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Authors: Andre, Sara E., Parker, John, and Briggs, Cheryl J.

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Effect of Temperature on Host Response to *Batrachochytrium dendrobatidis* Infection in the Mountain Yellow-legged Frog (*Rana muscosa*)

Sara E. Andre,[1,4] John Parker,[2] and Cheryl J. Briggs[3] [1]School of Veterinary Medicine, University of California, Davis, California 95616, USA; [2]Office of Laboratory and Animal Care, University of California, Berkeley, California 94720, USA; [3]Department of Ecology, Evolution and Marine Biology, University of California, Santa Barbara, California 93106-9610, USA; [4]Corresponding author (email: sandre@ucdavis.edu)

ABSTRACT: The pathogenic chytrid fungus *Batrachochytrium dendrobatidis*, which causes the disease chytridiomycosis, has been implicated in declines of amphibian populations throughout the world, including declines and extinctions of local populations of mountain yellow-legged frogs, *Rana muscosa*, in the California Sierra Nevada. Previous studies have shown *B. dendrobatidis* achieves its maximum growth rate in culture in the temperature range of 17–25 C, and exposure to very high temperatures can clear frogs of *B. dendrobatidis* infection. Here we present the results of a laboratory experiment in which experimentally infected *R. muscosa* tadpoles were followed through metamorphosis at temperatures of 17 and 22 C. All infected animals developed clinical disease within a similar time frame. However, frogs housed at 22 C exhibited a significantly lower mortality than those housed at 17 C. Within 35 days after metamorphosis, 50% of the frogs housed at 22 C died, while 95% of the frogs housed at 17 C died. Clinical signs subsided in the surviving frogs at 22 C, despite persistent infection. Because both temperatures are within the optimal thermal range for growth of *B. dendrobatidis*, we propose that the difference in outcome indicates the effect of temperature on the host's resistance to chytridiomycosis, rather than an effect on the fungus alone.

Key words: Amphibian, amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, chytridiomycosis, mortality, *Rana muscosa*, temperature.

Chytridiomycosis, the disease caused by the chytrid fungus *Batrachochytrium dendrobatidis*, has been implicated as the cause of sudden declines in several frog populations. Infected frogs have been found in Australia, Europe, Africa, New Zealand, Central America, South America, and North America (Berger, 1998; Muths et al., 2003; Lips et al., 2006). The mountain yellow-legged frog, *Rana muscosa*, which occurs only in high elevation areas of the California Sierra Nevada Mountains in western North America, has experienced recent population declines and local extinctions due to mortality caused by *B. dendrobatidis* (Rachowicz et al., 2006).

Batrachochytrium dendrobatidis infection is restricted to the outermost portion of the epidermis, and disease results when the fungal burden surpasses the tolerance of the host. In general, larval frogs are not susceptible to clinical disease due to their lack of dermal keratin. However, tadpoles of *Rana* spp. are equipped with minimal amounts of keratin in their mouthparts, a state which provides a focal site for *B. dendrobatidis* infection with no effect on activity or mortality. One exception is boreal toad (*Bufo boreus*) tadpoles, which have shown increased mortality after *Batrachochytrium* exposure (Blaustein et al., 2005). The subclinical infection of tadpoles provides a means for autoinfection at metamorphosis (Marantelli et al., 2004). In many species, *B. dendrobatidis* infection results in death of subadults and adults; however, the outcome of infection varies between populations and individuals. *Batrachochytrium dendrobatidis* has been demonstrated to be a highly virulent primary pathogen in some captive and wild populations, yet causes only subclinical to transient infection in other populations (Berger, 1998; Muths et al., 2003; Lips et al., 2006; Rachowicz et al., 2006). Given *B. dendrobatidis'* low genetic variability, geographic structuring, and host specificity (Morehouse et al., 2003), this variation in pathogenicity may reflect the

influence of both environmental and host factors.

The effect of temperature on *B. dendrobatidis* has recently received a great deal of attention (Pounds et al., 2006). In pure culture, *B. dendrobatidis* has the highest growth rate at temperatures between 17 to 25 C, but can grow and reproduce at temperatures spanning 4 to 25 C, allowing it to survive in overwintering hosts (Johnson and Speare, 2003; Piotrowski et al., 2004). In some species, infection prevalence has been found to be highest during cool temperature periods (Berger et al., 2004; Woodhams and Alford, 2005; Kriger and Hero, 2007). Carey et al. (2006) found that survival time after experimental *B. dendrobatidis* infection in boreal toads did not differ at temperatures between 12 C and 23 C. In another experiment with great barred frogs (*Mixophyes fasciolatus*), Berger et al. (2004) found all frogs exposed at 17 C and 23 C died following exposure to *B. dendrobatidis*, but half of the individuals exposed at 27 C survived. In addition, elevated temperatures of 27 C and 37 C have been shown to cure chytridiomycosis in certain species of frogs (Woodhams et al., 2003; Berger et al., 2004).

In this study, we investigate the effect of *B. dendrobatidis* on mountain yellow-legged frogs (*R. muscosa*) at two temperatures that are within the optimal thermal range for *B. dendrobatidis* growth in culture, as well as within the thermal range typically experienced by *R. muscosa* (Bradford, 1984).

Sixty *R. muscosa* tadpoles, approximately 1 yr of age and of undetermined sex (reared from egg masses collected in 2002 from Kings Canyon National Park, California, USA), were housed in the ectotherm facility at University of California, Berkeley (UCB), Berkeley, California, USA. The UCB campus is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and these animals were covered under a protocol approved by the UCB Institutional Animal Care and Use Committee.

Experimental infection was achieved, several months prior to this experiment, using the LJR089 strain of *B. dendrobatidis* cultured in 2002 from an infected *R. muscosa* from Sequoia National Park, California. Inoculation occurred following the protocol described by Rachowicz and Briggs (2007), in which 20-l tadpole tanks filled with 12 l of water were inoculated with approximately 1×10^7 zoospores (determined by hemocytometer counts). Over a period of 3 wk, each tank was inoculated a total of 15 times. All infected tadpoles were housed at 17 C during the inoculation period and during the following 2 months. Upon reaching Gosner stage 41, experimentally infected tadpoles and uninfected controls were divided into two temperature status treatments consisting of 20 experimentally infected individuals and 10 uninfected at each temperature (17 or 22 C).

Animals were individually housed in 5-l fiberglass aquaria and maintained in static dechloraminated water that was changed biweekly. Development was monitored and recorded four times a week using stages based on Gosner's anuran embryo staging tables (Gosner, 1960). Aquaria water levels were uniformly set according to the Gosner stage of the individual animal, and twice a week animals were moved to freshly prepared tanks. Prior to Gosner stage 42, tadpoles were kept in fully aquatic tanks. From Gosner stage 42 through 43, metamorphs were kept in tanks with approximately 2.5 cm of water and a folded paper towel. Upon entering Gosner stage 44, metamorphs were placed in tanks with only 1.2 cm of water. Tadpoles were fed rabbit pellets, and postmetamorphic animals were provided live crickets. Cricket consumption was monitored and recorded throughout the experiment. Infection status was confirmed via skin-scraping cytology prior to the start of the experiment and at various time points thereafter. Likewise, controls

were confirmed to lack detectable levels of pathogen prior to, and throughout, the experiment. The skin scraping technique (Drake et. al., 2007) involved using a wooden applicator stick or the blunt side of a scalpel to scrape epithelial cells from either the jaw sheaths or tooth rows of tadpoles, or from the ventral surface of the feet, legs, and abdomen of postmetamorphic frogs. The cells were spread onto a drop of water on a microscope slide and examined for the presence of *B. dendrobatidis* sporangia using a compound microscope at 100–400× magnification. Animals were euthanized upon reaching the experimental endpoint, defined as marked obtundation, non-ambulatory, lacking of righting response, or death.

The Kaplan-Meier method of survival function estimation was used to compare rates of death between groups, and a log-rank test was used to compare survival functions for equality. The Cox proportional-hazards regression model was used to estimate hazard rate ratios (HRR) and 95% confidence intervals (95% CI) comparing group effects. Any P values <0.05 were considered statistically significant.

Sixty frogs were initially included in the study. Two animals (one infected animal at 22 C and one uninfected control at 17 C) were excluded from the study due to unrelated mortality as tadpoles. One of the infected frogs in the 22 C room was euthanized 9 days after entering Gosner stage 45 due to an extreme hind limb edema believed to be unrelated to the fungal infection.

In all, the median survival time in infected frogs was 25 days, and the estimated survival probability of infected frogs 35 days after entering Gosner stage 45 was 26%. This survival was significantly less ($P<0.001$) than in uninfected frogs, which experienced no postmetamorphic mortality at either temperature. Among the infected frogs ($n=39$), there was a significant difference in mortality between the two temperature groups ($P=0.0036$; Fig. 1). At 17 C, only 5% of the infected frogs survived, the last death occurring on day 35; at 22 C, 50% of the infected frogs survived, the last death occurring on day 32. The rate of death among infected frogs in the higher temperature group was approximately one fifth that of the rate in the lower temperature groups (HRR= 0.20, 95% CI=0.086–0.48).

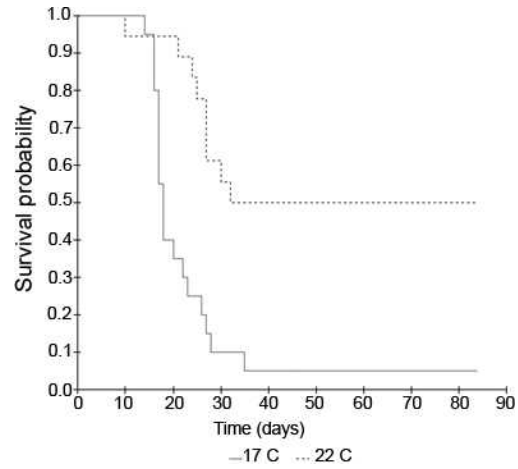

FIGURE 1. Survival probabilities of *Rana muscosa* infected with *Batrachochytrium dendrobatidis* at 17 and 22 C as a function of the number of days postmetamorphosis (onset of Gosner stage 45). Some frogs were censored due to death from other causes, or for remaining alive at the end of the experiment. No uninfected frogs died from chytrid infection (data not shown).

Excessive shedding was observed in all infected animals, at both temperatures, as compared to controls. Skin sloughed from infected animals appeared to be in much smaller pieces than the sloughed skin observed from healthy animals undergoing ecdysis. Other clinical signs of disease, including inappetence, lethargy, and reduced responsiveness, were also noted in all infected animals, but appeared, subjectively, more pronounced at the cooler temperature. Animals that survived beyond 35 days postmetamorphosis at 22 C regained their appetite and responsiveness and subsequently recovered from the disease, despite the persistence of infection. The single survivor at 17 C remained anorectic and unresponsive for the duration of the study.

The positive effects of higher ambient temperature on survival or recovery of frogs infected with *B. dendrobatidis* has previously been established (Woodhams et al., 2003; Berger et al., 2004). However, in those reports, these effects were demonstrated only when temperatures exceeded those considered optimal to sustain *B. dendrobatidis* growth. Consequently, the observed attenuation and resolution of disease in those reports may have been due to the elevated temperature's direct effect on the pathogen's survivability (Berger et al., 2004).

Providing that optimal growth of *B. dendrobatidis* in culture correlates with its capacity to produce disease, and all other factors are considered equal, the temperatures employed in this study should have had no effect on the pathogenesis. Thus, the difference in pathogenicity demonstrated in this study is attributable to an increased capability of the host to contend with infection. These results suggest the thermal optimum for *B. dendrobatidis* growth in culture may not necessarily determine its impact within particular amphibian populations.

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