Water Temperature and Chytridiomycosis in *Rana muscosa* Larvae

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Abstract The fungal pathogen *Batrachochytrium dendrobatidis*, also known as chytrid fungus, has been implicated in amphibian declines worldwide. Chytridiomycosis is a water-borne disease that interferes with osmoregulation in amphibian skin, and has the ability to rapidly wipe out populations. Laboratory studies have shown that exposing adult frogs to elevated temperatures can reduce or even eliminate their infection. My field study tests whether elevated temperatures can alter infection rates in frog larvae, which can be a vital reservoir for the pathogen. Seventy-two tadpoles were removed from alpine lakes and placed in small pools with a density of twelve tadpoles per pool. Half of the pools were left exposed to sunlight to allow the water to receive solar heating during the day, and the other half shaded to attempt to simulate temperature fluctuations similar to a lake. An eight-degree difference in peak temperatures was achieved between the two groups and a 3.5-degree difference in the averages, however no significant change in infection level was found after two weeks of treatment. These results suggest that higher or more sustained temperature increases are required to reduce infection in tadpoles.

Introduction

Many studies have demonstrated that populations of amphibians are experiencing decline on a global scale (Blaustein et al. 1993, Hecnar 1996, Alford and Richards 1999, Stuart et al. 2004). The loss of amphibians from communities is likely to create both direct and indirect effects on populations of other species, such as reduced community stability and species diversity. For example, studies have shown that the dramatic decline in amphibian populations significantly alters the trophic structure of food webs in alpine lakes (Finlay and Vredenburg manuscript 2005). Factors implicated in amphibian decline include habitat destruction (Gray and Smith 2005), introduced species (Vredenburg 2004), pesticide contamination (Davidson 2004, Fellers et al. 2004), acid rain (Watkins-Colwell and Watkins-Colwell 1998), increased UV radiation (Diamond et al. 2002), and disease (Greer et al. 2005, Hero and Morrison 2004). I focus on the fungal pathogen *Batrachochytrium dendrobatidis*, hereafter referred to as the 'chytrid fungus', which causes the emerging infectious disease chytridiomycosis in frogs. This disease has been implicated in the disappearance or decline of many frog populations in Europe, Australia, Central America, North America, and parts of Asia (Berger et al 1998). The chytrid fungus infects the keratinized tissue in the skin of amphibians, leading to secondary skin infections, internal desiccation, and interference with osmoregulation which normally occurs through the skin (Berger et al. 1998, Nichols et al. 2001). However, the exact mechanisms that cause mortality have not yet been determined. Amphibian larvae which carry the infection are not mortally affected until they metamorphose, although one study has shown that it may create a competitive disadvantage (Parris and Cornelius 2004).

Some amphibian populations previously decimated by chytrid infection have rebounded in recent years, displaying new resistance to the fungus (McDonald et al. 2005, Rollins-Smith et al. 2005). Their persistence could be the result of natural selection which allows for populations to develop immunological defenses to the fungus or a change in behavior to inhibit fungal growth. Not all populations recover, however, and the initial outbreak resulted in the extinction of some species (Pounds et al. 2005). These findings highlight the importance of developing management strategies to help populations survive the initial epidemic of chytrid infection, thereby providing the opportunity for more gradual adaptation and recovery (McDonald et al. 2005). Laboratory studies have been conducted to test methods for clearing frogs of chytrid infection. Some of these treatment methods could have management implications. A study by

Parker in 2002 shows that placing infected adult frogs in tanks containing the fungicide malachite green for twenty-four hours will rid the frogs of their infection. Unfortunately, this fungicide is highly carcinogenic and should not be used on a large scale or in delicate ecosystems. Another study conducted by Woodhams (2003) demonstrates that when infected frogs of the species *Litoris chloris* are subjected to periodic elevations in water temperature (up to 37°C), they have a greater chance of survival than those kept at room temperature. Parker also specifically tested the effects of temperature treatment of *Rana muscosa* adults (unpublished data). Chytrid is known to grow equally well at 18 or 23 degrees in isolated cultures, but the frog's infection level declined in this experiment at the higher end of this temperature spectrum. This result implies that the frogs may exhibit an immunological or metabolic response to the fungus as a result of these higher temperatures. However, no studies have tested whether elevated temperatures can effectively cure amphibians in the field. For my study, I have chosen to conduct a field experiment with the mountain yellow-legged frog, *Rana muscosa*, an aquatic species that lives at high elevation in the Sierra Nevada.

This experiment addresses the questions: "How will elevated water temperature affect chytrid load in *R. muscosa* larvae?" and "Can temperature be used to treat infected populations in the field as an aid to population recovery?" Using larvae as the developmental stage for intervention is relevant because of how chytrid affects *Rana muscosa* populations. In adult frogs, the infection is most prevalent on the feet and ventral regions of the animal near the inner thighs, which are important areas for respiration, water uptake and gas exchange. In the larvae, however, chytrid infection is found only in the mouthparts and its effects do not appear to be fatal at this stage. Individuals of this species usually remain in larval form for three to four years before they metamorphose, and thus the larvae can persist and carry the infection for several years after all the adults in the population have died from infection. If the larvae that remain can be cleared of the infection, there is a greater chance the site will be recolonized by individuals from the metapopulation.

Methods

My study site is Sixty Lake Basin (36.8186° N, 118.4251° W; 3,000- to 3,500-m elevation) in Kings Canyon National Park, CA (Figure 1). Within the Basin, there is a cluster of lakes in which chytrid exterminated all of the adult frog population during Winter 2004/5. The sizable

population of tadpoles that remain carry the infection in their mouthparts. The infection can be immediately detected by observing pigment gaps in their beaks (Rachowicz and Vredenburg 2004).

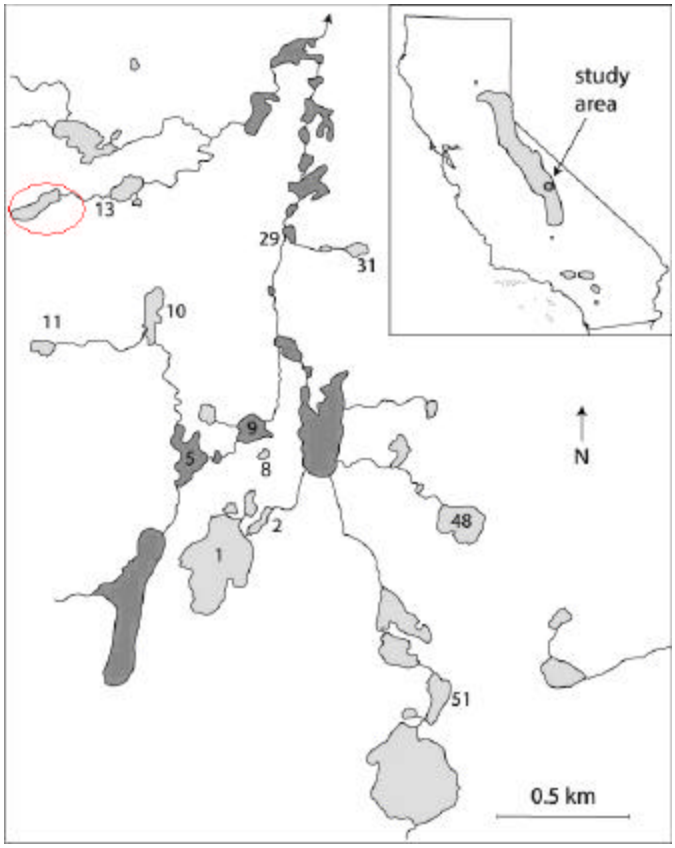

Figure 1: Map of Sixty Lake Basin. Several lakes have been numbered. The lake circled in red is my study site.
Source: Vredenburg, V.T. 2006, unpublished manuscript

I tested my hypothesis by creating two different treatments: one with water heated by the sun and one maintained at a cooler temperature. To contain the treatments and to exclude confounding variables such as lake currents, predators, and variations in tadpole density, I placed the tadpoles in inflatable rubber wading pools. These pools were approximately one and a half meters in diameter and placed at an average of fifteen meters away from the source lake. Sun-heated pools were placed in full sunlight on white granite surfaces. The control pools were shaded by tarps and tree branches and placed on leaf litter. Each temperature treatment (sun-heated/shaded control) had three replicate pools.

To set up the experiment, my partner and I inflated the pools and placed approximately 24 gallons of lake water in each, resulting in a water depth of about five centimeters. We added

about five liters of lake sediments to each pool after first sifting it using small hand nets to check for predatory insects that feed on tadpoles. The temperature of each pool was monitored throughout the experiment at one-minute intervals using ibuttons (Maxim Integrated Products, Sunnyvale, CA).

We caught seventy-two tadpoles from the study lake using dip nets. We swabbed their mouthparts using cotton swabs, which is a standard procedure for measuring chytrid infection load (Olsen et al. 2004). The swabs were allowed to dry and placed in labeled 1 µL screw-top test tubes. As we added the tadpoles to the pools, we also noted the Gosner stage (Gosner 1960), a standard procedure for categorizing the level of metamorphosis. We attempted to evenly distribute the stages throughout the pools by adding the tadpoles in the latest stage of development first to all the pools, then adding the next younger stage, and so on. Tadpole density was twelve tadpoles/pool.

The experiment lasted two weeks. We swabbed all tadpoles at the end of the experiment and released them into the source lake. As an additional control, twenty-five tadpoles from three locations in the source lake were also swabbed before and after the two-week period. We did this to verify that the changes observed in the pools were due to temperature alone.

The swabs were processed and analyzed using quantitative RealTime PCR on an Opticon 2 (Biocompare, Inc., South San Francisco, CA). The DNA was chemically extracted from the swabs and the resulting supernatant was diluted, plated, and run on the Opticon. The resulting numbers of genomic equivalents, or 'ge scores' detected by the machine were multiplied by eighty. This gives the number of chytrid zoospores that were on the animal, and this number is the equivalent of an individual's infection level. (Note: The DNA extraction procedure used to obtain twenty-six, or about two-thirds, of the ge scores for the shaded pools was inconsistent with that used on the other samples. A statistical comparison between the scores in this group that use both normal and inconsistent procedures show no difference, however, and therefore they are included in the data analysis.)

I analyzed the ge scores using a two-tailed Mann-Whitney nonparametric test. The infection levels from the shaded pools two weeks after the treatment period were compared to the initial levels from the tadpoles before the treatment. This ensures that the shaded pool served as a control. The shaded pool tadpoles' infection level was then compared to that from the sun-heated pools. I then lumped the infection data from both categories and compared this group to

the infection data from the twenty-five tadpoles sampled from the source lake after the two week period. I also compared the ibutton® temperature data from each group, looking at variation and mean temperatures.

Results

The infection levels of tadpoles in the sun-heated pools, shaded pools, and lake environment were found to be effectively equal. The Mann-Whitney test showed no significant (a=0.05) difference between the infection levels for the tadpoles in the sun-heated and the shaded pools (Figure 2), as well as no difference between the tadpoles included in the experiment and the tadpoles sampled in the lake after two weeks of treatment (Figures3, 4, 5).

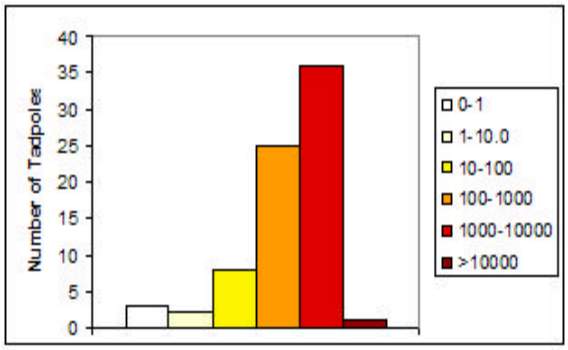

Figure 2: Infection distribution of lake tadpoles before treatment

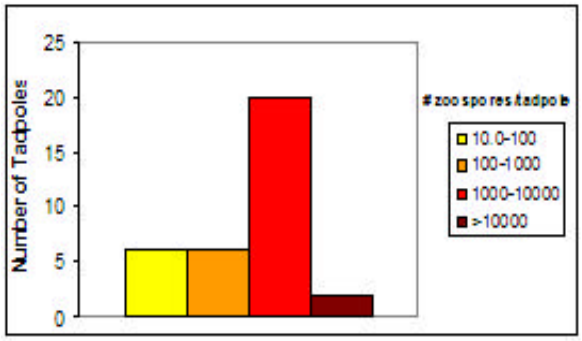

Figure 3: Infection distribution for tadpoles in sun-heated pools .

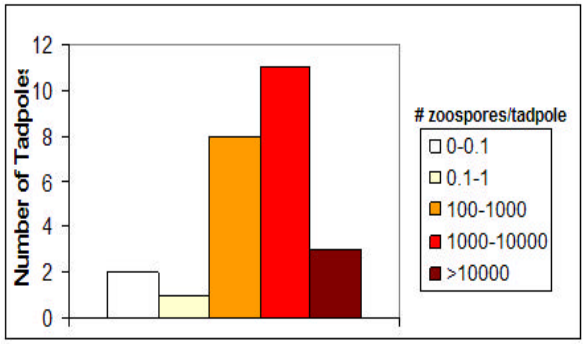

Figure 4: Infection distribution for tadpoles in shaded pools.

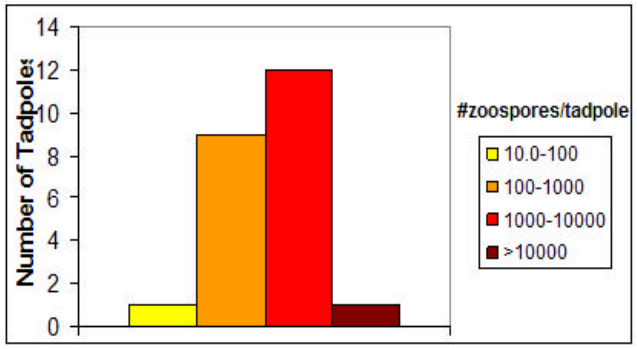

Figure 5: Infection distribution for tadpoles from the source lake.

The ibutton® data showed that the sun-heated pools reached a maximum temperature of 24°C while the shaded pools reached maximums of 13°C, which was lower the maximum temperatures measured in the lake (Figure 6). The water reached these peak temperatures for less than an hour before they would begin to drop again, fluctuating in accordance with the sun and daytime temperatures. A 3.5 degree difference in mean temperature was observed between the sun-heated and the shaded pools, and the mean temperature of the shaded pools was two degrees lower than that of the lake (Figure 7). The temperature in the shaded pools was consistently lower than that of the lake (the lake water was shallow, >0.5 meters, where we placed the ibuttons, which coincided with spots where we found large numbers of tadpoles).

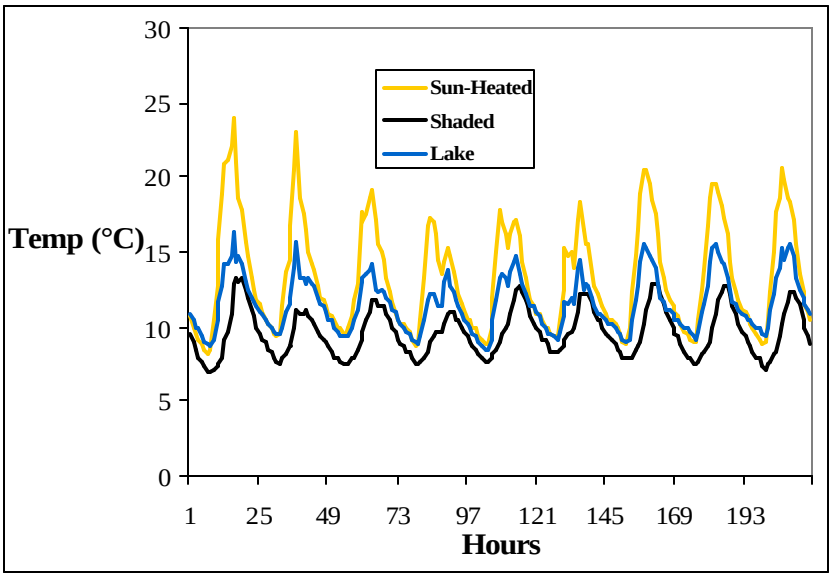

Figure 6: Daily fluctuations in temperature. Each peak represents the afternoon high, and each trough reflects the nighttime low.

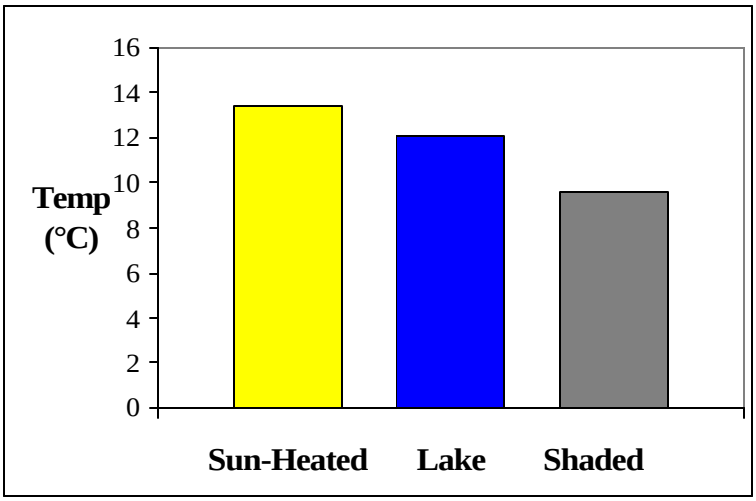

Figure 7: Comparison of mean temperatures between the 3 groups.

Discussion

Parker's experiment demonstrated that heat treatments needed to reach 23 degrees to affect chytrid infection (Parker unpublished data). My sun-heated pools reached this temperature, but it had no effect on the infection level. One possible explanation is that the use of larval subjects may have influenced the effectiveness of the treatment. Other laboratory experiments used adult frogs, which probably had a different immune or metabolic response to the temperature change. Also, tadpoles can sustain higher infection rates for longer periods than adult frogs, which would either die or be severely hampered by the infection levels detected in some individuals in this experiment (upwards of 10,000 zoospores). This ability may allow the larvae to hold the infection more effectively than adults thus making treatments less effective. The higher quantity of the pathogen reduces the chance that the treatment will have a significant impact on infection.

Two opposing forces could have also led to the lack of significant difference in infection levels. Chytrid infection tends to increase and spread as tadpoles reach later stages of metamorphosis. It is widely accepted that increased temperature accelerates tadpole development (Blaustein et al. 1999). If the increased temperatures in the experiment were decreasing fungal load, this reduction could have been counteracted by the higher infection level that accrues during metamorphosis.

Crowding did not appear to be a factor in larval infection levels. Rachowicz and Vredenburg (2004) demonstrate that chytrid infection can be transmitted from an infected to an uninfected individual. Tadpoles could gain a higher infection level from being in an enclosed space where water-borne zoospores from the other hosts can more easily infect or reinfect them. However I found no evidence of this in my experiment: tadpole infection did not increase in the enclosed, concentrated pool environment versus the diluted lake environment. An explanation could be that most of the tadpoles possessed such a high infection load that dilution would not have sloughed off the fungus faster than it could grow and renew the infection.

In the controlled laboratory environment, a constant temperature of 23 degrees could be maintained until the frogs showed a response in infection, but this was not possible in the field. The maximum temperature of 24 degrees reached in the sun-heated pool was only sustained for one or two hours. While this temperature has been shown to affect infection level, the fluctuation in temperature probably produced a different response in the frogs than was observed in the laboratory. There is little data on the physiological response of adult or larval frogs to the

chytrid infection, however, especially in relation to temperature. The temperature fluctuation in this experiment could have either prevented the frogs from experiencing a curative immune response to combat the fungus, or allowed the fungus to recover from any setbacks in growth. Since the mechanism of how elevated temperature induced the frogs to reduce their infection is unknown, it is difficult to speculate on whether the same response would appear in the larvae.

A possible way to offset the temperature fluctuation would be to increase the peak temperature achieved in the pools. One could create these conditions in the field by placing sun-reflectors facing the water (as in solar ovens) or using black rubber holding pools. This setup would create temperatures higher than 23 degrees for a larger fraction of time. The resulting effects on the frog larvae and the pathogen might create a difference in infection levels.

A greater temperature elevation and duration of treatment would be required to show a change in infection level in *R. muscosa* tadpoles. New experimental designs could be constructed and applied as a treatment. Information on infected frogs' physical response to temperature should be an avenue of further study. If we wish to avoid serious declines in anuran diversity, scientists must continue to create methods for curbing the devastating effects of this recently emerged, relatively little-studied pathogen. Temperature treatment remains a possible nontoxic option to stem the severity of chytrid outbreaks in the world's frog populations.

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