

EMERGING INFECTIOUS DISEASE AS A PROXIMATE CAUSE OF AMPHIBIAN MASS MORTALITY

LARA J. RACHOWICZ,^{1,5} ROLAND A. KNAPP,² JESS A. T. MORGAN,³ MARY J. STICE,¹ VANCE T. VREDENBURG,¹
JOHN M. PARKER,^{1,4} AND CHERYL J. BRIGGS¹

¹Department of Integrative Biology, University of California, Berkeley, California 94720-3140 USA

²Sierra Nevada Aquatic Research Laboratory, University of California, HCR 79, Box 198, Mammoth Lakes, California 93546 USA

³Department of Plant and Microbial Biology, University of California, Berkeley, California 94720-3102 USA

⁴Office of Laboratory and Animal Care, University of California, 203 Northwest Animal Facility, Berkeley, California 94720-7150 USA

Abstract. A newly discovered infectious disease of amphibians, chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis*, is implicated in population declines and possible extinctions throughout the world. The purpose of our study was to examine the effects of *B. dendrobatidis* on the mountain yellow-legged frog (*Rana muscosa*) in the Sierra Nevada of California (USA). We (1) quantified the prevalence and incidence of *B. dendrobatidis* through repeat surveys of several hundred *R. muscosa* populations in the southern Sierra Nevada; (2) described the population-level effects of *B. dendrobatidis* on *R. muscosa* population abundance; and (3) compared the mortality rates of infected and uninfected *R. muscosa* individuals from pre- through post-metamorphosis using both laboratory and field experiments. Mouthpart inspections conducted in 144 and 132 *R. muscosa* populations in 2003 and 2004, respectively, indicated that 19% of *R. muscosa* populations in both years showed indications of chytridiomycosis. Sixteen percent of populations that were uninfected in 2003 became infected by 2004. *Rana muscosa* population sizes were reduced by an average of 88% following *B. dendrobatidis* outbreaks at six sites, but at seven *B. dendrobatidis*-negative sites, *R. muscosa* population sizes increased by an average of 45% over the same time period. In the laboratory, all infected *R. muscosa* developed fatal chytridiomycosis after metamorphosis, while all uninfected individuals remained healthy. In the field experiment in which *R. muscosa* tadpoles were caged at infected and uninfected sites, 96% of the individuals that metamorphosed at infected sites died vs. 5% at the uninfected sites. These studies indicate that chytridiomycosis causes high mortality in post-metamorphic *R. muscosa*, that this emerging disease is the proximate cause of numerous observed *R. muscosa* population declines, and that the disease threatens this species with extirpation at numerous sites in California's Sierra Nevada.

Key words: amphibian decline; *Batrachochytrium dendrobatidis*; chytridiomycosis; emerging infectious disease; population decline; *Rana muscosa*; Sierra Nevada.

INTRODUCTION

There has been a recent explosion of interest in the ecology of infectious disease, due in part to an increased appreciation of the impact of disease on natural populations. Documented population-level outcomes of host–parasite interactions in wildlife populations include cyclic fluctuations in host abundance (Hudson et al. 1998), declines in host populations (Berger et al. 1998, Daszak et al. 2000), and host extinction (Cunningham and Daszak 1998, Daszak and Cunningham 1999). However, evidence for infectious diseases causing extirpations and extinctions in wildlife is sparse (Cun-

ningham and Daszak 1998, Daszak and Cunningham 1999, de Castro and Bolker 2005).

A new infectious disease, chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis*, has been discovered very recently (Berger et al. 1998, Longcore et al. 1999) and has already been implicated in the decline of amphibian populations and species extinctions worldwide (Berger et al. 1998, Bosch et al. 2001, Bradley et al. 2002, Hopkins and Channing 2003, Lips et al. 2003a, 2004, Muths et al. 2003, Ron et al. 2003). Most of the supporting evidence for this assertion is based on the detection of amphibians infected with *B. dendrobatidis* at die-off sites (e.g., Berger et al. 1998, Green and Kagarise-Sherman 2001, Lips et al. 2003a, Bell et al. 2004, La Marca et al. 2005). These studies have typically been retrospective and restricted to describing the presence of *B. dendrobatidis*-infected amphibians at a limited number of sites in a relatively small area. Laboratory studies show that chytridiomy-

Manuscript received 14 October 2005; accepted 28 November 2005; final version received 3 January 2006.
Corresponding Editor: K. Wilson.

⁵Present address: Resources Management and Science, Yosemite National Park, 5083 Foresta Road, P.O. Box 700, El Portal, California 95318 USA.
E-mail: lara_rachowicz@nps.gov

cosis can cause mortality in post-metamorphic individuals of some species (Berger et al. 1998, Nichols et al. 2001, Rachowicz and Vredenburg 2004), but field studies that follow individuals in order to estimate the mortality rates of specific amphibian life stages in environments with and without *B. dendrobatidis* are lacking. Following infected and uninfected individuals in the field is extremely important to understanding the role of the disease as a causal factor of mortality in natural populations. To further establish a causal link between chytridiomycosis and widespread population decline, studies are needed to determine whether disease progression in controlled laboratory conditions without environmental cofactors present is similar to disease manifestation in the field. As Carey et al. (2003) point out, the relevance of laboratory results to the interactions among *B. dendrobatidis*, environmental conditions, and the amphibian host in the field has not yet been addressed. Despite the growing body of literature about the impact of chytridiomycosis on amphibians, the prevalence, incidence, and effect of chytridiomycosis on species across their ranges remain poorly understood.

In this study, we examined the effects of chytridiomycosis on mountain yellow-legged frogs (*Rana muscosa*) in the Sierra Nevada of California. *Rana muscosa* is endemic to the mountains of California and adjacent Nevada where it was once one of the most common amphibians. *Rana muscosa* has declined severely over the last half century and is now absent from at least 80% of historic localities (Bradford et al. 1994, Drost and Fellers 1996, Jennings 1996). Although the best-documented cause of population declines in *R. muscosa* is the presence of nonnative trout (Bradford 1989, Bradford et al. 1993, Knapp and Matthews 2000, Vredenburg 2004), recent observations suggest that chytridiomycosis may be an increasing threat to this species (Fellers et al. 2001, Rachowicz and Vredenburg 2004).

The objectives of this study were as follows: (1) use repeat surveys of disease status at hundreds of *R. muscosa* populations in the southern Sierra Nevada (where the most robust populations of *R. muscosa* remain; Knapp and Matthews 2000) to quantify the prevalence and incidence of *B. dendrobatidis*; (2) at a subset of these survey sites, describe the effect of *B. dendrobatidis* on *R. muscosa* populations; (3) follow individual *R. muscosa* from pre- through post-metamorphosis in the laboratory and at uninfected and infected field sites to determine whether the progression and outcome of chytridiomycosis under natural conditions are comparable to those observed in the laboratory.

METHODS

Prevalence and incidence of chytridiomycosis in R. muscosa populations

Oral chytridiomycosis in *R. muscosa* tadpoles results in depigmentation of mouthparts, including jaw sheaths and tooth rows, with no enhanced mortality (Fellers et al. 2001, Rachowicz and Vredenburg 2004). Therefore,

we used the degree of tadpole jaw sheath depigmentation to describe the prevalence of chytridiomycosis in *R. muscosa* (Knapp and Morgan 2006). In both 2003 and 2004, we visited all 579 *R. muscosa* populations in Sequoia-Kings Canyon National Park (a general description of these sites is provided in Knapp and Matthews [2000]) and quantified the extent of tadpole jaw sheath depigmentation. Based on previous research results indicating that the extent of depigmentation of upper jaw sheaths provides a much more reliable indication of *B. dendrobatidis* infection than the depigmentation of either lower jaw sheaths or tooththrows (see Knapp and Morgan [2006] and Appendix A for details), our mouthpart inspections focused solely on upper jaw sheaths. We examined upper jaw sheaths with a 10× hand lens and estimated the percentage of the upper jaw sheath length that was pigmented for each tadpole (0–100%).

We restricted mouthpart inspections to tadpoles of Gosner stages 30–40 (Gosner 1960, Rachowicz and Vredenburg 2004) and at each site sought to inspect the mouthparts of at least 10 tadpoles. Because we were sometimes unable to capture 10 tadpoles, we included in our analyses all sites at which we inspected mouthparts of at least five individuals (range = 5–31 tadpoles, mean = 11; total number of tadpoles inspected = 3396). This included 144 sites in 2003 and 132 sites in 2004. Because the prevalence of mouthpart depigmentation in tadpoles at each *B. dendrobatidis*-positive site generally exceeds 50% ($n = 12$ sites, range = 27–100% of tadpoles per site with mouthpart depigmentation, mean = 81%; R. A. Knapp, unpublished data), inspections of even five tadpoles per site would usually provide an accurate assessment of *B. dendrobatidis* presence/absence. For example, when the prevalence of mouthpart depigmentation is 50%, inspection of only five tadpoles would provide a 97% chance of encountering at least one tadpole with mouthpart depigmentation.

Rana muscosa have an unusually long tadpole stage that lasts 2–4 yr (Zweifel 1955). As a consequence, *R. muscosa* tadpoles spend several months each year overwintering in ice-covered lakes. Previous research on *R. muscosa* indicated that prolonged exposure to near-freezing (e.g., winter) temperatures can also cause tadpole mouthpart depigmentation (Rachowicz 2002). Although the mechanism underlying this effect remains unknown, we minimized the chances that observed jaw sheath depigmentation was a consequence of winter temperatures by conducting all surveys in mid-summer and at least three weeks after ice-out. To minimize the chances of surveyors spreading *B. dendrobatidis* between sites, we disinfected all survey gear between each site using a 0.1% solution of quaternary ammonia (Johnson et al. 2003).

For each site, we averaged the upper jaw sheath pigmentation estimates across all inspected tadpoles to create a site-specific jaw sheath pigmentation score. Sites were categorized as *B. dendrobatidis*-negative when the jaw sheath pigmentation score was 100% and *B.*

dendrobatidis-positive when the jaw sheath pigmentation score was $<100\%$. The reliability of this categorization to indicate the presence/absence of *B. dendrobatidis* was confirmed by polymerase chain reaction (PCR) assays (overall misclassification rate = 8%; see Knapp and Morgan [2006] and Appendix A for details).

*Effect of chytridiomycosis on wild
R. muscosa populations*

To describe the effect of chytridiomycosis on *R. muscosa* populations in the field, we compared *R. muscosa* population trajectories at *B. dendrobatidis*-positive vs. *B. dendrobatidis*-negative sites. In addition to using tadpole mouthpart depigmentation to assess *B. dendrobatidis* presence/absence, we also used microscopic and molecular assays (hereafter referred to as assays) to detect *B. dendrobatidis* in *R. muscosa* tadpoles and frogs, found in the keratinized mouthparts and skin cells, respectively. These assays have evolved through time as increasingly sensitive methods were developed. In general, cytology (Berger et al. 1998, Rachowicz and Vredenburg 2004) and histology (Fellers et al. 2001, Nichols et al. 2001) were used prior to 2002, and PCR (Boyle et al. 2004; see Appendix B for additional details) was used from 2002 to 2004. Although the use of less sensitive cytological and histological assays could have resulted in a higher rate of false-negatives for assays conducted prior to 2002, in all cases results from microscopic and molecular assays were in agreement with the results from tadpole mouthpart inspections. Therefore, any bias caused by the lower sensitivity of the microscopic assays compared to the molecular assays is unlikely to have influenced our results and conclusions.

The sites we chose for the comparison of *R. muscosa* population trajectories at *B. dendrobatidis*-positive vs. *B. dendrobatidis*-negative sites were those for which we had data on *R. muscosa* abundance and *B. dendrobatidis* presence/absence from at least four years between 1996 and 2004 (in some cases the sites were visited more than once per year). This included 13 total sites, 12 in Sequoia-Kings Canyon National Park and one in the adjacent John Muir Wilderness. All 12 sites from Sequoia-Kings Canyon National Park were also included in the chytridiomycosis study described above (see *Prevalence and incidence of chytridiomycosis in R. muscosa populations*). Six of the 13 sites were categorized as *B. dendrobatidis*-positive based on *R. muscosa* being *B. dendrobatidis*-positive during later site visits but *B. dendrobatidis*-negative during early site visits. For three of the six sites, *B. dendrobatidis* absence during early site visits was based on mouthpart inspections of ≥ 5 tadpoles and results from assays conducted on one or more specimens. For the remaining three sites, *B. dendrobatidis* absence during early site visits was based solely on mouthpart inspections of ≥ 5 tadpoles. For all six sites, *B. dendrobatidis* presence during later site visits was based on mouthpart inspections of ≥ 5 tadpoles and results from assays conducted on one or more speci-

mens. Seven of the 13 sites were categorized as *B. dendrobatidis*-negative based on *R. muscosa* being free of *B. dendrobatidis* during every site visit. For all seven sites, the *B. dendrobatidis*-negative status was based on mouthpart inspections of ≥ 5 tadpoles during each site visit. Results from tadpole mouthpart inspections were supplemented with the results from assays conducted on one or more specimens collected during either one (five of seven sites) or two of the surveys (two of seven sites).

Visual encounter surveys (Crump and Scott 1994) for *R. muscosa* included the entire lake or pond shoreline and the first 100 m of each inlet and outlet stream. During surveys, we recorded the abundances of live and deceased *R. muscosa* adults, subadults, and tadpoles (for additional details on survey methodology, see Knapp and Matthews [2000] and Knapp [2005]). During the summer, all three life stages are highly visible during shoreline surveys because post-metamorphic *R. muscosa* spend the majority of their time on shore immediately adjacent to water and tadpoles are found primarily in near-shore shallows (Bradford 1984). In addition, tadpoles are present throughout the summer (and during all other seasons) due to the unusual longevity of this life stage in *R. muscosa*. As a consequence, counts of all life stages are highly repeatable (Knapp and Matthews 2000, Vredenburg 2004), with $\sim 50\%$ of the individuals in a population being counted (R. A. Knapp, unpublished data).

*Mortality of R. muscosa individuals due to
chytridiomycosis: laboratory studies*

The laboratory portion of this study involved two experiments. In both, individual infected and uninfected *R. muscosa* were followed through time to assess the impact on individuals. Sample sizes for both experiments were small because *R. muscosa* is a threatened species and in decline.

Mortality rates of naturally infected animals (laboratory experiment 1).—To quantify mortality rates of *R. muscosa* with and without chytridiomycosis, we collected 10 tadpoles found to be infected with *B. dendrobatidis* (hereafter referred to as infected) and five tadpoles without *B. dendrobatidis* (referred to as uninfected) and followed them through metamorphosis. Naturally infected tadpoles were collected from one water body in the Toiyabe National Forest and four water bodies in Sequoia National Park. Prior to collection, *R. muscosa* tadpoles from the same populations were found to be *B. dendrobatidis*-positive based on assays conducted on one or more specimens per site. At the time of collection, tadpoles for this experiment were at Gosner stages 35–38 (metamorphosis happens during Gosner stages 42–45; Gosner 1960) and showed jaw sheath depigmentation characteristic of *B. dendrobatidis* infection (as described in *Prevalence and incidence of chytridiomycosis in R. muscosa populations*). Late-stage tadpoles were used because younger *R. muscosa* tadpoles likely were not yet infected (Rachowicz and

Vredenburg 2004). Uninfected tadpoles were collected from one water body in Kings Canyon National Park. During the two years prior to these collections, 15 tadpoles from this water body tested negative for *B. dendrobatidis* using assays. At the time of collection, tadpoles for this experiment were at Gosner stages 35–40 and showed no jaw sheath depigmentation.

Collected tadpoles were transported to the animal care facility at the University of California, Berkeley, where they were individually housed in 5-L plastic containers filled with 4 L of aerated, charcoal-filtered water maintained at 17°C. Individuals received 12 h of light and dark per day. Tanks were changed twice weekly, and tadpoles were fed rabbit chow ad libitum. Once tadpoles reached stage 42 and began to metamorphose, the amount of water in each container was reduced to 0.25 L and further reduced to 0.125 L when tadpoles reached stage 44. Beginning at stage 45, 10 1–2-week-old crickets were added to each tank for food twice weekly.

Mortality rates of experimentally infected animals (laboratory experiment 2).—Because it is possible that differences in mortality in laboratory experiment 1 may be due to factors other than *B. dendrobatidis* infection status, all tadpoles in laboratory experiment 2 came from the same frog metapopulation so that the only difference between control and treatment animals was infection status and not source population. In laboratory experiment 2, uninfected tadpoles were reared in the laboratory from egg masses collected from two connected water bodies in Kings Canyon National Park, both of which had no known history of chytridiomycosis based on two years of repeated tadpole mouthpart inspections and assays of three specimens. In the laboratory, egg masses were placed individually into separate 20-L plastic containers, each filled with 12 L of aerated, charcoal-filtered water. Once eggs hatched, tadpoles were maintained as described above (see *Mortality rates of naturally infected animals ...*). When tadpoles reached Gosner stages 31–36, 20 were randomly selected from their cohort and placed singly into 5-L plastic containers. Ten tadpoles were randomly assigned to the chytrid-inoculation treatment and 10 to the control treatment. Over the next 30 d, each container was inoculated 20 times with either *B. dendrobatidis* zoospores (chytrid-inoculation treatment, $n = 10$; aquatic flagellated zoospore is the mode of transmission, Longcore et al. [1999]) or a control inoculum (control treatment, $n = 10$), and all 20 tadpoles were then followed through metamorphosis. The *B. dendrobatidis* strain used was isolated from a *R. muscosa* tadpole collected from Sequoia National Park in October 2002 following techniques described by Longcore et al. (1999). Inoculum was prepared by dispensing 1 mL of *B. dendrobatidis* culture, suspended in a 1% tryptone broth, onto a 1% tryptone agar plate and incubating for 7–10 d at room temperature. The plate was then flooded with 10 mL of sterile distilled water for 30–45 min to release zoospores (Longcore et al. 1999). For each

inoculation, 1 mL of the 10-mL zoospore solution was added directly into the water of each container in the chytrid-inoculation treatment. Based on measurements made using a hemacytometer, each inoculation contained an average of 1.3×10^6 zoospores. Control inoculum was prepared following the same methods but without *B. dendrobatidis* in the broth.

Data collection during laboratory experiments.—All tadpoles were examined five times each week beginning when they were at approximately Gosner stage 40. At each examination, we recorded Gosner stage and any clinical signs of disease (lethargy, anorexia, excessive shedding of skin; Nichols et al. 2001). All individuals shed skin regardless of disease status during and following metamorphosis and, thus, we removed skin from each container at least once, stained the skin with eosin and methylene blue (Dip Stain Kit, Volu-Sol, Salt Lake City, Utah, USA), and examined each piece for the presence of *B. dendrobatidis* (Nichols et al. 2001). For experiment 2 only, the number of crickets eaten per animal of the 10 crickets offered was recorded twice per week at stages 45 and 46. Individuals were considered moribund when they did not right themselves within 5 s once turned onto their dorsal surface. Moribund animals were euthanized with an overdose of buffered 0.1% tricaine methanesulfonate (MS-222) prior to preservation. Individuals found moribund or deceased were preserved for postmortem examination in 10% buffered formalin for experiment 1 and in 95% ethanol for experiment 2. To give control animals ample opportunity to display any signs of illness, we ended both studies more than two weeks after all individuals in the infected group were found moribund or deceased. Gross and histological examinations were performed on nine preserved post-metamorphic individuals from the naturally infected group (experiment 1) and on seven preserved post-metamorphic individuals from the chytrid inoculation treatment (experiment 2), as described below in *Postmortem evaluation*.

Data analysis.—Fisher exact tests were used to compare mortality between the infected and uninfected groups in both laboratory studies. To compare growth rates of animals in infected and uninfected treatments, the number of days each individual was alive in each Gosner stage was calculated for all individuals in both studies by recording the Gosner stage for five consecutive days each week. If an individual grew into a new stage or died during the two days each week not observed, one day was added to the previous stage and one to the next to estimate the number of days in each stage. The mean number of days spent at each stage (41–45) was compared between groups (naturally infected vs. uninfected and experimentally infected vs. uninfected) with two-tailed *t* tests. Additionally, the mean number of days spent at each stage from both infected groups combined and both uninfected groups combined were tested with two-tailed *t* tests. The cut-off level of significance was 0.01, the family-wise error rate deter-

TABLE 1. Characteristics of the water bodies in which the cage experiments were performed (see *Mortality of R. muscosa individuals due to chytridiomycosis: field study*).

Location name	Jurisdiction†	Infection status	Elevation (m)	Water temperature (°C)‡
Tuolumne 1	YNP	infected	3176	14.4 (0.06)
Tuolumne 2	YNP	infected	3237	14.0 (0.06)
Humphreys Basin 1	JMW	infected	3443	16.5 (0.09)
Mt. Whitney	SNP	infected	3610	13.0 (0.05)
Humphreys Basin 2	JMW	uninfected	3583	13.4 (0.07)
Sixty Lake Basin 3	KCNP	uninfected	3290	17.1 (0.08)
Sixty Lake Basin 10	KCNP	uninfected	3380	15.5 (0.09)

† Abbreviations: YNP, Yosemite National Park; JMW, John Muir Wilderness; SNP, Sequoia National Park; KCNP, Kings Canyon National Park. All sites are located in the Sierra Nevada.

‡ Data are given as mean with SE in parentheses. Temperature was measured once per hour from 29 July to 14 September 2003.

mined using a Bonferroni adjustment for repeated tests. For laboratory experiment 2, the mean number of crickets eaten per feeding by infected vs. uninfected individuals was compared at stages 45 and 46 with two-tailed *t* tests with a Bonferroni adjustment for multiple testing (cut-off value of 0.025).

Mortality of R. muscosa individuals due to chytridiomycosis: field study

We followed individually caged *R. muscosa* tadpoles through metamorphosis at uninfected and infected field sites to determine whether the progression and outcome of chytridiomycosis in the field is comparable to that observed in the laboratory. Sites used in this study ranged from Yosemite National Park in the central Sierra Nevada to Sequoia National Park in the southern Sierra Nevada (Table 1). Infected sites were chosen based on the presence of *R. muscosa* mass mortality events of post-metamorphic individuals observed during the previous year. The presence of *B. dendrobatidis* was confirmed at all infected sites through assays performed on one or more *R. muscosa* specimens. Uninfected sites had high densities of *R. muscosa* tadpoles and post-metamorphic individuals, and they had no known history of chytridiomycosis based on 2–4 years of mouthpart inspections and assays performed on one or more *R. muscosa* specimens. All experimental lakes were fishless.

In early summer 2003, we collected 10 *R. muscosa* tadpoles at Gosner stage 40 or early stage 41 at each site. Late-stage tadpoles were selected to increase the likelihood that any fungal infection was well established and to increase the chances that the individual would metamorphose during the experiment. Tadpoles at infected sites had depigmented jaw sheaths, and tadpoles at uninfected sites had fully pigmented jaw sheaths. Each tadpole was placed individually in a cage in the lake from which it was collected. The cages were situated 60–90 cm apart along the lake shoreline and at locations in each water body where tadpoles naturally congregated. Cages consisted of 142 × 30 cm plastic mesh cylinders (mesh size = 1.5–3 mm²), reinforced by plastic-coated wire and held in place by wood dowels. Each cage was placed perpendicular to the shoreline and

partially submerged such that two-thirds of each cage was under water (tadpole and frog habitat) and one-third was out of the water (additional frog habitat). Three handfuls of lake substrate were placed in each mesh cage to weight them down to the lake bottom at the submerged end and further simulate natural conditions. One temperature logger was attached to the bottom of one cage per site, and these loggers recorded temperature once per hour from 29 July to 14 September 2003.

Approximately every two weeks from mid-July until late September (a total of five or six visits per site), caged individuals were examined and substrate in cages was replenished. During each examination, we recorded whether the animal was alive, moribund, or deceased, its Gosner stage, and clinical signs of disease. Moribund individuals were euthanized, and all deceased individuals were preserved in 95% ethanol for postmortem examination. The study was terminated in late September when water temperatures approached 0°C. At the end of the study, any remaining live individuals from infected sites and a subset of live individuals from control sites were euthanized. Gross and histologic examinations were performed on seven preserved individuals found deceased or moribund from infected sites and eight from uninfected sites (described below in *Postmortem evaluation*). Remaining animals found deceased could not be evaluated due to postmortem decomposition.

To eliminate the possibility that measured mortality rates were confounded by the different rearing conditions experienced by tadpoles from different lakes before they were put in cages, it would have been preferable to use tadpoles from a common source for transplanting into each study lake. However, that was not possible because of federal land research permit restrictions regarding translocations of *R. muscosa*. Additionally, we were not able to quantify the effect of lake type (infected, uninfected) on the morbidity or mortality of early-stage tadpoles. The design of our experiment necessitated that tadpoles complete metamorphosis by the end of the summer (tadpoles would not survive over winter within cages). Because *R. muscosa* has a multiyear tadpole stage, we were therefore

restricted to starting our experiment with late-stage tadpoles.

Data analysis.—The proportion of individuals that metamorphosed and the proportion that metamorphosed and died at each site were calculated and compared between infected and uninfected sites using Wilcoxon rank-sum tests. The mean Gosner stage of individuals in the cages at uninfected and infected sites was calculated for each survey date. We included only the individuals that eventually metamorphosed in these Gosner stage means in order to have a comparison to laboratory experiments, in which all tadpoles metamorphosed. The mean temperature was calculated for each site, and we compared the temperatures at infected and uninfected sites (two-tailed Wilcoxon rank-sum test).

Field data were not complete enough to determine precisely how many days the caged animals lived post-metamorphosis because the cages were monitored approximately every two weeks. However, we estimated the minimum and maximum days that a recovered, deceased animal likely survived post-metamorphosis based on its stage when last observed alive, its stage when found deceased, and laboratory data on rates of metamorphosis. Tadpoles that metamorphosed developed at the same rate in the laboratory and field experiments (see *Results: Mortality of R. muscosa individuals due to chytridiomycosis: field study*); therefore, laboratory data provided reasonable approximations of the length of time at each stage in the field. For example, if an animal at stage 46 was found deceased in its cage at the time of the survey (t) and was stage 44 at the previous survey, $t - 14$ d, the animal lived post-metamorphosis for a maximum of 13 d (i.e., it reached stage 46 and died on day $t - 13$) and a minimum of 0 d (i.e., it reached stage 46 and died on day t). However, because the laboratory data showed that animals spent 7 d on average at stage 45, estimated maximum time at stage 46 could be reduced to $13 - 7 = 6$ d. In this manner, the minimum and maximum number of possible days alive post-metamorphosis was estimated for each animal that completed metamorphosis. The median minimum number and median maximum number of days alive post-metamorphosis was calculated for each site.

Postmortem evaluation

Necropsies were conducted in order to determine whether chytridiomycosis was the sole cause of death in animals from the experiments. Only carcasses with complete to partial retention of ventral or webbed skin were evaluated postmortem. Scrape cytology smears of superficial integument obtained from ethanol-fixed carcasses were rehydrated and evaluated unstained using standard light microscopy. Specimens were considered infected if they contained multiple epithelial cells with intracellular *B. dendrobatidis* thalli (Pessier et al. 1999). Animals were considered clinically uninfected if they lacked thalli-like inclusions in numerous epithelial cells scraped from three different anatomical locations

(ventrum, thigh, and toe webbing). The coelomic cavity was incised for inspection of viscera, and representative tissues from all visceral organs were processed for histologic examination. Paraffin-embedded tissues were sectioned (5 μ m thickness) and stained with hematoxylin and eosin (H & E).

RESULTS

Prevalence and incidence of chytridiomycosis in R. muscosa populations

In 2003 and 2004, 28 of 144 (19.4%) and 25 of 132 (18.9%) of the tadpole populations, respectively, were found to have jaw sheath pigmentation scores $<100\%$, a level of depigmentation indicative of chytridiomycosis (Appendix A). The similar prevalence of *B. dendrobatidis*-positive sites in both years might be interpreted to indicate that few of the sites that were *B. dendrobatidis*-negative in 2003 became *B. dendrobatidis*-positive in 2004. However, *R. muscosa* populations in Sequoia-Kings Canyon National Park generally decline to extirpation or near-extirpation within one year following a *B. dendrobatidis* outbreak (described below in *Effect of chytridiomycosis on wild R. muscosa populations*). For example, of the 28 *R. muscosa* populations that were classified as *B. dendrobatidis*-positive in 2003, only seven (25%) were still large enough in 2004 for us to conduct tadpole mouthpart inspections (six of the seven sites had jaw sheath pigmentation scores in 2004 indicative of chytridiomycosis). Therefore, despite similar proportions of *R. muscosa* populations being categorized as *B. dendrobatidis*-positive in 2003 and 2004, the majority of populations (67%) found to be *B. dendrobatidis*-positive in 2004 actually represented new outbreaks. To characterize the actual rate at which uninfected *R. muscosa* populations became infected between 2003 and 2004, we focused our analysis on the 75 populations that were categorized as *B. dendrobatidis*-negative in 2003 and for which we were able to conduct tadpole mouthpart inspections again in 2004. Of these 75 populations, 12 were found to be *B. dendrobatidis*-positive in 2004. Therefore, in Sequoia-Kings Canyon National Park, the incidence of newly infected *R. muscosa* populations between 2003 and 2004 was 16% (12/75).

Effect of chytridiomycosis on wild R. muscosa populations

Trends in *R. muscosa* population sizes were markedly different at *B. dendrobatidis*-positive vs. -negative sites. At all six *B. dendrobatidis*-positive sites, population sizes (total number of tadpoles + post-metamorphic frogs) decreased between the first and last surveys conducted during the study period (Fig. 1a–f). Of these six *R. muscosa* populations, three were extirpated following the disease outbreak (Fig. 1a, e, f) and two were reduced to a single individual (Fig. 1b, d). At the sixth site (Fig. 1c), 13 post-metamorphic individuals and >200 tadpoles were still present during the 2004 survey. However, the disease outbreak at this site did not start until September

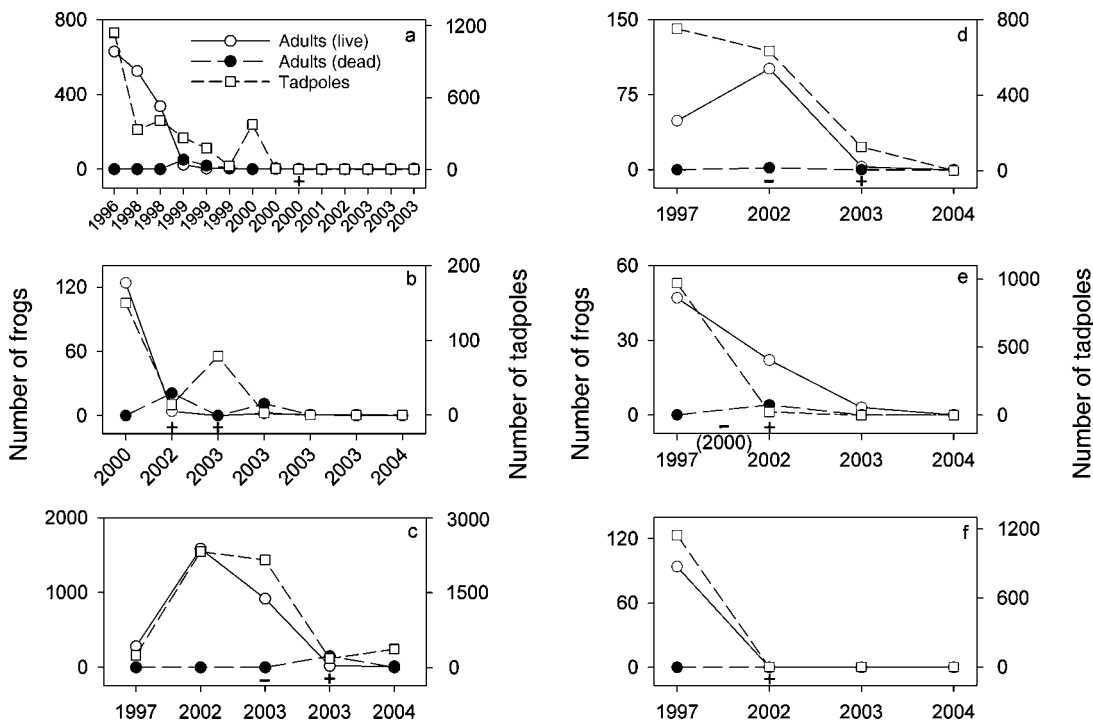


FIG. 1. The number of post-metamorphic and larval *Rana muscosa* counted between 1996 and 2004 at each of six sites categorized as *Batrachochytrium dendrobatidis*-positive. Disease status was determined for all sites using tadpole mouthpart inspections, and symbols above the x-axis (+ or -) indicate the surveys in which *R. muscosa* specimens were collected and confirmed to be *B. dendrobatidis*-positive (+) or negative (-) based on cytology, histology, and/or polymerase chain reaction tests. The repetition of years along the x-axis reflects surveys done on different dates in the same year. The "2000" label along the x-axis in panel (e) appears in parentheses because only chytrid inspections were conducted; no amphibian survey was conducted on this date at this site. Sites shown in each panel are located in the following basins in the Sierra Nevada: (a) Humphreys Basin, (b) Hitchcock Lakes Basin, (c) and (d) Pinchot Basin, (e) Woods Lake Basin, and (f) Amphitheatre Basin.

2003 and, therefore, likely had not had sufficient time to run its full course by the time of the 2004 survey. At the seven *B. dendrobatidis*-negative sites (Fig. 2a-g), only three populations showed evidence of decline between the first and last survey (Fig. 2a, d, e), and, at the time of the last survey, all seven *B. dendrobatidis*-negative sites continued to harbor relatively large populations of both tadpoles and post-metamorphic individuals. The difference between the *B. dendrobatidis*-positive and *B. dendrobatidis*-negative sites in the proportion of populations showing declines was statistically significant (6 of 6 vs. 3 of 7; one-tailed Fisher exact test, $P = 0.049$). The percentage change in *R. muscosa* total population size between the first and last surveys was also significantly different between the *B. dendrobatidis*-positive and -negative sites (-88% vs. +45%; one-tailed Wilcoxon rank-sum test, $Z = 2.37$, $P = 0.009$).

The mean number of deceased frogs found per survey was significantly higher at *B. dendrobatidis*-positive than *B. dendrobatidis*-negative sites (7.4 vs. 0.3, respectively; one-tailed Wilcoxon rank-sum test, $Z = 1.8$, $P = 0.03$). At the *B. dendrobatidis*-positive sites, deceased frogs were found at five of the six sites ($n = 2-151$ per survey) and during 11 of 37 surveys (30%; Fig. 1a-e). At all five sites at which deceased frogs were found, the initial

discovery of deceased frogs coincided with a precipitous decline in *R. muscosa* numbers. Deceased frogs were also found occasionally at the *B. dendrobatidis*-negative sites, but were uncommon when present ($n = 1-3$ per survey) and were found at only three of seven sites (Fig. 2b, d, f) and during four of 27 surveys (15%). The presence of deceased frogs at the three *B. dendrobatidis*-negative sites was not associated with any obvious declines in the *R. muscosa* populations. Deceased tadpoles were never found at any *B. dendrobatidis*-positive or -negative sites.

Mortality of R. muscosa individuals due to chytridiomycosis: laboratory studies

Mortality rates.—In experiment 1, all 10 of the field-collected naturally infected individuals metamorphosed; seven died soon thereafter and three became moribund. In contrast, the five uninfected field-collected individuals metamorphosed, appeared clinically healthy, and survived (Table 2). The mortality rates between the infected and uninfected treatments were significantly different (one-tailed Fisher exact test, $P < 0.001$).

In experiment 2, three tadpoles in the chytrid inoculation treatment were excluded from further analysis because two did not become infected despite being inoculated with *B. dendrobatidis* and one became

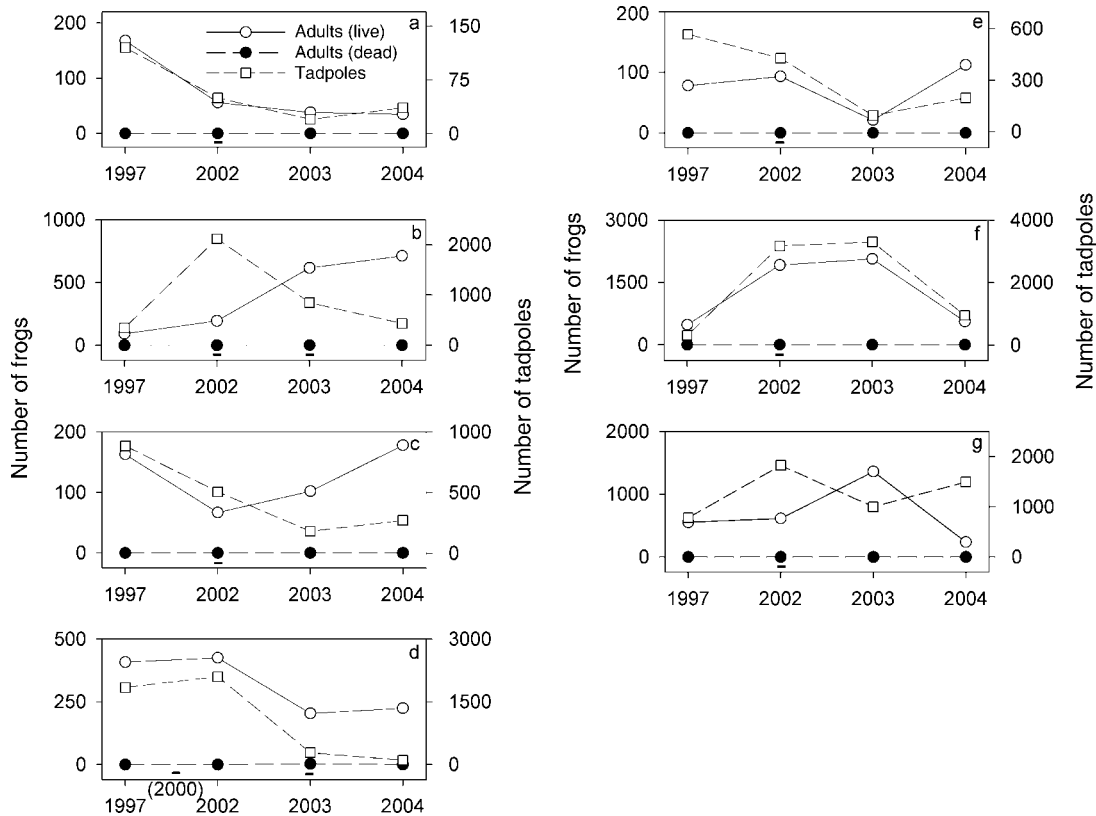


FIG. 2. The number of post-metamorphic and larval *Rana muscosa* counted between 1997 and 2004 at each of seven sites categorized as *Batrachochytrium dendrobatidis*-negative. Disease status was determined for all sites using mouthpart inspections, and minus (-) symbols above the x-axis indicate the surveys in which *R. muscosa* specimens were collected and found to be *B. dendrobatidis*-negative based on cytology, histology, and/or polymerase chain reaction tests. Sites shown in each panel are located in the following basins: (a) Dusy Basin, (b) Evolution Basin, (c) to (e) Sixty Lake Basin, and (f) and (g) Barrett Lake Basin.

ill and was euthanized before metamorphosis (apparently due to a severe dilation of the stomach). Of the seven remaining individuals in the chytrid inoculation treatment, all seven showed clinical signs of chytridiomycosis and either became moribund ($n=6$) or died ($n=1$) following metamorphosis. All 10 individuals in the control treatment metamorphosed, appeared clinically healthy, and survived (Table 2). The mortality rates between the infected and uninfected treatments were significantly different (one-tailed Fisher exact test, $P < 0.001$).

In both experiments 1 and 2, *B. dendrobatidis* was found in shed skin samples from all infected individuals and not found in skin from any control individuals. Postmortem evaluations found that chytridiomycosis was the only cause of morbidity and mortality observed in the nine infected individuals examined from the naturally infected group (experiment 1) and seven infected individuals examined from the inoculated group (experiment 2), and lesions were restricted to the integumentary system.

Feeding rate of metamorphosing *R. muscosa* in experiment 2 was unaffected by infection status during Gosner stage 45 but affected during stage 46, when

animals were fully metamorphosed. During Gosner stage 45, uninfected individuals ate on average 2.2 crickets per feeding and the infected individuals ate 1.9 crickets (two-tailed t test, $P = 0.64$). However, at stage 46, the uninfected individuals ate on average 11.1 crickets per feeding and the infected individuals ate 2.0 crickets, a significant difference (two-tailed t test, $P < 0.001$).

The mean number of days individuals remained at each Gosner stage (41–45) was not significantly different between the infected and uninfected groups (tested for each laboratory experiment and for both experiments combined) using two-tailed t tests with a significance level adjusted for multiple tests (Fig. 3a). At stage 46, uninfected individuals remained alive much longer than infected individuals; they were followed for means of 73 (naturally infected study) and 137 d (chytrid inoculation study; Table 2).

Mortality of R. muscosa individuals due to chytridiomycosis: field study

Of the 70 individual *R. muscosa* in the caging experiment, eight disappeared, possibly as a result of escape or of predation by aquatic insects, and were

TABLE 2. Comparison of the number of tadpoles that metamorphosed, mortality of post-metamorphic individuals, and number of days alive post-metamorphosis for uninfected and infected *Rana muscosa* individuals in the laboratory observational study, laboratory experiment, and field experiment.

Experiment and site	No. metamorphosed	No. deceased	No. days alive
A) Uninfected <i>Rana muscosa</i>			
Laboratory experiment 1 (observational)			
Naturally uninfected	5 (5)	0 (5)	73 (2.4)
Laboratory experiment 2 (experimental)			
Controls	10 (10)	0 (10)	137 (4.7)
Field experiment			
Humphreys Basin 2	7 (9)	0 (7)	19–25
Sixty Lake Basin 3	6 (6)	0 (6)	27–35
Sixty Lake Basin 10	9 (9)	1 (9)	16–30
B) Infected <i>Rana muscosa</i>			
Laboratory experiment 1 (observational)			
Naturally uninfected	10 (10)	10 (10)	12 (3.8)
Laboratory experiment 2 (experimental)			
Inoculated	7 (7)	7 (7)	16 (6.0)
Field experiment			
Tuolumne 1	4 (10)	4 (4)	0–25
Tuolumne 2	6 (10)	5 (6)	0–20
Humphreys Basin 1	7 (8)	7 (7)	0–15
Mt. Whitney	7 (10)	7 (7)	0–16

Notes: The number metamorphosed represents the number of tadpoles followed to the end of the study that metamorphosed, and the number deceased represents the number of metamorphosed individuals followed to the end of the study that died or became moribund; numbers in parentheses represent total number followed. For laboratory studies, data are the mean (with SE in parentheses) number of days alive post-metamorphosis. For the field experiment, data given are the median minimum number and median maximum number of days alive post-metamorphosis. The number of days alive for uninfected individuals was truncated by the termination of the respective studies.

excluded from further analyses. Of the remaining 62 individuals, $36\% \pm 9.9\%$ (means \pm SE) did not metamorphose at each of the four infected sites and $7\% \pm 7.4\%$ did not metamorphose at each of the three uninfected sites (Table 2). This difference was not statistically significant (two-tailed Wilcoxon rank-sum test, $P = 0.075$). Of the individuals that metamorphosed, $96\% \pm 4.3\%$ died at each infected lake and $4\% \pm 3.7\%$ died at each uninfected lake (Table 2; one-tailed Wilcoxon rank-sum test, $P < 0.001$). The mean water temperatures of infected and uninfected sites were not significantly different (Table 1; two-tailed Wilcoxon rank-sum test, $P = 0.63$).

Growth rate appeared similar for uninfected and infected laboratory and field individuals (Fig. 3b). In all groups, metamorphosis (stages 42–45) took approximately four weeks to complete. The median, minimum, and maximum estimated numbers of days alive post-metamorphosis at the infected field sites were similar to those for infected individuals in the two laboratory studies (Table 2).

Batrachochytrium dendrobatidis zoospores were found in all the deceased metamorphic individuals that underwent postmortem evaluations from the infected sites, and no evidence for any other cause of death was found in these animals. *Batrachochytrium dendrobatidis* was not found in any individuals that underwent postmortem evaluations from uninfected sites. Besides the presence of *B. dendrobatidis*, histologic examinations of visceral organs and gross examinations did not reveal any differences between the infected and uninfected

animals. Thus, *B. dendrobatidis* was presumed to be the causative agent of mortality.

DISCUSSION

Our results suggest that *B. dendrobatidis* is widespread in *R. muscosa* populations in the southern Sierra Nevada, the only portion of the historic range where large populations of this declining amphibian still exist. The prevalence of chytridiomycosis in the study populations was 19% in the two study years, and 16% of populations that were uninfected in 2003 became infected by 2004 (incidence). Furthermore, the outcomes of our laboratory and field experiments and field surveys indicate that *B. dendrobatidis* causes high mortality in post-metamorphic *R. muscosa*. Specifically, tadpoles infected with *B. dendrobatidis* survived to metamorphosis, after which they rapidly became moribund or died while uninfected control individuals remained healthy following metamorphosis; postmortem exams implicated chytridiomycosis as the cause of death. Infected tadpoles that were individually housed in the laboratory remained infected after metamorphosis, indicating that individuals maintain chytridiomycosis between the tadpole and post-metamorphic stages without contact with other infected individuals or any fungal source other than their own. Additionally, the results of our field surveys of infected and uninfected populations through time revealed a pattern of decline in infected populations that was congruent with our experimental results, characterized by post-metamorphic animals disappearing and larvae surviving. Thus, the results of all our studies suggest that chytridiomycosis is likely the

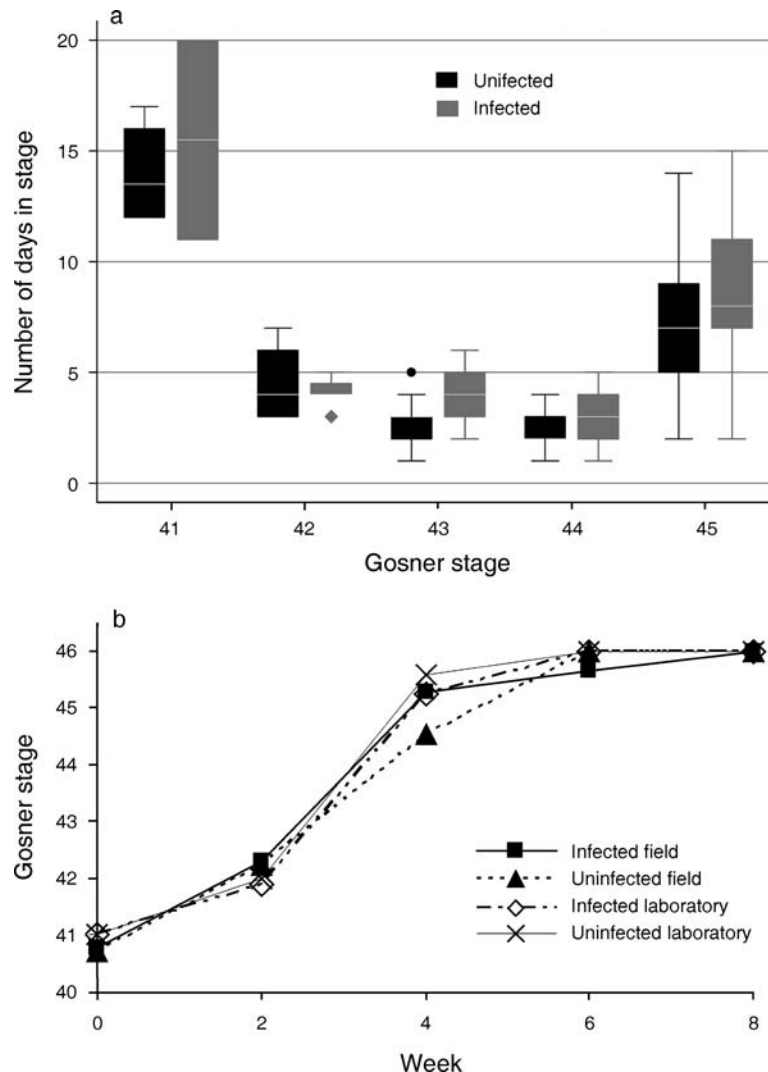


FIG. 3. (a) Number of days (mean \pm SE) *Rana muscosa* individuals from the naturally infected and experimentally infected laboratory studies combined remained in Gosner stages 41–45 in the absence and presence of *Batrachochytrium dendrobatidis* infection. (b) The mean Gosner stage of all individuals that metamorphosed in the field and laboratory studies in the absence and presence of *B. dendrobatidis* infection over eight weeks.

proximate cause of recently observed mass mortalities and is a major contributor to the ongoing population decline of *R. muscosa* in the Sierra Nevada. Our study contributes to the growing body of evidence demonstrating that infectious diseases can have major impacts on the population biology of wildlife (reviewed in Daszak et al. [2000]).

The pattern of amphibian decline and the population dynamics documented in *B. dendrobatidis*-infected *R. muscosa* populations have been observed in population decline episodes in numerous other amphibian species, such as in Australia and North and Central America where post-metamorphic individuals disappeared, tadpoles remained longer than adults, and populations were extirpated (Scott 1993, Berger et al. 1998, Lips 1999). Additionally, similar to the disease process seen in our

experiments with *R. muscosa*, chytridiomycosis epidemics in captive populations of other amphibian species is associated with infected tadpoles surviving to metamorphosis and then experiencing high mortality 1–3 weeks following metamorphosis (Berger et al. 1998, Mazzoni et al. 2003).

Our study is unique in that we demonstrate the negative impact of a pathogen on the population biology of a declining amphibian species by performing studies of individuals and populations in both the presence and absence of *B. dendrobatidis*, the causative pathogen, through time. We surveyed multiple populations of *R. muscosa* over a large spatial scale and over multiple years and performed field and laboratory experiments to quantify disease-induced mortality rates by following individuals through the disease process. Most previous

studies that implicate *B. dendrobatidis* as a proximate cause of amphibian mortality utilized the detection of *B. dendrobatidis* on deceased and dying amphibians as evidence for past declines (e.g., Berger et al. 1998, Bosch et al. 2001, Muths et al. 2003).

Our work with *R. muscosa* contributes to the growing body of evidence that species-level differences in susceptibility exist; some species are very susceptible in the wild and captivity, whereas others are not. For example, dendrobatid frogs appear to be susceptible to chytridiomycosis in experimental laboratory conditions (Nichols et al. 2001) as well as captive conditions at the National Park Zoo in Washington, D.C., USA, where they had been successfully reared for several years before a sporadic mortality event occurred due to chytridiomycosis (Longcore et al. 1999, Pessier et al. 1999). Bullfrogs (*Rana catesbeiana*) appear to be relatively resistant to the clinical aspects of *B. dendrobatidis* infection in the wild and also resistant in captivity, whereas dendrobatid frogs infected under identical experimental conditions were susceptible (Daszak et al. 2004). Additionally, captive *Xenopus* frogs show variability in susceptibility; *X. laevis* are known to have mild *B. dendrobatidis* infections yet no outbreaks or reports of clinical chytridiomycosis have been observed, whereas *X. tropicalis* succumb to the disease under similar conditions (Parker et al. 2002). Although our study showed that chytridiomycosis in *R. muscosa* populations in the southern Sierra Nevada nearly always resulted in host extirpation, other studies have reported persistence of amphibian populations in the wild despite *B. dendrobatidis* infection (Davidson et al. 2003, Daszak et al. 2004, Retallick et al. 2004, Weldon et al. 2004). For example, in *Taudactylus eungellensis*, a frog species that catastrophically declined in the mid-1980s, *B. dendrobatidis* persisted at stable, endemic levels from 1994 to 1998 in remnant populations (Retallick et al. 2004). In the northern Sierra Nevada, we have observed the persistence of a small number of infected *R. muscosa* populations (Briggs et al. 2005). Future monitoring of *R. muscosa* populations that have undergone severe declines in the Sierra Nevada should be conducted to assess the possibility of future frog-pathogen coexistence. Understanding the causes of between- and within-species variability in disease outcome is critical for the conservation of amphibian populations; variability in environmental and immune system factors have been proposed (Rollins-Smith et al. 2002, Lips et al. 2003b).

Our study shows the devastating effects and important implications of *B. dendrobatidis* infection on an already-threatened amphibian species. However, key aspects of the biology and effects of *B. dendrobatidis* that are crucial for conservation remain poorly understood. In particular, we do not yet fully understand the dispersal ability of *B. dendrobatidis*, an ability that has allowed this fungus to colonize even the most remote areas of the Sierra Nevada. The discovery of amphibian carriers (e.g., *Rana catesbeiana*, *Xenopus laevis*; Daszak

et al. 2004, Weldon et al. 2004) suggests that disease dispersal can occur not only by transmission from an infected individual to an individual of the same species, but also through contact between susceptible species and carrier species. Although *R. catesbeiana* and *X. laevis* are not present in the high elevation Sierra Nevada, these carriers have been introduced to California, including to Yosemite National Park, and may spread the fungus by introducing it to sympatric native species (Rachowicz et al. 2005). No evidence has been found for a nonaquatic or resting stage, although these have been found in other Chytridiomycete species (Longcore et al. 1999) and could play an important role in dispersal.

Additionally, we do not know definitively whether *B. dendrobatidis* has had a long association with amphibians worldwide or whether this broad geographic association is the result of the recent spread of the fungus. Although some evidence supports the hypothesis of a recent spread (Morehouse et al. 2003), this is an area of continued investigation both across the range of *R. muscosa* in the Sierra Nevada (J. A. T. Morgan and J. Taylor, unpublished data) and around the world. A better description of the historic and current range of this emerging infectious fungus would help address this key question (Rachowicz et al. 2005). An alternative to the recent spread hypothesis for the emergence of *B. dendrobatidis* implicates an environmental cofactor (e.g., chemical contaminants, climate change, increased UV-B radiation) that increased the susceptibility of amphibians to chytridiomycosis by compromising their immune system or changing host-pathogen dynamics (Daszak et al. 1999, Carey et al. 2003, Rachowicz et al. 2005). Our results suggest that *B. dendrobatidis* is capable of causing high mortality and population decline without the presence of cofactors because field mortality rates and disease progression were similar to those in the laboratory in which cofactors were not present (although captivity itself may be a stressful environment for amphibians, resulting in compromised immunity; Wright and Whitaker 2001). Additionally, our necropsies of infected *R. muscosa* did not find any additional opportunistic pathogens, which often signify immunosuppression (Carey et al. 2003). Similarly, necropsies of other species infected with *B. dendrobatidis* found a lack of evidence for immunosuppression (Berger et al. 1998).

The best documented cause of declines in *R. muscosa* until now was the introduction of nonnative fish into the Sierra Nevada. Fish introductions contributed to major declines in this species as a consequence of direct predation (Bradford 1989, Knapp and Matthews 2000, Vredenburg 2004, Knapp 2005) and habitat fragmentation (Bradford et al. 1993). Our results clearly indicate, however, that even those *R. muscosa* populations found in habitats that remain in their historic fishless condition are now being extirpated by *B. dendrobatidis*. We conclude that the remaining *R. muscosa* populations are seriously threatened with extirpation by previous fish introductions

and now chytridiomycosis, creating a highly uncertain future for this declining amphibian species.

ACKNOWLEDGMENTS

This work was supported by NIH grant R01 ES12067 to C. Briggs from the National Institute of Environmental Health Sciences as part of the NSF/NIH Ecology of Infectious Disease Program and by the following grants to R. Knapp: NSF grants DEB-9629473 and DEB-00754448, National Park Service Task Agreements 8550-002 and J8550-02-A003. R. Knapp thanks the many field assistants who helped conduct the amphibian and disease surveys, especially Peter Kirchner, Jesse Moore, Casey Ray, and Ericka Spies. L. Rachowicz thanks all the animal care and field assistants, especially Rosa Schneider and Tate Tunstall for their invaluable help. We also thank Craig Moritz, Rob Bingham, Martha Hoopes, and John Taylor for valuable discussions and comments; Wayne Getz, Wayne Sousa, and David Wake for helpful comments on a draft of this manuscript; Brian Stacy for supportive pathologic evaluations; Helen Diggs for veterinary oversight; Alex Hyatt for help with the real-time PCR assay; and Joyce Longcore for assistance in culturing *Batrachochytrium dendrobatidis*.

LITERATURE CITED

- Bell, B. D., S. Carver, N. J. Mitchell, and S. Pledger. 2004. The recent decline of a New Zealand endemic: How and why did populations of Archey's frog *Leiopelma archeyi* crash over 1996–2001? *Biological Conservation* **120**:189–199.
- Berger, L., et al. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences (USA)* **95**:9031–9036.
- Bosch, J., I. Martínez-Solano, and M. Garcia-Paris. 2001. Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biological Conservation* **97**:331–337.
- Boyle, D. G., D. B. Boyle, V. Olsen, J. A. T. Morgan, and A. D. Hyatt. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms* **60**:141–148.
- Bradford, D. F. 1984. Temperature modulation in a high-elevation amphibian, *Rana muscosa*. *Copeia* **1984**:966–976.
- Bradford, D. F. 1989. Allotopic distribution of native frogs and introduced fish in high Sierra Nevada lakes of California: implications of the negative impact of fish introductions. *Copeia* **1989**:775–778.
- Bradford, D. F., D. M. Graber, and F. Tabatabai. 1994. Population declines of the native frog, *Rana muscosa*, in Sequoia and Kings Canyon National Parks, California. *Southwestern Naturalist* **39**:323–327.
- Bradford, D. F., F. Tabatabai, and D. M. Graber. 1993. Isolation of remaining populations of the native frog, *Rana muscosa*, by introduced fishes in Sequoia and Kings Canyon National Parks, California. *Conservation Biology* **7**:882–888.
- Bradley, G. A., P. C. Rosen, M. J. Sredl, T. R. Jones, and J. E. Longcore. 2002. Chytridiomycosis in native Arizona frogs. *Journal of Wildlife Diseases* **38**:206–212.
- Briggs, C. J., V. T. Vredenburg, R. A. Knapp, and L. J. Rachowicz. 2005. Investigating the population-level effects of chytridiomycosis: an emerging infectious disease of amphibians. *Ecology* **86**:3149–3159.
- Carey, C., A. P. Pessier, and A. D. Peace. 2003. Pathogens, infectious disease, and immune defenses. Pages 127–136 in R. D. Semlitsch, editor. *Amphibian conservation*. Smithsonian Books, Washington, D.C., USA.
- Crump, M. L., and N. J. Scott. 1994. Visual encounter surveys. Pages 84–92 in W. R. Heyer, M. A. Donnelly, R. W. McDiarmid, L.-A. C. Hayek, and M. S. Foster, editors. *Measuring and monitoring biological diversity: standard methods for amphibians*. Smithsonian Institution Press, Washington, D.C., USA.
- Cunningham, A. A., and P. Daszak. 1998. Extinction of a species of land snail due to infection with a microsporidian parasite. *Conservation Biology* **12**:1139–1141.
- Daszak, P., L. Berger, A. A. Cunningham, A. D. Hyatt, D. E. Green, and R. Speare. 1999. Emerging infectious diseases and amphibian population declines. *Emerging Infectious Diseases* **5**:735–748.
- Daszak, P., and A. A. Cunningham. 1999. Extinction by infection. *Trends in Ecology and Evolution* **14**:279.
- Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2000. Emerging infectious diseases of wildlife—threats to biodiversity and human health. *Science* **287**:443–449.
- Daszak, P., A. Striemy, A. A. Cunningham, J. E. Longcore, C. C. Brown, and D. Porter. 2004. Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. *Herpetological Journal* **14**:201–207.
- Davidson, E. W., M. Parris, J. P. Collins, J. E. Longcore, A. P. Pessier, and J. Brunner. 2003. Pathogenicity and transmission of chytridiomycosis in tiger salamanders (*Ambystoma tigrinum*). *Copeia* **2003**:601–607.
- de Castro, F., and B. Bolker. 2005. Mechanisms of disease-induced extinction. *Ecology Letters* **8**:117–126.
- Drost, C. A., and G. M. Fellers. 1996. Collapse of a regional frog fauna in the Yosemite area of the California Sierra Nevada, USA. *Conservation Biology* **10**:414–425.
- Fellers, G. M., D. E. Green, and J. E. Longcore. 2001. Oral chytridiomycosis in the mountain yellow-legged frog (*Rana muscosa*). *Copeia* **2001**:945–953.
- Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* **16**:183–190.
- Green, D. E., and C. Kagarise-Sherman. 2001. Diagnostic histological findings in Yosemite toads (*Bufo canorus*) from a die-off in the 1970s. *Journal of Herpetology* **35**:92–103.
- Hopkins, S., and A. Channing. 2003. Chytrid fungus in Northern and Western Cape frog populations, South Africa. *Herpetological Review* **34**:334–336.
- Hudson, P. J., A. P. Dobson, and D. Newborn. 1998. Prevention of population cycles by parasite removal. *Science* **282**:2256–2258.
- Jennings, M. R. 1996. Status of amphibians. Pages 921–944 in *Sierra Nevada ecosystem project: final report to Congress*. University of California, Centers for Water and Wildland Resources, Davis, California, USA.
- Johnson, M. L., L. Berger, L. Phillips, and R. Speare. 2003. Fungicidal effects of chemical disinfectants, UV light, desiccation and heat on the amphibian chytrid *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms* **57**:255–260.
- Knapp, R. A. 2005. Effects of nonnative fish and habitat characteristics on lentic herpetofauna in Yosemite National Park, USA. *Biological Conservation* **121**:265–279.
- Knapp, R. A., and K. R. Matthews. 2000. Non-native fish introductions and the decline of the mountain yellow-legged frog from within protected areas. *Conservation Biology* **14**:428–438.
- Knapp, R. A., and J. A. T. Morgan. 2006. Tadpole mouthpart pigmentation as an accurate indicator of chytridiomycosis, an emerging disease of amphibians. *Copeia* **2006**:188–197.
- La Marca, E., et al. 2005. Catastrophic population declines and extinctions in Neotropical harlequin frogs (Bufonidae: *Ateolopus*). *Biotropica* **37**:190–201.
- Lips, K. R. 1999. Mass mortality and population declines of anurans at an upland site in western Panama. *Conservation Biology* **13**:117–125.

- Lips, K. R., D. E. Green, and R. Papendick. 2003a. Chytridiomycosis in wild frogs from southern Costa Rica. *Journal of Herpetology* **37**:215–218.
- Lips, K. R., J. R. Mendelson, III, A. Munoz-Alonso, L. Canseco-Marquez, and D. G. Mulcahy. 2004. Amphibian population declines in montane southern Mexico: resurveys of historical localities. *Biological Conservation* **119**:555–564.
- Lips, K. R., J. D. Reeve, and L. R. Witters. 2003b. Ecological traits predicting amphibian population declines in central America. *Conservation Biology* **17**:1078–1088.
- Longcore, J. E., A. P. Pessier, and D. K. Nichols. 1999. *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia* **91**:219–227.
- Mazzoni, R., A. A. Cunningham, P. Daszak, A. Apolo, E. Perdomo, and G. Speranza. 2003. Emerging pathogen of wild amphibians in frogs (*Rana catesbeiana*) farmed for international trade. *Emerging Infectious Diseases* **9**:995–998.
- Morehouse, E. A., T. Y. James, A. R. D. Ganley, R. Vilgalys, L. Berger, P. J. Murphy, and J. E. Longcore. 2003. Multilocus sequence typing suggests the chytrid pathogen of amphibians is a recently emerged clone. *Molecular Ecology* **12**:395–403.
- Muths, E., P. S. Corn, A. P. Pessier, and D. E. Green. 2003. Evidence for disease-related amphibian decline in Colorado. *Biological Conservation* **110**:357–365.
- Nichols, D. K., E. W. Lamirande, A. P. Pessier, and J. E. Longcore. 2001. Experimental transmission of cutaneous chytridiomycosis in dendrobatid frogs. *Journal of Wildlife Diseases* **37**:1–11.
- Parker, J. M., I. Mikaelian, N. Hana, and H. E. Diggs. 2002. Clinical diagnosis and treatment of epidermal chytridiomycosis in African clawed frogs (*Xenopus tropicalis*). *Comparative Medicine* **52**:265–268.
- Pessier, A. P., D. K. Nichols, J. E. Longcore, and M. S. Fuller. 1999. Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp.) and White's tree frogs (*Litoria caerulea*). *Journal of Veterinary Diagnostic Investigation* **11**:194–199.
- Rachowicz, L. J. 2002. Mouthpart pigmentation in *Rana muscosa* tadpoles: seasonal changes without chytridiomycosis. *Herpetological Review* **33**:263–265.
- Rachowicz, L. J., J.-M. Hero, R. A. Alford, J. W. Taylor, J. A. T. Morgan, V. T. Vredenburg, J. P. Collins, and C. J. Briggs. 2005. The novel and endemic pathogen hypotheses: competing explanations for the origin of emerging infectious diseases of wildlife. *Conservation Biology* **19**:1441–1448.
- Rachowicz, L. J., and V. T. Vredenburg. 2004. Transmission of *Batrachochytrium dendrobatidis* within and between amphibian life stages. *Diseases of Aquatic Organisms* **61**:75–83.
- Retallick, R. W. R., H. McCallum, and R. Speare. 2004. Endemic infection of the amphibian chytrid fungus in a frog community post-decline. *PLOS Biology* **2**:e351. [Online, doi: 310.1371/journal.pbio.0020351.]
- Rollins-Smith, L. A., J. K. Doersam, J. E. Longcore, S. K. Taylor, J. C. Shamblin, C. Carey, and M. A. Zasloff. 2002. Antimicrobial peptide defenses against pathogens associated with global amphibian declines. *Developmental and Comparative Immunology* **26**:63–72.
- Ron, S. R., W. E. Duellman, L. A. Coloma, and M. R. Bustamante. 2003. Population decline of the Jambato Toad *Atelopus ignescens* (Anura: Bufonidae) in the Andes of Ecuador. *Journal of Herpetology* **37**:116–126.
- Scott, N. J. 1993. Postmetamorphic death syndrome. *Froglog* **7**:1–2.
- Vredenburg, V. T. 2004. Reversing introduced species effects: experimental removal of introduced fish leads to rapid recovery of a declining frog. *Proceedings of the National Academy of Sciences (USA)* **101**:7646–7650.
- Weldon, K. M., L. H. du Preez, A. D. Hyatt, R. Muller, and R. Speare. 2004. Origin of the amphibian chytrid fungus. *Emerging Infectious Diseases* **10**:2100–2105.
- Wright, K. M., and B. R. Whitaker. 2001. Amphibian medicine and captive husbandry. Krieger, Malabar, Florida, USA.
- Zweifel, R. G. 1955. Ecology, distribution and systematics of frogs of the *Rana boylii* group. University of California Publications in Zoology **54**:207–292.

APPENDIX A

A description of the reliability of jaw sheath depigmentation as an indicator of chytridiomycosis (*Ecological Archives* E087-096-A1).

APPENDIX B

A description of standard and real-time polymerase chain reaction assays for *Batrachochytrium dendrobatidis* (*Ecological Archives* E087-096-A2).