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PATHOGENICITY OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN TWO THREATENED CALIFORNIA AMPHIBIANS: *RANA DRAYTONII* AND *AMBYSTOMA CALIFORNIENSE*

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***ABSTRACT*.—Infection by the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, can be lethal but the effects of infection are species-specific. California, with the highest level of amphibian endemism in the United States, also harbors the greatest number of at-risk species, but few taxa occurring there have been tested for the effects of *B. dendrobatidis* infection. For this reason, I examined the consequences of infection in two threatened California species: *Rana draytonii* and *Ambystoma californiense*. Consistent with previous reports, both species were found to be susceptible to infection, but no animals died and all infected animals survived the 18-month study. Comparisons of skin slough rates for *A. californiense* revealed that infected salamanders sloughed at three times the rate of uninfected salamanders, a pattern that may have long-term energetic costs. My results indicate that long-term studies are needed to understand the population-level consequences of sublethal infection by *B. dendrobatidis*.**

Key Words.—*Ambystoma californiense*; amphibian chytridiomycosis; *Batrachochytrium dendrobatidis*; California Red-legged Frog; California Tiger Salamander; *Rana draytonii*

INTRODUCTION

Conservation International lists California, encompassing 70% of the California Floristic Province, as one of the 34 global biodiversity hotspots (Myers et al. 2000; Mittermeier et al. 2005; Brooks et al. 2006). Biodiversity hotspots are sites where "exceptional concentrations of endemic species are undergoing exceptional loss of habitat" (Myers et al. 2000). California amphibians exhibit the highest levels of endemism found in the United States (Stebbins 2003; Rissler et al. 2005), as 31 of the 59 species occur exclusively within the state. Twenty-five of the state's 37 recognized salamander species occur nowhere else in the world (Stebbins 2003; Rissler et al. 2006). The fact that California has the greatest number of endangered species (309) in the United States underscores its high biodiversity. Increased human population growth and the related changes in land use kindle species endangerment (Wilson 1992; Dobson et al. 1997; Wilcove et al. 1998).

Eight California amphibian species receive federal protection and three additional species are candidates for listing (U. S. Fish and Wildlife Service. 2007. Online resource: USFWS Threatened and Endangered Species System (TESS). Available at: http://ecos.fws.gov/tess_public/StateListing.do?state=CA&status=candidate.; U. S. Fish and Wildlife Service. 2008. Online resource: USFWS Threatened and Endangered Species System (TESS). Available at: http://ecos.fws.gov/tess_public/StateListing.do?state=CA&status=listed.). California lists nine amphibians as either threatened or endangered and fully protects three species (CNDDB [California Natural Diversity Database. Biogeographic Data Branch. 2007. State and federally listed endangered and threatened animals of California. Online resource: http://www.dfg.ca.gov/biogeodata/cnddb/pdfs/TEAnimals.pdf.]). Federal and state regulations currently protect two taxa: the Desert Slender Salamander (*Batrachoseps aridus*), and Santa Cruz Long-toed Salamander (*Ambystoma macrodactylum croceum*). California has identified 25 amphibians as Species of Special Concern (Jennings and Hayes 1994; CNDDB 2007). With the single exception of *Rana muscosa*, which occurs primarily in protected habitat (USFWS 1999), historic habitat loss and anthropogenic habitat alterations are the primary factors driving the decline of these species (Jennings and Hayes 1994; USFWS 2007; USFWS 2008).

Though habitat loss and alteration are indisputably key factors affecting amphibian populations in California and across the globe (Jennings and Hayes 1994; Alford and Richards 1999; Stuart et al. 2004), numerous other factors may negatively impact amphibians (Fisher and Shaffer 1996; Alford and Richards 1999; Davidson et al. 2002). Forty-eight percent of global amphibian declines are "enigmatic," because we know of no specific causative agent and many of these declines are occurring in areas with minimal anthropogenic influences (Drost and Fellers 1993; Daszak et al. 2004b; Stuart et al. 2004). Since the mid 1980s, when global declines of amphibians were

first recognized, investigators focused on testing hypotheses addressing these enigmatic declines. These hypotheses include increased UV-B radiation (Blaustein et al. 1994), pesticides (Davidson et al. 2001; Sparling et al. 2001; Davidson et al. 2002), climate change and introduced species (Hayes and Jennings 1986; Gamradt and Kats 1996; Kupferberg 1997). Few, however, seriously considered infectious disease as a potential factor.

That oversight changed when Laurance et al. (1996) postulated that an unidentified disease caused recent declines of Australian amphibian populations, leading to the identification of chytridiomycosis in 1998 (Berger et al. 1998). Amphibian chytridiomycosis is a disease caused by the zoosporic fungal pathogen *Batrachochytrium dendrobatidis* ("BD"; Berger et al. 1998; Longcore et al. 1999). Amphibian chytridiomycosis is a causal agent in the decline of native ranids, bufonids, and hylids in Panama (Lips 1998; Lips 1999), Australia (Berger et al. 1998) and the United States (Carey et al. 1999; Morrell 1999; Bradley et al. 2002). All amphibians seem to be susceptible to infection; however, effects of BD infection differ greatly among host species. Some species succumb to overt disease leading to mortality; whereas, others although testing positive for the pathogen, remain relatively asymptomatic (Davidson et al. 2003; Daszak et al. 2004a). It is therefore critical that each amphibian species be examined individually for their vulnerability to BD.

The bulk of research on amphibian chytridiomycosis currently focuses on areas outside the United States (US), although a few studies to determine the effects of the infection on selected North American amphibians exist (Davidson et al. 2003; Parris 2004; Rachowicz and Vredenburg 2004; Blaustein et al. 2005). BD infection attacks many California amphibians: *Ambystoma californiense*, *A. macrodactylum croceum*, *Bufo californicus*, *B. canorus*, *Pseudacris regilla*, *Rana aurora*, *R. boylii*, *R. cascadae*, *R. catesbeiana*, *R. draytonii*, *R. muscosa, Spea hammondii* and *Taricha torosa torosa* (Vredenburg and Summers 2001; Padgett-Flohr and Longcore 2005; Padgett-Flohr and Longcore 2007). However, research on species vulnerability to BD within the state has been largely restricted to *R. muscosa* (Fellers et al. 2001; Rachowicz and Vredenberg 2004; Briggs et al. 2005), a montane species that occurs in the higher elevations of the Sierra Nevadas (1,300-3,700 m). BD in *R. muscosa* [*sensu stricto*] is highly virulent and the pathogen may be responsible for recent population declines in extant and re-introduced *R. muscosa* populations in the Sierras (Rachowicz et al. 2006).

Currently, no regulatory policies exist to address the potential impacts of pathogens to listed amphibian species, as little epizootiological information is available to assist in management decisions and guide formulation of policies. Additional stressors such as pathogens may have dire consequences to protected species because reduced, fragmented, and isolated wildlife populations can experience extreme reductions or local extinctions during an epizootic event (Warner 1968; van Riper III et al. 1986; Anderson and May 1986; Berger et al. 1998). To develop comprehensive recovery actions for listed amphibian species, agencies need species-specific information on the risks posed by pathogens. Therefore, my objective was to begin to fill this gap by assessing the effects of BD infection for two federally listed California species: *Rana draytonii*, the largest native frog in the state, and *Ambystoma californiense*, an endemic salamander.

Focal species.—The USFWS listed *A. californiense* (California Tiger Salamander; "CTS") as a Threatened species on 4 August 2004 (USFWS 2004), 10 years after California listed it as a state Species of Special Concern in 1994 (CNDDB 2007). *Ambystoma californiense* is a lowland endemic historically restricted to the grasslands and lower foothill regions (< 600 m) of central and northern California. *Ambystoma californiense* requires aquatic breeding and upland aestivation habitats, both of which were plentiful in the Central Valley and foothills of California before the 1850s. Habitat loss and alterations of groundwater tables associated with increased human developments reduced the range of this species by ~55% (Jennings and Hayes 1994).

Rana draytonii (California Red-legged Frog; "CRLF"), federally listed as a Threatened species on 23 May 1996 (USFWS 1996), is also a California state Species of Special Concern. *Rana draytonii* is endemic to the state and Baja California (Linsdale 1932; Jennings and Hayes 1994; Shaffer et al. 2004). Historically, *R. draytonii* was found in most foothill and coastal drainages west of the Sierra-Cascade Mountains at elevations below 1,500 m (Jennings and Hayes 1994; USFWS 1996). The species ranged from Shasta County to Tulare County at the eastern edge of its distribution, across the Great Central Valley and from Mendocino County south to Baja California on the western edge. By early 1990s, *R. draytonii* had disappeared from an estimated 70% of its former range (Jennings 1988; Jennings and Hayes 1994).

Rana draytonii is highly aquatic, generally found in or near water. These frogs typically inhabit perennial ponds but occasionally inhabit low-velocity, low gradient (< 2%) streams. Adults normally become sexually mature in two (♂) to three (♀) years and can live > 10 years. The late maturation and comparatively moderate clutch size of this species (800-5,000 eggs/female) make it highly susceptible to extirpation. Overexploitation in the late 1800s, habitat loss and alteration, the introduction of Bullfrogs (*Rana catesbeiana*) and other aquatic predators, and historic

timber harvest probably contributed to *R. draytonii* population declines (Moyle 1973; Hayes and Jennings 1986; Jennings 1988; Jennings and Hayes 1994).

MATERIALS AND METHODS

In spring 2006, I collected 30 CTS larvae and 12 CRLF larvae from Joseph D. Grant County Park, Santa Clara, California, USA (elev. 650 m). I reared larvae individually in 9.5-L aquaria with 6 L of filtered, dechlorinated tap water, which was aerated to ensure adequate oxygenation. I provided a diet of Tubifex worms (*Tubifex tubifex*) for CTS larvae and boiled lettuce (Romaine hearts) for CRLF larvae. Post-metamorphosis, I re-located all amphibians to individual covered 9.5-L aquaria and subdivided each one into aquatic and terrestrial compartments. The terrestrial compartment had a coconut shell refuge. Throughout the experiment, I used new moistened vinyl gloves to handle post-metamorphic frogs and I sterilized all equipment prior to and between handling of animals using a 20% bleach solution. I fed all post-metamorphic animals appropriately sized crickets dusted with vitamin and calcium supplements on a bi-weekly basis and I cleaned the aquaria weekly. I housed the animals in an environmentally controlled chamber held at 19.5˚C, the mid-range of the optimal temperatures for BD growth (Longcore et al. 1999; Piotrowski et al. 2004) and maintained them on a photoperiod of 12D:12L.

BD exposure.—Following metamorphosis, and before experimental exposure to BD, I tested each animal for BD infection via PCR analysis. I obtained samples by rubbing a cotton swab $\geq$15 times over all ventral surfaces, including the bottoms of the feet. I stored assay swabs in 70% ethanol and shipped them to Pisces Molecular (2200 Central Avenue, Suite F, Boulder, CO 80301, USA). PCR analysis followed the procedure outlined in Annis et al. (2004) with the following modifications: we increased the 35-cycle to 45-cycle, increased the annealing temperature from 60˚F to 65˚F, and increased the [Mg^{2+}] from 1.5 mM to 3.5 mM. Modifications were made based on experimental trials that showed these adjustments in the PCR procedure resulted in higher sensitivity (J. Wood, K. Rogers, L. Livo and J. Epp, unpubl. data). Each PCR run included controls of positive DNA, negative DNA, and contamination detection. The PCR assay is highly specific for the BD ribosomal RNA Intervening Transcribed Sequence and the test is very sensitive as it will detect the presence of < 10 BD zoospores in a 2µl sample (Annis et al. 2004).

I found that all CTS were BD-free before experimental exposure, but six of the 12 CRLF metamorphs were positive for BD, indicating that CRLF larvae were infected prior to collection in the field. Therefore, I observed CRLF for progression of field-acquired infection; whereas, BD exposure of CTS occurred in the laboratory.

Pathogen culture followed the standardized protocol described in Longcore et al. (1999). I grew BD isolate (JEL270) provided by Dr. Longcore on 1% tryptone agar incubated at 23ºC. JEL270 was isolated from infected *R. catesbeiana* collected from Pt. Reyes, California. I flooded each of 12 agar plates with 2-3 mL of sterile distilled water and decanted after 30 min to collect zoospores. Mean zoospore concentration in the resulting solution (1.64 x 10^6/mL) was calculated by counting zoospores contained in 1.0 mL of solution using a hemocytometer.

Prior to exposing CTS to BD, I weighed each individual and randomly assigned them to one of three treatment groups with 10 animals per group: (1) control (0 zoospores/mL); (2) low dose (1 x 10^3 zoospores/mL), or (3) high dose (1 x 10^6 zoospores/mL). For exposure to BD zoospores, I individually housed each animal for 24 hrs in a coded 118-mL plastic container with a top perforated with 10-mm holes. Each container held the minimum volume of water that enabled contact with all ventral body parts, but allowed the animal to keep its upper torso and head above the surface. I released a quantity of inoculum sufficient to achieve target zoospore concentration directly into the water in the container. At the end of the 24-hr exposure period, I transferred all animals into individual long-term housing aquaria that were coded on their bottoms, and then randomly arranged them using a double-blind protocol.

Temperature.—Although the chamber was environmentally controlled and the temperature was consistently maintained at 19.5˚C, three and a half months into the study, intrusion by an unknown saboteur caused a reduction in temperature from 19.5˚C to 3˚C (hereafter the "cold snap") for as much as 24 hours. Thereafter, I observed the animals closely for physiological responses to the reduced temperature and for the potential response of the pathogen *in situ*. Following the emplacement of additional security measures, I was able to maintain the chamber at 19.5˚C post-cold snap for the remainder of the study.

BD-diagnosis and post-exposure monitoring.—I monitored amphibians for approximately 3 hrs every 2-3 days to assess for any behavioral changes commonly associated with BD infection including lethargy, inappetance, loss of righting reflex, and avoidance of the ponded area in the housing units. I also collected skin sloughed from each CTS and CRLF continuously throughout the study. To assess and follow infection

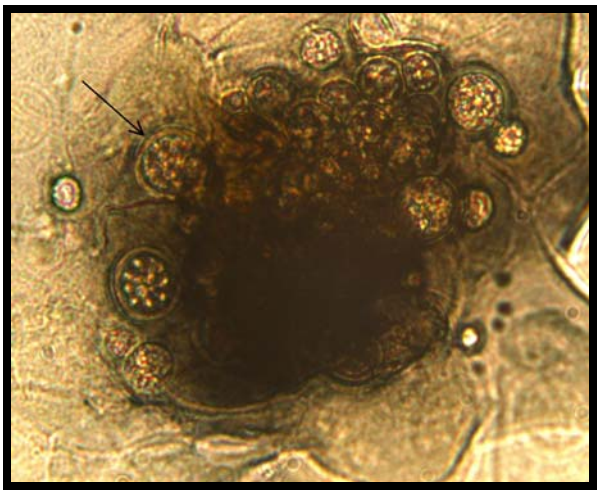


FIGURE 1. *Batrachochytrium dendrobatidis* thalli with zoospores in *Ambystoma californiense* skin slough. Wet-mount slide preparation viewed at 400×. BD thalli *in situ* were easily viewed via light microscopy even at lower magnifications (Fig. 2) and were characterized by areas that were highly melanized. BD thalli were present in focal points on the epidermis and were characterized by thick cell walls, and a granular appearance when filled with zoospores (arrow). Photographed by Gretchen Padgett-Flohr.

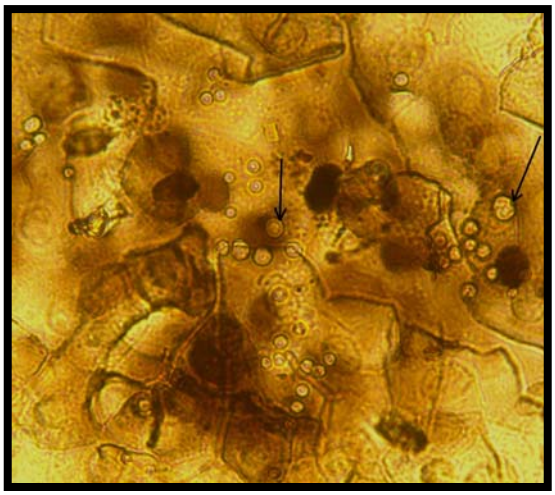


FIGURE 2. Wet-mount slide preparation viewed at 200×. *Batrachochytrium dendrobatidis* thalli are present as heavily walled spheres (arrows) showing numerous discharge papillae *in situ* in *Ambystoma californiense*. Focal points of hyperplasia and increased melanization were associated with BD infection points. Photographed by Gretchen Padgett-Flohr.

status, I used light microscopy (400×) to visually examine a wet-mount slide of each skin slough for BD thalli (Fig. 1; Longcore et al. 2007). My visual assessment also included characterizing the physical appearance of BD cells *in situ* for each amphibian species. I supplemented light microscopy diagnosis of control animals with PCR assay to reduce the likelihood of false negatives (recording an animal as uninfected that actually had a low level of infection). PCR analysis was conducted three times during the 18-mo course of the study.

Statistical Analysis.—I used SAS/STAT® software, Version 9.1.2 (2004) for Windows to conduct a two-sample *t*-test ($\alpha = 0.05$) to test the hypothesis that sloughing frequency differed between infected versus uninfected CTS and further tested for differences in sloughing frequencies of both groups in response to the cold snap. I also tested the hypothesis that weight gains in infected and uninfected CTS and CRLF did not differ using a two-sample *t*-test ($\alpha = 0.05$).

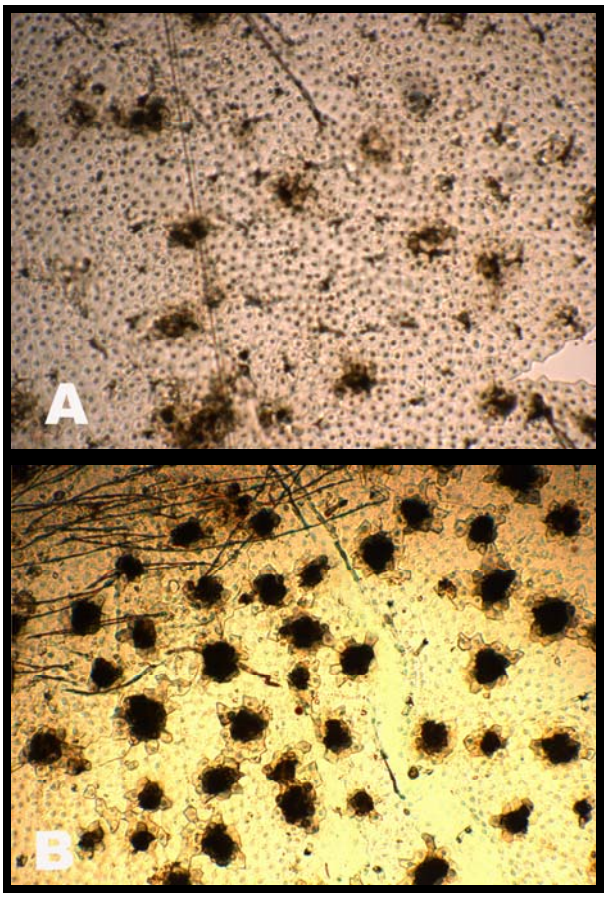
RESULTS

California Tiger Salamander.—All exposed CTS in both low-dose and high-dose groups became infected with BD and tested positive for the pathogen within 24 days post-exposure; however, none of the animals died or displayed clinical signs of disease within 18-mos post-exposure. Control animals remained negative for BD, and infected CTS did not lose their infection over time, consistently testing positive for the pathogen via light microscopy over the 18 mos (Fig. 1 and 2). Mean gain in mass per animal during the 18-mo post-exposure period of monitoring was 15.6 g (SD $\pm$ 2.9 g), and I found no difference in gain between the infected groups and the control group ($t = 1.25$, $df = 28$, $P = 0.22$).

Infected CTS had a higher rate of skin sloughing than uninfected animals ($t = 4.24$, $df = 28$, $P = 0.0002$). Infected CTS sloughed whole skins approximately every two to three days; whereas, uninfected CTS sloughed whole skins approximately once every one to two weeks.

Skin sloughing in the period immediately following the cold snap increased significantly for both infected and uninfected animals ($t = 2.98$, $df = 28$, $P = 0.0059$). Yet, even at the overall increased rate of sloughing, infected CTS still sloughed at a significantly higher rate than uninfected animals ($t = 3.47$, $df = 58$, $P = 0.0001$). Infected CTS sloughed whole skins daily following the cold snap, and uninfected CTS sloughed skins two to three times weekly.

I found that distinct melanized areas with concentrations of colonial BD thalli uniquely characterized the BD infection (Fig. 3). These distinct ("focal") points of infection occurred throughout the skin slough, and I also observed an increase in the number, density, and distribution of focal BD infection points in infected animals post-cold snap (Fig 3). Focal infection points doubled post-cold snap and the areas of focal infection increased in size, density, and distribution (Fig. 3). Four weeks post-cold snap, slough rates returned to pre-cold snap rates and focal points of infection also



IGURE 3. Wet-mount slide preparations (40×) of California Tiger Salamander (*Ambystoma californiense;* CTF) skin slough collected from CTF #6 pre-cold snap (A) and post-cold snap (B). Dark areas are focal *Batrachochytrium dendrobatidis* infections that increased in size and density in response to the drop in temperature. Photograph by Gretchen Padgett-Flohr.

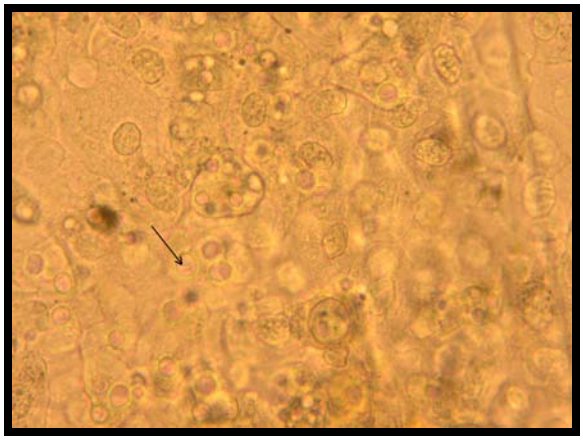


FIGURE 4. Wet-mount slide preparation (400X) of California Red-legged Frog (*Rana draytonii*; CRLF) skin slough showing *Batrachochytrium dendrobatidis* thalli with septae (arrow). BD infection in CRLF differed from that seen in California Tiger Salamander as the thalli were individually dispersed over the tissue as opposed to the colonial appearance of thalli in the CTS (see Fig. 1-3). Photograph by Gretchen Padgett-Flohr.

decreased.

California Red-legged Frog.—All CRLF survived and none of the six infected frogs displayed clinical signs of disease or lost their infections over time. Average (± SD) weight gained per animal over the 18-month period was 33.1 ± 16.5 g and I found no difference in mass gain between the infected group and the control group ($F = 0.43$, df = 10, $P = 0.5275$).

California Red-legged Frogs typically consumed most of the slough before it could be retrieved, making CRLF skin slough difficult to obtain. I had minor success at viewing CRLF skin slough and found that the appearance of BD thalli in CRLF differed from what I observed in CTS. The tissue contained randomly dispersed BD thalli, which lacked a colonial appearance and lacked areas of increased melanization (Fig. 4). CRLF skin slough deteriorated so quickly (< 24 hours) that it was often unusable for wet-mount slide examination. Because of this, I had to verify that animals remained infected or disease free by using PCR analysis on three occasions during the study. CRLF responded physiologically to the cold snap with increased melanization throughout their bodies. Normal coloration gradually returned within 3-4 weeks post-cold snap.

DISCUSSION

My experimental results indicate that CRLF and CTS are susceptible to infection with BD, but infection does not lead to mortality or an overt disease state under the conditions present within the laboratory. These results support the findings of other researchers who suggested that BD infection outcomes are species specific (Davidson et al. 2003; Blaustein et al. 2005; Woodhams and Alford 2005) and further emphasizes the necessity for species-specific testing. Both Davidson et al. (2003) and I studied ambystomatid salamanders, but observed different long-term responses to infection.

Davidson et al. (2003) found that *A. tigrinum* from southern Arizona could rid themselves of BD infection and speculated that rapid sloughing might contribute to that species' ability to shed infections. Although infected CTS in my study exhibited a similar high rate of sloughing, they did not lose their infections. Whether this pattern reflects differences related to phylogeny (two different ambystomatid taxa were involved) or the environmental conditions among the laboratory exposures is unclear. Histological findings indicating that infection status remained unchanged through the course of my study appear to exclude the possibility that animals shed infections only to become reinfected by zoospores in the microenvironment of their holding tanks.

I observed clinical signs of infection in CTS (increased sloughing rates and hyperplasia), yet I saw none of the behavioral changes commonly associated with pathogenically compromising BD infection. Species that are mortally affected by BD generally exhibit inappetance, lethargy, loss of righting reflex, avoidance of water, and abnormal postures (Daszak et al. 1999; Banks and McCracken 2002; Bradley et al. 2002). I found that infected CTS and CRLF continued to eat well, swim in their ponds, and move about their tanks in a consistent manner. Mortality in highly vulnerable species typically occurs fairly quickly (< 60 days) in infected post-metamorphic animals (Bosch et al. 2001; Bradley et al. 2002; Berger et al. 2005; Carey et al. 2006). Although slough consumption by CRLF prevented me from making useful assessments of infection loads in that species, most infected salamanders harbored moderate to heavy infections (Longcore et al. 2007). However, no eventual mortality occurred in either species over the 18-mo study period, despite the moderate-heavy infection loads and excessive skin sloughing in the CTS.

California Red-legged Frogs and CTS appear capable of carrying BD. These results imply that either BD was never lethal to these species in the past, or that evolution on the part of BD or the amphibians has reduced its impact. Host-parasite relationships may evolve towards a benign interaction with limited or no virulence (Anderson and May 1982); alternatively, an evolutionary arms race may stabilize virulence (Wakelin 1994; Mocarski 2002). To date, sequencing of BD genetics demonstrates no evidence of rapid BD evolution and suggests the pathogen is a clonal form with inconsequential genetic variation across the globe (Morehouse et al. 2003; Morgan et al. 2007). Hence, it seems more likely that strong selection for BD resistance in California amphibians is more likely than vice versa, and that the level of resistance observed in my study represents the outcome of selection acting since the first record of BD in California. Aquatic amphibians, producing relatively large numbers of larvae, lend themselves to rapid adjustments to environmental conditions and challenges via selection acting on populations. Conversely, the fast generation time of the pathogen may place it at a selective advantage relative to amphibians and thus, the relatively asymptomatic state I observed in both species in this study may result from pre-existing, species-specific properties, such as anti-microbial peptides or other as yet unidentified immune system factors.

In my study, the added stressor of extreme cold (3˚C) induced a physiological response in all CTS, as evidenced by the increased rate in sloughing and increased infection load of infected animals, but those salamanders still did not succumb to disease. Given the response of the pathogen in the CTS to the short period of extreme cold in my experiment, studies on BD must be conducted at much lower temperatures than those that are currently reported in the literature (12˚C-23˚C) (Davidson et al. 2003; Blaustein et al. 2005; Carey et al. 2006; Rachowicz et al. 2006). Most studies testing species for susceptibility to BD take place within the reported optimal range for growing BD in culture (Longcore et al. 1999), but do not reflect the range of temperatures experienced by amphibians in the wild. Kriger and Hero (2006) found that BD prevalence peaked on their study site in Australia when mean air temperatures fell below 19.4˚C and further, that prevalence was still high at 12.3˚C. Cold weather and overwintering is a source of stress that compromises the amphibian immune system (Carey 1993; Maniero and Carey 2004; Raffel et al. 2006) and die-off events have been reported in the western United States (Bradley et al. 2002) during the cooler months.

Though the species in this study were behaviorally asymptomatic when infected and infection did not lead to mortality, long-term impacts from BD infection cannot be ruled out. Significant diseases are those that reduce either survival or reproduction (Wobeser 2006). Disease agents often reduce fitness as they increase the energy costs, thus creating a trade-off in energy allocations because energy is usually a limited resource in the wild. Immune system activation requires energy (Buttgereit et al. 2000; McCallum and Trauth 2007). For example, lymphocyte stimulation causes an immediate 35% increase in cellular oxygen consumption (Buttgereit et al. 2000). Increased energy allocated to repair and maintenance is unavailable for growth and reproduction. When antigenically challenged, reproductive male *Acris crepitans* cease spermatogenesis, demonstrating that they shifted resources from reproduction to mounting an immune response (McCallum and Trauth 2007). Hence, BD infection in CTS may affect lifelong fitness because excessive skin sloughing could be energetically expensive leading to depleted fat reserves necessary for breeding. This could either limit reproductive effort or if sufficiently severe, prevent the animals from becoming reproductive. *Acris crepitans* from the above trade-off study ceased calling behavior post-inoculation with a novel antigen, and gular patch coloration faded (M. McCallum pers. comm.). This suggests a direct reduction in testosterone output. Immunochallenged amphibians may exhibit decreased fecundity (McCallum and Trauth 2007) such that non-lethal pathogens or toxins may have unobvious long-term population impacts. Thus, my results, in concert with those previous investigations, provide impetus for long-term studies on sublethal effects of pathogens on amphibian populations.

My study does not identify BD as a key threat to either listed species; however, the ranges of CRLF and CTS are very broad, encompassing much of California

and a variety of climate regimes which could influence the susceptibility of these amphibians to BD. A further concern is that other species typically co-occurring with CRLF and CTS (e.g. *Rana boylii*, *Spea hammondii*) may be highly susceptible. Furthermore, that CRLF and CTS may be refractory to BD does not eliminate threats from other amphibian pathogens in the environment (Drury et al. 1995; Mao et al. 1999; Davis et al. 2007; Nieto et al. 2007), most of which are still unknown. It is therefore critical that translocations or re-introductions of amphibians in California be carried out with extreme caution, if at all, because moving amphibians involves concomitant transport of their resident pathogens and parasites.

The unique fauna of the California biodiversity hotspot is already at risk from many factors, and disease is now a recognized part of the problem. This requires regulatory policies to prevent the spread of pathogens in wild populations as part of recovery plans for listed amphibian. To achieve this, I recommend: (1) All permits for work around wetlands should include strict decontamination requirements for all equipment (including vehicles); (2) establishment of an accessible state-wide disease tracking system via state and federal permits that require immediate reporting of amphibian disease occurrences by species and location; (3) establishment of a quarantine policy that restricts public and private access to areas associated with BD-related amphibian declines; (4) mandatory disease (not just BD) testing of amphibians at donor and recipient sites before relocation or re-introduction activities; (5) establishment of state-wide amphibian disease monitoring programs; and (6) mandatory voucher specimen collection as part of all permitted research for retrospective examination of amphibians for disease.

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