Table 2).

The percentage of body mass stored in the bladder was determined by comparing individual bladder volumes against bladder empty body mass (expressed as percent body mass per time interval of rehydration; 15, 60, or 120 min). This measure increased with time during rehydration for control and infected subjects at both one week and five months after their initial inoculation (Tables 2, 3). Body mass stored in the bladder did not differ between the control and infected frogs at either one week or five months after inoculation (Table 3). However, body mass stored in the bladder showed a trend of decline in infected subjects one week after inoculation (Table 2), similar to urine accumulation rate.

DISCUSSION

Batrachochytrium dendrobatidis, a pathogenic chytrid fungus implicated in global amphibian declines, is thought to affect frogs by impeding physiological functions of the skin or by releasing toxins into the host (Berger et al., 1998). We found that dehydration and gas exchange rates in *L. raniformis* were not influenced by infection with a virulent strain of Bd. Rates of rehydration, however, were reduced during the initial phase of infection, when subjects inoculated with Bd exhibited clinical signs of chytridiomycosis, but not later after these subjects recovered. Although they became aclinical, experimental subjects never cleared themselves of the infection during the study.

The clinical signs of chytridiomycosis in *L. raniformis* are consistent with those observed in other species (Bosch et al., 2001; Nichols et al., 2001; Raverty and Reynolds, 2001), but the signs occurred earlier and persisted only for a short period of time (ten days after exposure to Bd zoospores). Because most subjects recovered, we were able to opportunistically compare skin function of the same infected subjects while they exhibited clinical signs of chytridiomycosis (one week after inoculation) with those they showed later when they were aclinical (five months after inoculation). The recovery shown in our rehydration measures, coinciding with the recovery in outward clinical signs initially shown by infected subjects, clearly indicates that frogs ceased to be affected by their Bd infection. Similarly,

Table 2. Mean (± SE) Rates of Rehydration and Urine Accumulation ($mg.g^{-1}\cdot min^{-1}$), and Percentage of Body Mass Stored in the Bladder per Time Period or Interval, for Control and Bd-infected *L. raniformis*, One Week and Five Months after Inoculation with Zoospores.

		1 Week		5 Months	
	Time period or interval (min)	Control	Infected	Control	Infected
Rehydration	0–60	1.20 ± 0.08	0.98 ± 0.06	1.11 ± 0.17	1.01 ± 0.07
	60–120	0.34 ± 0.09	0.55 ± 0.08	0.40 ± 0.09	0.39 ± 0.18
	120–240	0.16 ± 0.11	0.34 ± 0.05	0.08 ± 0.03	0.16 ± 0.05
Urine accumulation	0–60	0.42 ± 0.06	0.30 ± 0.04	0.43 ± 0.03	0.45 ± 0.05
	60–120	0.22 ± 0.06	0.10 ± 0.02	0.24 ± 0.08	0.16 ± 0.03
	120–240	0.18 ± 0.05	0.10 ± 0.03	0.19 ± 0.05	0.17 ± 0.09
% of body mass stored in the bladder	15	0.60 ± 0.08	0.44 ± 0.05	0.59 ± 0.06	0.64 ± 0.07
	60	1.20 ± 0.36	0.54 ± 0.11	1.22 ± 0.36	0.89 ± 0.17
	120	1.90 ± 0.56	1.01 ± 0.28	1.20 ± 0.55	1.79 ± 0.38

Table 3. Changes in Rates of Rehydration and Urine Accumulation, and Percentage of Body Massed Stored in the Bladder during Rehydration, Over Time and Treatment (Control vs. Bd-infected *L. raniformis*) as Analyzed by Repeated-Measures ANOVA. Measurements were made at one week and five months after inoculation with Bd zoospores. Percent change of measurements, as indicated, were estimated across the repeated measures by a linear polynomial. Significant results are shown in bold.

	1 Week							5 Months						
		Time			Time*Treatment				Time			Time*Treatment		
	F	df	*P*	%	*F*	df	*P*	*F*	df	*P*	%	*F*	df	*P*
Rehydration	58.70	2, 18	**<0.001**	92	4.33	2, 18	**<0.05**	30.09	2, 16	**<0.001**	94	0.02	2, 16	0.984
Urine accumulation	15.13	2, 18	**<0.001**	82	0.12	2, 18	0.837	21.08	2, 16	**<0.001**	80	0.64	2, 16	0.539
% of body mass stored in the bladder	4.67	2, 18	**<0.05**	98	0.72	2, 18	0.447	9.94	2, 16	**<0.001**	97	0.23	2, 16	0.795

Voyles et al. (2007) did not detect significant differences in plasma electrolytes in *L. caerulea* that were infected, but aclinical, relative to uninfected controls.

Clinical signs associated with Bd infection, as reported to date, typically occur many days to weeks after inoculation (Berger et al., 1998, 2005; Nichols et al., 2001; Carey et al., 2006; Woodhams et al., 2007). As infected frogs showed clinical signs so rapidly, before the reproductive cycle of Bd could commence, we suspect that the initial invasion of the fungus into the epidermis may be responsible for the clinical signs observed. Rosenblum et al. (2008) identified increased gene expression for fungalysin metallopeptidases and serine proteases during the sporangial life stage of Bd. These peptides may have a functional role in Bd pathogenicity, causing clinical signs of chytridiomycosis, but further research is required to demonstrate this. The early clinical signs of infection observed here suggest a new mechanism by which Bd contributes to morbidity and mortality that should be considered when examining disease progression in other amphibians.

Recovery from chytridiomycosis might occur more often than is commonly believed (e.g., the salamander *Ambystoma tigrinum* and frogs *Rana yavapaiensis*, *Rana boylii*, and *Leiopelma archeyi*; Davidson et al., 2003; Bishop et al., 2009). The immunological responses underlying frogs' recovery are beyond the scope of this study; nonetheless, our results demonstrate that subjects can survive even high Bd inoculate levels. Innate immune responses typically offer rapid, non-specific protection through a variety of mechanisms including the production of anti-microbial peptides, and these confer immunity to Bd on some amphibians (Rollins-Smith et al., 2002). In our study, infected subjects recovered after first exhibiting clinical signs. Such a time lag in response is more consistent with an adaptive immune response in which immunological receptors specific to Bd antigens are generated (Richmond et al., 2009; Robert and Ohta, 2009). Indeed, recent studies suggest that adaptive immune responses enable individuals to survive diseases that have been implicated in amphibian population declines (Barribeau et al., 2008; Teacher et al., 2009). Further research on the immunological differences between species that are susceptible and resistant to Bd species is needed. Quite possibly, species that are resistant to Bd show physiological responses similar to those that we document here for *L. raniformis*.

Because we analyzed skin that frogs sloughed into their surroundings, we were unable to precisely determine the distribution, or intensity of infection, of Bd sporangia and zoosporangia over the body of infected subjects. Previous studies found that Bd preferentially infects regions of the skin that frogs typically have in contact with the substrate: the digits, hind limbs and ventral abdominal region (Berger et al., 1998; Pessier et al., 1999). In our study, the ventral abdomen, hind limbs, and digits were primarily exposed to Bd zoospores whenever subjects hydrated in their water reservoirs. Subjects sloughed skin profusely in these areas, as would be expected if these were the primary sites of Bd infection.

Metabolism.—Infection with Bd may influence gas exchange through the skin of amphibians (Berger et al., 1998; Carey, 2000). Our results, however, are not consistent with this hypothesis. The presence of Bd sporangia and zoosporangia in the skin of subjects did not significantly affect their metabolic rates at either one week or five months, even when individuals displayed clinical signs of chytridiomycosis. The primary sites for cutaneous gas exchange are the buccal region and epidermal surfaces of the frog exposed to a gaseous environment (Shoemaker et al., 1992); yet the primary sites for Bd infection tend to be those body surfaces in contact with the substrate, where conditions are more anoxic (Berger et al., 1998; Pessier et al., 1999).

Rates of carbon dioxide production were on average lower one week after infection. However, the general, non-significant, trend for a reduction in $\dot{V}CO_2$ of the infected frogs relative to the controls probably is not due to interference with cutaneous respiration, because no alteration in breathing pattern (pulmonary compensation) was observed between the two treatments (Whitford and Hutchison, 1966; Gottlieb and Jackson, 1976; Boutilier, 1988; Ultsch, 1996; Reid et al., 2000). Rather, the difference in CO_2 production between our control and infected subjects may represent an increase in fat metabolism (via an energetic investment in immunological response to Bd infection) during periods of chytridiomycosis (Ganong, 1997). Similar to our results, Voyles et al. (2007) observed a declining trend in blood CO_2 in infected *L. caerulea*.

Possibly, effects of Bd infection on rates of gas exchange might be masked by delayed acclimation of subjects to testing apparatus and methods. However, our measurements are consistent with those recorded by Cree (1984), conducted on the same species, which gives us confidence in the robustness of our procedures.

Water balance.—Infection with Bd also has been suggested to affect amphibians by interfering with physiological func-

tions of the skin associated with water balance (Berger et al., 1998; Carey, 2000). Similar to respiration, EWL in amphibians primarily occurs from the body surfaces exposed to a gaseous environment, and thus the presence of Bd sporangia and zoosporangia in the skin seems unlikely to impede dehydration by a physical blockage. Infection with Bd had no influence over the rate of dehydration of *L. raniformis* at either one week or five months after infection.

Given that ventral skin is the primary site both of Bd infection and rehydration in amphibians (Shoemaker et al., 1992; Berger et al., 1998; Pessier et al., 1999), rehydration and electrolyte uptake would be the most likely forms of skin function to be impeded by chytridiomycosis. Pronounced cutaneous water balance responses to dehydration and abilities to rehydrate rapidly in shallow water previously have been demonstrated in *L. raniformis* (Cree, 1985b). By contrast, in our study, subjects' ability to rehydrate was impeded one week after their initial inoculation with zoospores, when infected subjects displayed signs of chytridiomycosis. Voyles et al. (2009) found that rates of sodium and potassium uptake were reduced in *L. caerulea* with clinical chytridiomycosis relative to aclinical infected and uninfected controls. Together, our studies demonstrate that Bd blocks osmoregulatory function of the skin in frogs showing clinical signs of chytridiomycosis.

Infected *L. raniformis* rehydrated more slowly than the controls during the first hour. In the subsequent three hours, control frogs rehydrated more slowly than infected subjects, which is attributable to their having achieved hydration rapidly. The ability of infected subjects to accumulate urine and store fluids in the bladder also was reduced, although not significantly, during rehydration, which reflects the impeded rehydration rate. Yet, five months after being infected with Bd, when infected frogs were aclinical, no difference in the rehydration rate of experimental and control subjects was detected. Our results, like those of Voyles et al. (2009), demonstrate that osmotic skin function remains normal in infected frogs as long as they continue to be aclinical.

Plasma osmolality may have been reduced, and sodium transport across the cutaneous membrane disrupted, in infected subjects (Voyles et al., 2007) soon after inoculation when they demonstrated clinical signs. A reduction in plasma electrolytes may disrupt normal cutaneous osmoregulation. In turn, reduced plasma osmolality and electrolyte conditions, particularly hyponatremia (low sodium) and hypokalemia (low potassium), may interfere with cell membrane and neuromuscular function, causing clinical chytridiomycosis and mortality in non-resistant individuals and species (Voyles et al., 2009). The reduced rehydration rate that we observed in frogs showing signs of chytridiomycosis is consistent with this hypothesis. Whether the physiological effects of Bd on osmotic regulation that we and Voyles et al. (2009) observed reflect physical blockage, due to hyperkeratosis, or the action of toxic secretions (Berger et al., 1998; Pessier et al., 1999; Rosenblum et al., 2008), or both, requires further study.

Conclusions.—Our study contributes further insights into how amphibians are affected by infection with Bd, a pathogenic fungus associated with global amphibian declines. Infected *L. raniformis* initially exhibited signs of chytridiomycosis, but most survived despite being infected. Early clinical signs may have been due to Bd invasion of the skin. The progression of clinical signs followed by apparent recovery appeared similar to an adaptive immune response. We examined the effect of Bd on skin function when infected subjects exhibited clinical signs of chytridiomycosis and again later when aclinical. We found no detectable effects of Bd on respiratory metabolism or dehydration rates during the early phase of infection, nor any effects in aclinical subjects. However, during the early phase of infection, measurable inhibitory effects on rehydration rate and affiliated measures (urine accumulation and the percentage of body mass stored in the bladder) were associated with clinical signs of infection. When infected frogs were aclinical, no decrement in rehydration rate was observed. Reduced rates of rehydration in subjects with clinical signs of chytridiomycosis may be due to hyperkeratosis of the epidermis causing a physical blockage, reduced plasma electrolytes, or toxic secretory compounds.

Further research examining the relationship between infection with Bd and water balance in amphibians should focus on how the intensity of infection influences rehydration. Moreover, measurements of skin function physiology on subjects selectively exposed to compounds isolated from Bd, rather than infective Bd zoospores, would likely contribute to clarifying the mechanism by which Bd affects amphibians.

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