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Publication Date
2006

Hydrological Regimes, Pond Morphology, and Habitat Use:
Predicting the Impact of an Emerging Aquatic Pathogen

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Abstract

Declines in amphibian populations have been reported throughout the world in recent years. Chytridiomycosis, a disease of amphibians caused by the chytrid fungus, *Batrachochytrium dendrobatidis*, is one of a number of factors that have been shown to contribute to these population declines. *B. dendrobatidis* is associated with rapid population declines and local extinctions of populations of mountain yellow-legged frog, *Rana muscosa*, in some areas of the California Sierra Nevada mountains, however in other areas *R. muscosa* populations are persisting in the presence of this fungal pathogen. We conducted field surveys and laboratory experiments to investigate several factors that may explain these different population-level outcomes of *B. dendrobatidis*. Our field surveys revealed that *R. muscosa* at persistent sites are carrying very low fungal loads, which lead to sub-clinical infections. At these sites, *B. dendrobatidis* is having no detectable effect on adult *R. muscosa* survival (although subadults may be experiencing high disease-induced mortality), and some adults are apparently losing the infection. At sites experiencing frog population crashes, a large fraction of *R. muscosa* adult are carrying extremely high fungal loads, and very few infected adults are surviving over the course of a summer, or between years. We found no consistent differences in temperature profiles between the two types of sites. Our laboratory experiments detected no difference in transmission rate or virulence of fungal strains from persistent versus die-off sites. We are investigating differences in susceptibility of frogs from the two types of sites in an experiment currently underway.

Introduction and Problem Statement

Declines in amphibian populations have been reported throughout the world in recent years (Blaustein & Wake 1995; Alford & Richards 1999; Houlahan et al. 2000). Disease is one of a number of factors that has been implicated in these declines (Blaustein et al. 1994; Laurance et al. 1996; Berger et al. 1998; Daszak et al. 1999; Daszak et al. 2003; Young et al. 2001; Wright & Whitaker 2001; Lips et al. 2003; Pounds et al. 2006) along with habitat loss (Fisher & Shaffer 1996), climate change (Pounds 1999), chemical pollution (Davidson et al. 2001), introduction of exotic species (Knapp & Matthews 2000), and increased ultraviolet radiation (Pechmann et al. 1991). Chytridiomycosis is a potentially fatal disease of amphibians caused by the chytrid fungus *Batrachochytrium dendrobatidis*, which has appeared recently in the aquatic habitats of California and throughout the world.

Rana muscosa, mountain yellow-legged frog, is a native Californian frog that exists almost entirely on protected federal lands in California at high elevations (up to 3700m) (Knapp & Matthews 2000). Although this species was once one of the most common vertebrates in the high elevation aquatic ecosystems of the Sierra Nevada and the Transverse Range of southern California (Grinnell & Storer 1924; Zweifel 1955), it has become increasingly rare during the past century (Jennings 1994). During most of the

last century, the best-documented cause of the decline of *R. muscosa* was the introduction on non-native trout (Bradford 1989). Since the 1890's, trout have been stocked throughout the range of *R. muscosa* to create recreational fisheries, and in different portions of the Sierra Nevada trout now occupy 33-90% of available habitat (Knapp & Matthews 2000). Trout prey on *R. muscosa* tadpoles and adults, and adults avoid laying eggs in waterbodies containing trout. Today, the distribution of remaining *R. muscosa* populations and trout are strongly non-overlapping (Knapp & Matthews 2000), and large-scale fish removal efforts in Sequoia & Kings Canyon and Yosemite National Parks are currently being proposed by the National Park Service to protect this and other native aquatic species (D. Graber, pers. com.).

In the late 1990's a new threat emerged to the persistence *R. muscosa* populations in the Sierra Nevada, and to amphibian populations throughout the world: a new fungal pathogen, *Batrachochytrium dendrobatidis*, which leads to the disease chytridiomycosis. The disease caused by *Batrachochytrium dendrobatidis* is called chytridiomycosis. Whether the emergence of chytridiomycosis as a world-wide disease of amphibians is due to the recent geographic spread of the pathogen, or due to the increased susceptibility of amphibians and/or increased virulence of a fungus that was already present, is a topic of active current research (Daszak et al. 2000; Berger et al. 1999; Morehouse et al. 2003; Rachowicz et al. 2005). Preliminary genetic evidence points towards a recent human-caused spread of a novel pathogen (Morehouse et al. 2003), however this evidence is not yet conclusive. There has been speculation that a few species of frogs that have been purposely introduced throughout the world, which can carry the disease without succumbing to its lethal effects, may have led to the anthropogenic spread of the disease. Bullfrogs (*Rana catesbeiana*) introduced into many parts of the world as a food source, and *Xenopus lavis*, which is used extensively in laboratory studies, are two potential carrier species (Mazzoni et al. 2003; Parker et al. 2002).

Batrachochytrium targets substrates containing keratin in amphibians. Tadpoles contain keratin in their mouthparts, while keratin is present throughout the skin of adult frogs. *Batrachochytrium* is a waterborne pathogen. It infects frogs through a mobile zoospore stage, which has a single posterior flagellum, allowing the zoospore to swim to a suitable substrate containing keratin. Because keratin is present only in the mouthparts of tadpoles, but throughout the skin in adult frogs, the impact of the disease on individuals is stage-specific. Chytridiomycosis has little to no effect on tadpoles, although tadpoles can become infected and transmit the disease to other tadpoles and to adults (Rachowicz & Vredenburg 2004). The impact of chytridiomycosis on adult frogs varies greatly between species. Mortality rates of 100% in post-metamorphic frogs have been reported in many species (Berger et al. 1998; Longcore et al. 1999; Nichols et al. 2001), while infected adults of other species appear to be able to carry and spread the disease without experiencing negative effects (e.g. Mazzoni et al. 2003). The impact of chytridiomycosis on individuals within a species, has been found to depend on temperature and on the amount of time that adults spend in the water (Berger et al. unpublished manuscript).

In the Sierra Nevada mountains, we have observed massive die-offs of *R. muscosa* in Sequoia & Kings Canyon National Parks (R. Knapp, L. Rachowicz, pers. obs). Each year since 2000, populations in several lakes in the parks have undergone a pattern of die-off that is typical of chytridiomycosis: Adult frogs are the first to disappear, with multiple

chytrid-positive dead adult frogs observed. *R. muscosa* has a multi-year tadpole stage, and individuals can remain in this stage for 2-4 years. Infected tadpoles can survive in infected lakes until they reach metamorphosis, at which point they die. Extinction of the local population occurs after all tadpoles pass through metamorphosis. This pattern has been repeated in numerous lakes in Sequoia & Kings Canyon National Parks, and over the past few years *R. muscosa* has virtually gone extinct from some complete watersheds in that park (R. Knapp, unpublished data).

The pattern is different at a number of infected lakes in Yosemite National Park. At a number of sites in Yosemite and Little Indian Valley, north of the park, populations of *R. muscosa* have persisted with the disease for at least 8 years. Those sites contain relatively low densities of adults. Virtually 100% of tadpoles are infected, but successful reproduction occurs each year, and new cohorts of tadpoles have been observed every year. L. Rachowicz has shown in a field experiment that tadpoles in both of these types of sites die during metamorphosis, in a way similar to those in the laboratory (L. Rachowicz, unpublished data). However, in her experiment, the tadpoles and metamorphs were confined to cages, and were not able to escape from the aquatic environment and therefore remained exposed to chytrid zoospores throughout metamorphosis.

The most obvious differences between the sites in Sequoia & Kings Canyon National Parks in which die-offs are occurring, and the sites in Yosemite in which *R. muscosa* populations are persisting with infection, are the pond morphology and the topography of the regions. The differences in topography result in differences in the habitat use by *R. muscosa* that could affect their risk of encountering and succumbing to this water-borne disease. The topography of the Sequoia-Kings Canyon sites consists mainly of discrete lakes surrounded by granite bedrock, and the adults spend the majority of their time in the lakes, or basking on the surrounding rocks. The Yosemite sites in which the frogs are persisting include extensive marsh and stream areas with emergent vegetation, in addition to lakes. At these sites the frogs are not confined to isolated lakes, and may be able to escape from areas with high zoospore concentration. In addition, frogs that occur in marshy areas may be experiencing higher temperature regimes that are not optimal for the growth of *Batrachochytrium*. Changes to the hydrological regime in these different areas, due to environmental change, could affect the impact of this water-borne pathogen on native amphibian populations.

Objectives

We investigated two classes of hypotheses as to why the fungal pathogen is having different outcomes on frog populations in different California watersheds. The first hypothesis that we investigated is that differences in the pond morphology and topography of the landscape in different areas result in the frogs using the habitat differently at the different sites, altering their risk of acquiring and succumbing to the disease. Frogs that spend most of their time aggregated in the main lakes and ponds at each site, and in colder temperature habitats, are at greater risk from the disease. Frog die-offs due to the disease in the Sierra are occurring mainly in areas consisting of deep

lakes surrounded by granite bedrock, where the adult frogs spend the majority of their time in the lakes. The sites at which the frogs are persisting with the fungus include extensive marsh and stream areas with emergent vegetation, in addition to lakes. At these sites the frogs are not confined to isolated lakes, and may be able to escape from areas with high concentrations of zoospores (the infectious stage of the fungus by which the disease is spread). The second hypothesis that we investigated is that differences in the transmission, infectivity, and/or virulence of the fungal strains, or differences in susceptibility of the frog genotypes at the different types of sites, are leading to the observed differences in the impact of the fungal pathogen.

Procedure

Our study included both field and laboratory components: In the field, a survey of *B. dendrobatidis* infected sites experiencing *R. muscosa* die-offs and infected sites where the frog populations are persisting with the pathogen was conducted. This provided detailed information on the progression of the disease and its impact on *R. muscosa* populations. Field radio-tracking and mark-recapture studies were performed to determine if frogs are using the habitat differently in the different types of sites, and if they are being exposed to higher temperatures at sites at which the populations are persisting with the disease. Laboratory experiments were carried out to compare the ecological characteristics of *Batrachochytrium* strains from die-off and persisting sites. Transmission rate, infectivity (as measured by zoospore release rate), and virulence of pathogen strains collected from die-off and persisting sites will be compared. Laboratory experiments are still underway to determine if there are genetic differences in susceptibility to the disease in the frog genotypes from die-off and persisting sites.

Field Surveys: During the summers of 2004 and 2005 we conducted detailed surveys at three sites in the Sierra Nevada mountains experiencing *R. muscosa* die-offs due to chytridiomycosis (in Sequoia National Park), and at three sites with *B. dendrobatidis* present with persistent *R. muscosa* populations (in Yosemite National Park and in National Forest Service land to the north). In the high Sierra, *R. muscosa* have a relatively short summer season in which they are active, with ice-free days spanning from mid-June through late September. Over the course of the summer, each site was visited at least 3 times, and the following variables were quantified on each visit:

- Abundance and stage-structure of *R. muscosa* populations was quantified through visual counts (Knapp & Matthews 2000; Knapp et al. 2001). Presence of dead and dying individuals of each stage was also recorded.

- Infection status of a sample of *R. muscosa* individuals at each site was determined using a newly-developed real-time PCR (quantitative PCR) protocol to determine the infection status (presence/absence of *B. dendrobatidis*) and infection level (fungal loads) of *R. muscosa* individuals. This protocol involves non-destructive swabbing of the frog skin, and therefore can be used repeatedly on the same frog individuals. (technique described in Boyle et al. 2004).

- Presence, abundance, and stage-structure of all other amphibian species was recorded, and chytrid infection status was determined as described above. Other amphibians that were present at the sites include *Hyla regila* (pacific treefrog), *Bufo boreas* (western toad), *Bufo canorus* (Yosemite toad), and *Ambystoma macrodactylum* (long-toed salamander).

- Microhabitat characteristics including pond, stream, or marsh depth, substrate composition, surface area, temperature profile, amount of terrestrial vegetation on shoreline, presence or abundance of aquatic predators (fish, garter snakes) were recorded.

- At sites where marking was feasible (i.e. at low population density, persistent sites) we marked adult *R. muscosa* using PIT tags (passive integrated transponder), and recaptured and re-swabbed the same individuals over the course of the summer 2004 and 2005. A passive integrative transponder (PIT) is a radio-frequency identification tag that consists of an electromagnetic coil, tuning capacitor and microchip encased in glass. It is small (10 x 2.1 mm, 0.05 g) and carries a 10 digit hexadecimal number that is read with a portable scanner. PIT tags are commonly used by herpetologists, and others to permanently mark free ranging animals (Heyer et al. 1994). This method has proven to be very reliable with amphibians, and has had minimal impact on the survival of study animals (Heyer et al. 1994).

- To quantify habitat use and range of temperatures experienced by individual frogs, we used radio telemetry to reliably track a subset of the frogs at two persistent sites. Once per visit each radio-tracked frog was located and the water temperature at the frog's location was recorded. The frog was then captured, weighed, measured, and a skin swab was taken for PCR processing to determine chytrid infection status. Radios were removed at the end of each summer.

Laboratory Experiments: From winter through summer 2004 we conducted a laboratory experiment to investigate the ecological differences in fungal pathogen strains collected from infected frogs in die-off and persistent sites. We experimentally inoculated uninfected tadpoles that had been raised from eggs in the laboratory with a known amount of zoospores of a fungal strain from either a die-off site or a persistent site. Each week we inspected the tadpoles for signs of infection (to determine differences in transmission of the different strains), and measured the rate of release of infectious zoospores (to determine differences in infectivity of infected tadpoles). We followed the tadpoles through metamorphosis to record differences in survival of the individuals exposed to the different strains.

Antimicrobial peptides released from the skin of frogs are part of their innate immune response, and some peptides have been shown to be effective at killing *B. dendrobatidis* in laboratory cultures. From fall 2004 through spring 2005, we conducted an experiment to determine if the antimicrobial peptides that are released from *R. muscosa* are effective in protecting *R. muscosa* from *B. dendrobatidis*. Differences in the antimicrobial peptide response might help explain the different outcomes of the fungal pathogen observed in different parts of the Sierra Nevada mountains. Through this experiment we also quantified the dose-response curve of *R. muscosa* to *B. dendrobatidis*. *R. muscosa* subadults were exposed to specific quantified doses of *B. dendrobatidis* zoospores, and

monitored to determine the fraction that became infected, and the fate of the infected individuals. In some treatments, the antimicrobial peptides were removed from the frogs prior to exposure to *B. dendrobatidis*.

During the summer of 2005, *R. muscosa* egg masses were collected from die-off sites and infected but persistent sites. An experiment is currently underway that involves a fully factorial design in which we are exposing late-stage *R. muscosa* tadpoles from the two different types of sites to *B. dendrobatidis* strains from each of those sites. We are recording fraction infected, rate of release of zoospores from infected individuals, and survival of infected individuals. That experiment is still underway, so we are unable to report the results here.

Results

Field Surveys: We found that infected frogs at die-off sites carried very high fungal loads (Figure 1), while at persistent sites the infected frogs experienced only low to moderate infections (Figure 2). Interestingly, at the persistent infected sites, some adult *R. muscosa* were found to lose the infection between the start and end of the summer, and many infected adults were found to survive over the winter (Figure 3). We found no difference between the recapture success of infected and uninfected adults, which suggests that the presence of B. dendrobatidis is not affecting the survival of adult Rana muscosa at the persistent sites. Additional evidence (Rachowicz et al. in press) suggests that there is, however, a high level of mortality due to chytridiomycosis during metamorphosis.
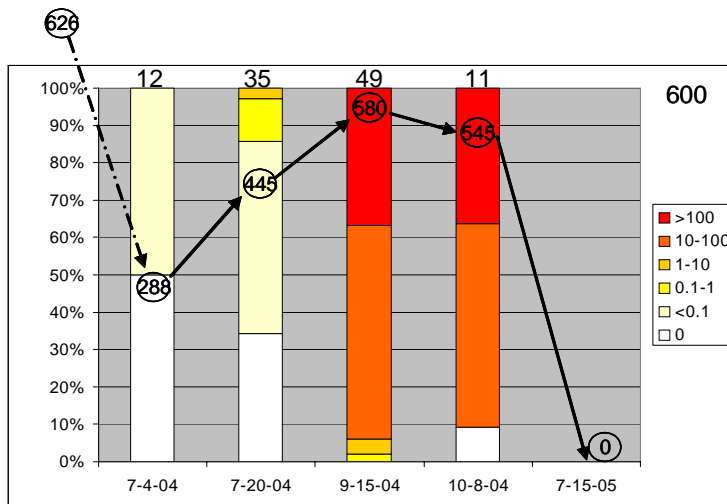

Figure 1. Change in frog population size and *B. dendrobatidis* fungal load in postmetamorphic *Rana muscosa* at one of the die-off sites (Milestone Basin, Sequoia National Park, CA). Bars show distribution of fungal loads in the *R. muscosa* population at each point in time. The fungal loads are quantified as "genomic equivalents" from quantitative PCR of swabs. Deeper red indicates higher fungal loads, and white indicates uninfected individuals. The sample sizes are shown above the bars, and the frog population counts are shown in the circles connected by lines. The population started out

with a very low level of infection in early summer 2004. In autumn 2004, a large fraction of the population was infected with very high fungal loads. By early summer 2005, no post-metamorphic *R. muscosa* remained in the lake.
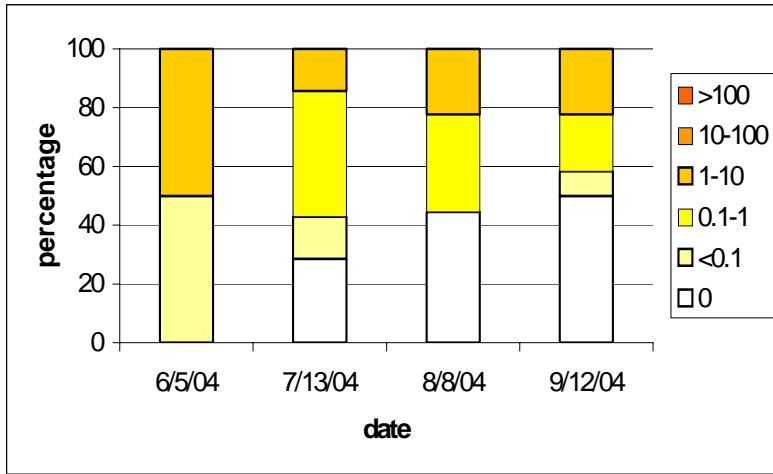

Figure 2. Change in *B. dendrobatidis* fungal load in postmetamorphic *Rana muscosa* at one of the persistent sites (Mono Pass, Yosemite National Park). As in Figure 1, bars show distribution of fungal loads in postmetamorphic individuals in the *R. muscosa* population at each point in time. Deeper red indicates higher fungal loads, and white indicates uninfected individuals. Individuals in this population retain a low fungal load through time, and a large fraction of individuals are uninfected at most points in time.
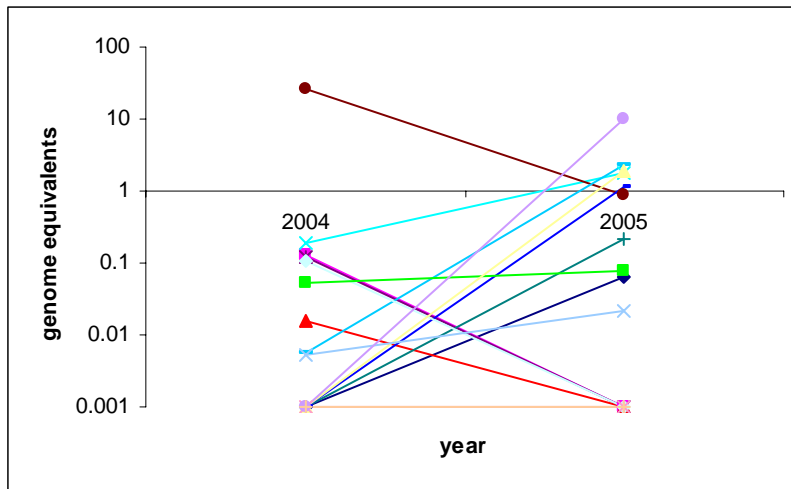

Figure 3. Change in fungal load between years in individual *R. muscosa* adults at one of the persistent sites (Mono Pass, Yosemite National Park, CA). Shown are the fungal loads of individuals that were PIT-tagged in 2004 and recaptured at least once in 2005. A value of 0.001 is added to all genome equivalent values, to allow uninfected individuals to be included on the logarithmic scale. Therefore, a genome equivalent value of 0.001 should be interpreted as "uninfected". This reveals that infected adults are surviving

between years, and that infected adults are losing their infection through time (while other uninfected individuals are becoming infected).

Laboratory Experiments: In the experiment conducted from winter through summer 2004 we found that transmission of the fungus to tadpoles requires very high doses of zoospores, but we measured no differences between the fungal strains in transmission, virulence, infectivity, or tadpole survival. Thus, so far we have found no evidence that differences in fungal strains are responsible for the different population-level impacts of the disease at the different sites. The factorial experiment currently underway, which crosses frog genotypes from the persistent and die-off sites with *B. dendrobatidis* genotypes from the two types of sites, will provide additional data on this hypothesis.

We found that *R. muscosa* individuals increased their peptide production following exposure to *B. dendrobatidis*. It was predicted that if antimicrobial peptides served to defend the frogs against the disease, then individuals from which peptides had been removed prior to exposure would be more likely to become infected. However, we found absolutely no difference between the fractions of individuals that became after peptide removal versus those that were exposed to the same dose without prior peptide removal. This suggested that the antimicrobial peptides are not sufficient to protect *R. muscosa* from chytridiomycosis in even a simple experimental situation.

Conclusions

The fungal pathogen, *Batrachochytrium dendrobatidis*, which causes the disease, chytridiomycosis, is resulting in different population-level outcomes in mountain yellow-legged frog (*Rana muscosa*) populations in different regions of the California Sierra Nevada. We conducted field surveys and laboratory experiments to investigate several factors that may explain these different population-level outcomes of *B. dendrobatidis*. Our field surveys revealed that *R. muscosa* at persistent sites are carrying very low fungal loads, which lead to sub-clinical infections. At these sites, *B. dendrobatidis* is having no detectable effect on adult *R. muscosa* survival (although subadults may be experiencing high disease-induced mortality), and some adults are apparently losing the infection. At sites experiencing frog population crashes, a large fraction of *R. muscosa* adult are carrying extremely high fungal loads, and very few infected adults are surviving over the course of a summer, or between years. We found no consistent differences in temperature profiles between the two types of sites. Our laboratory experiments detected no difference in transmission rate or virulence of fungal strains from persistent versus die-off sites. We found that *R. muscosa* individuals increased their antimicrobial peptide production following exposure to *B. dendrobatidis*, but our laboratory experiment suggests that these antimicrobial peptides are not sufficient to protect *R. muscosa* from chytridiomycosis.

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