The ISME Journal (2009) 3, 818–824
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www.nature.com/ismej

ORIGINAL ARTICLE

Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus

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Emerging infectious diseases threaten human and wildlife populations. Altered ecological interactions between mutualistic microbes and hosts can result in disease, but an understanding of interactions between host, microbes and disease-causing organisms may lead to management strategies to affect disease outcomes. Many amphibian species in relatively pristine habitats are experiencing dramatic population declines and extinctions due to the skin disease chytridiomycosis, which is caused by the chytrid fungus *Batrachochytrium dendrobatidis*. Using a randomized, replicated experiment, we show that adding an antifungal bacterial species, *Janthinobacterium lividum*, found on several species of amphibians to the skins of the frog *Rana muscosa* prevented morbidity and mortality caused by the pathogen. The bacterial species produces the anti-chytrid metabolite violacein, which was found in much higher concentrations on frog skins in the treatments where *J. lividum* was added. Our results show that cutaneous microbes are a part of amphibians' innate immune system, the microbial community structure on frog skins is a determinant of disease outcome and altering microbial interactions on frog skins can prevent a lethal disease outcome. A bioaugmentation strategy may be an effective management tool to control chytridiomycosis in amphibian survival assurance colonies and in nature.

The ISME Journal (2009) **3,** 818–824; doi:10.1038/ismej.2009.27; published online 26 March 2009
Subject Category: microbe–microbe and microbe–host interactions
Keywords: *Batrachochytrium dendrobatidis*; bioaugmentation; chytridiomycosis; *Rana muscosa*; violacein

Introduction

Infectious diseases are emerging at an increasing rate (Daszak *et al.*, 2000). Some of these diseases pose a direct threat to humans, such as HIV, whereas diseases of wildlife threaten global biodiversity and therefore indirectly affect human welfare. One reason that infectious diseases are emerging at a more rapid rate is that ecological relationships have been altered in ways that favor disease transmission (Daszak *et al.*, 2000; Keesing *et al.*, 2006). For example, changes in the geographic distribution of pathogens and hosts can lead to disease outbreaks, as has occurred with West Nile virus (Allan *et al.*, 2009). Alteration of the ecological structure of communities is also implicated in disease outbreaks. For example, the incidence of the bacterially caused Lyme disease in the human population increased when the relative abundance of alternative hosts declined (LoGiudice *et al.*, 2003) and it may depend on the community structure of competitive microbes within tick vectors (Clay *et al.*, 2006).

The community structure of microbes on and within hosts is associated with resistance to disease (Dethlefsen *et al.*, 2007). For example, antifungal microbes can be important in disease resistance in a variety of species (Gil-Turnes *et al.*, 1989; Gil-Turnes and Fenical, 1992; Currie *et al.*, 1999; Kaltenpoth *et al.*, 2005; Scarborough *et al.*, 2005; Scott *et al.*, 2008). Disruption of antifungal microbial communities is likely to lead to a breakdown of the protective effects of beneficial microbes and may

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Received 27 January 2009; revised 23 February 2009; accepted 23 February 2009; published online 26 March 2009

lead to disease emergence (Dethlefsen *et al.*, 2007; Belden and Harris, 2007). On a more positive note, methods to restore protective microbial communities may provide a way to manage wildlife diseases in nature.

An emerging infectious disease, chytridiomycosis, is a major factor responsible for extinction of amphibian species, one-third of which are threatened with extinction (Stuart *et al.*, 2004; Lips *et al.*, 2006; Skerratt *et al.*, 2007). Caused by the chytrid species *Batrachochytrium dendrobatidis* (Bd), this skin disease is linked to population declines and extinctions of over 200 amphibian species in areas unaffected by habitat loss (Longcore *et al.*, 1999; Skerratt *et al.*, 2007). Since its description in 1999, we have learned a considerable amount about the pathogen's life history, physiology, population genetics, its responses to host immunity and how it causes death (Morehouse *et al.*, 2003; Piotrowski *et al.*, 2004; Berger *et al.*, 2005; Rollins-Smith and Conlon, 2005; Morgan *et al.*, 2007; Voyles *et al.*, 2007; Woodhams *et al.*, 2007a, b, 2008). Much less is known about how to control or manage the disease in nature. We propose that community interactions between amphibians' skin microbes and Bd can be manipulated in a way to enhance disease resistance in the sense of reducing the impact of the disease.

We have identified a number of cutaneous bacteria of amphibians that inhibit Bd *in vitro* and are likely to be a part of amphibians' innate immune system (Harris *et al.*, 2006; Woodhams *et al.*, 2007b). In addition, using a 'bacterial removal' experiment, we have shown that the naturally occurring cutaneous microbial community on red-backed salamanders, *Plethodon cinereus*, is a determinant of disease outcome (Becker and Harris, under review). In the current study, we establish a model for the use of microbes to protect individual amphibians against chytridiomycosis. We have previously shown that amphibian skin microbes produce antifungal metabolites that inhibit Bd (Brucker *et al.*, 2008a, b). The bacterial species used in this study, *Janthinobacterium lividum*, produces the antifungal and anti-Bd metabolite violacein (Brucker *et al.*, 2008b). The amphibian study species used was the mountain yellow-legged frog, *Rana muscosa*, from high elevation ponds in the Sierra Nevada mountains. Chytridiomycosis has caused population declines and extinctions of many populations of this frog species (Rachowicz *et al.*, 2006). When Bd arrives in some populations, it causes near-total mortality of the population. However, populations that persist with the pathogen have a higher proportion of individuals with culturable antifungal bacterial isolates than populations that decline once Bd arrives (Woodhams *et al.*, 2007b). Using a randomized and replicated experiment, we added an anti-Bd bacterial species, *J. lividum*, to the skins of *R. muscosa*, which resulted in reduced morbidity and mortality associated with chytridiomycosis.

Materials and methods

Thirty-one *R. muscosa* juveniles were obtained from a laboratory colony, raised from field-collected eggs, at the University of California, Berkeley. Each frog was placed into its own autoclaved plastic container containing approximately 200 ml of Provosoli medium (Wyngaard and Chinnappa, 1982) and was randomly assigned a position on metal racks in a temperature-controlled room set at 17 °C with a 12 h light cycle. Containers were cleaned with 10% bleach and autoclaved twice a week. Each frog was fed five crickets weekly. Animal care protocols were approved by the University of California, Berkeley's and James Madison University's Animal Care and Use Committee.

We performed a replicated, randomized experiment with three groups of *R. muscosa*: (1) Bd, exposure to Bd zoospores; (2) bacteria, exposure to *J. lividum* and (3) Bacteria + Bd, exposure to both *J. lividum* and Bd zoospores. Eighteen frogs were used in this experiment with the sample size for each treatment group being six frogs, with each frog in a separate container. There was no difference in mass among the three treatment groups at the beginning of the experiment (analysis of variance (ANOVA): d.f. = 2.15, F = 0.943, $P = 0.411$). A preliminary experiment with another 10 juvenile *R. muscosa* was conducted to obtain a growth rate under standard laboratory conditions. This estimate was used as a comparison for growth rates estimated from the 18 frogs in the three manipulated treatment groups. Three other juveniles were used to estimate the concentration of violacein on individuals that were not subjected to any experimental manipulations.

On 22 January 2008, 18 individuals were treated with a 25 ml solution of 3% hydrogen peroxide for 45 s and immediately rinsed with sterile Provosoli medium before returning to new containers. This procedure reduces the number of bacterial species on the skin, thereby minimizing community interactions between *J. lividum* and other cutaneous bacterial species. Therefore, we could standardize initial conditions on the frogs' skins and more readily attribute treatment effects to the presence or absence of *J. lividum*. After a period of 24 h, each frog in the Bacteria and the Bacteria + Bd treatments was inoculated with *J. lividum* in individual containers. The *J. lividum* strain used was obtained from the skin of the salamander *Hemidactylium scutatum* collected in the George Washington National Forest, Rockingham Co., VA, USA. This strain was chosen because its anti-Bd metabolites violacein and indole-3-carboxaldehyde have been characterized (Brucker *et al.*, 2008b). A pure culture of *J. lividum* was incubated for 72 h at room temperature in 1% tryptone while being continuously stirred. The culture was then centrifuged at 4500 *g* for 10 min. The supernatant was discarded and the pellet was resuspended in sterile Provosoli

medium. The resulting solution was diluted to obtain a concentration of 26.25×10^6 cells per ml. Frogs were placed in autoclaved 120 ml GladWare containers (Glad Products Co., Oakland, CA, USA) with 15 ml of the bacterial solution for 30 min. During this time, frogs in the Bd treatment were exposed to medium alone. Individuals were then placed into sterile containers for 48 h to allow *J. lividum* to establish on the skin of the inoculated frogs.

At this time, individuals in the Bd and the Bacteria + Bd treatments were exposed to Bd zoospores. This day was considered day 1 of the experiment. Immediately before the exposure, all frogs were weighed. Frogs were individually placed in 120 ml GladWare containers with 300 zoospores suspended in 15 ml of Provosoli medium for 24 h. Alternatively, frogs in the Bacteria treatment were exposed to medium alone. The Bd strain JEL 215 isolated from *R. muscosa* was used for exposure. Cultures of the isolate were maintained in 1% tryptone and transferred weekly. The zoospore solution used to inoculate the frogs was prepared by growing zoospores on plates containing 1% tryptone and 1% agar. After a period of approximately 72 h, the plates were flooded with 6 ml of Provosoli medium. After 20 min, the remaining solution was removed by pipette. The zoospore concentration was determined by a hemacytometer and diluted to 20 zoospores per ml.

After Bd exposure and the initial weight measurement, frogs were weighed approximately every 2 weeks. Sampling for *J. lividum* and for Bd was completed on 29 January 2008 and approximately every 2 weeks after this date. After rinsing each individual in sterile Provosoli medium twice, frogs were swabbed 10 times on their ventral surface with a rayon swab (Medical Wire and Equipment, Corsham, Wiltshire, UK) on the dorsal surface of the abdomen, legs and feet to detect Bd presence and to estimate abundance. The same procedure was carried out to assay for the presence of *J. lividum*. Swabs were immediately frozen at −20 °C until further processing occurred. Swabs from 22, 61 and 139 days after exposure to Bd were sent to Pisces Molecular (Boulder, CO, USA) for DNA extraction and for an estimate of Bd abundance using TaqMan PCR. Swabs from days 5, 19, 47 and 139 were assayed for the presence of *J. lividum* using PCR. DNA was extracted from the swabs using a Qiagen DNeasy Blood and Tissue kit (Germantown, MD, USA) with the manufacturer's protocol. DNA obtained from the swabs was amplified using the *J. lividum*-specific primers JlivF (5′-TACCACGAATTGCTGTGCCAGTTG-3′) and JlivR (5′-ACACG CTCCAGGTATACGTCTTCA-3′). These primers were designed using the *J. lividum* strain SKVTC8 violacein pigment gene obtained from GenBank (accession no. EU732703.1). PCR reactions (35 μl) contained 0.2 μM of each primer, 0.2 mM dNTPs, 2.5 U Taq polymerase with 1 × buffer (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris; Fisher Scientific, Pittsburg, PA, USA). The amplification conditions were as follows: 4 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 60 s at 60 °C and 2 min at 72 °C. The amplification was completed with a final extension for 10 min at 72 °C. DNA from a pure culture of *J. lividum*, which was the same strain used in the experiment, was extracted with a MoBio Microbial Ultra Clean DNA kit (Carlsbad, CA, USA) and amplified along with extracted swabs to act as a positive control. The amplification products were separated by electrophoresis through 2% agarose and stained with ethidium bromide (5 μg ml^{-1}). Presence or absence of a band around 500 bp was determined for each swab using ultraviolet light. On the 140th day of the experiment (12 June 2008), frogs were killed with carbon dioxide. The concentration of violacein on the skin was determined according to the method of Brucker *et al*. (2008b).

Differences in means were tested with ANOVA unless data were not normally distributed, in which case a nonparametric Wilcoxon test was used. A repeated-measures ANOVA was used to test for differences in the number of zoospores over time as a function of treatment. One value for zoospore equivalents in the Bacteria treatment was an extreme outlier (Dixon's test: $r_{10} = 1.0$, $n = 6$, $P < 0.01$) and was not considered in the analysis. Frequency differences were tested with Fisher's exact test, and survival differences were tested with a Mantel–Cox log-rank test.

Results

Application of anti-Bd bacteria to uninfected frogs

Placing frogs into a solution with *J. lividum* led to successful colonization of the skin. Primers specific to *J. lividum* indicated that all individuals in the experiment had *J. lividum* on their skins, whether or not they were exposed to a bath of this bacterial species. However, the metabolite produced by *J. lividum*, violacein, was found only on the frogs bathed in the *J. lividum* solution (see below), whereas frogs not treated had no detectable violacein on their skins. This result suggests that bathing in *J. lividum* increased population densities to the point where the secondary metabolite violacein was produced.

Survival

Adding *J. lividum* to uninfected frogs prevented lethal effects of the disease (Figure 1a). Five of six frogs in the Bd treatment died, and the sixth frog displayed symptoms of advanced-stage chytridiomycosis. No mortality occurred in the Bacteria and the Bacteria + Bd treatments (Mantel–Cox log-rank test: d.f. = 2, $\chi^2 = 15.877$, $P = 0.0004$).

Change in weight

Frogs in the Bd treatment did not grow and lost some weight on average (Figure 1b). Frogs that were

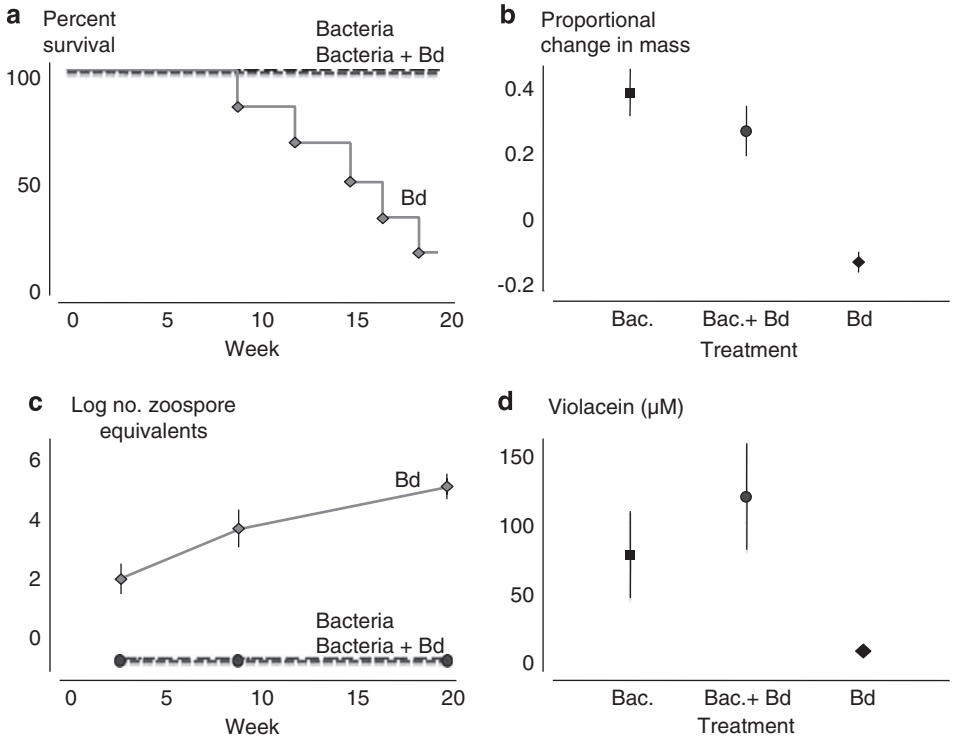

Figure 1 The effect of treatment group on (**a**) survival, (**b**) proportional growth of frogs, (**c**) the mean number of zoospore equivalents of *B. dendrobatidis* and (**d**) violacein concentration on frog skins. Error bars represent + 1 s.e. (black, Bacteria treatment; green, Bacteria + Bd treatment; orange, Bd treatment). A full colour version of this figure is available at *The ISME Journal* online.

inoculated with *J. lividum* or that were inoculated with *J. lividum* before exposure to Bd grew and gained about 33% of body mass (ANOVA: d.f. = 2.15, F = 17.71, $P = 0.0001$). Frogs in these later two treatments had a daily growth rate that was the same as the growth rate of 10 unmanipulated frogs in a preliminary experiment (see Materials and methods, ANOVA: d.f. = 2.19, F = 0.81, $P = 0.461$), suggesting that bacteria addition alone did not affect growth rate and that bacterial addition before exposure to Bd prevented weight loss that was associated with Bd infection.

Bd abundance

The number of zoospore equivalents measured by qPCR is an index of Bd abundance. Zoospore equivalents on frogs in the Bd treatment increased exponentially as the experiment progressed (Figure 1c). Frogs in the Bacteria and the Bacteria + Bd treatments had no detectable zoospore equivalents on their skins from the day of the first sample (day 19) until our last sample (day 139), which was a dramatic difference from the Bd treatment (repeated-measures ANOVA for the treatment × time interaction: d.f. = 4.28, Wilks' $\lambda = 0.179$, $P = 0.0001$). Furthermore, the bacteria added to frog skins in the Bacteria + Bd treatment prevented successful establishment by Bd zoospores (Kruskal–Wallis test—day 19: d.f. = 2, $\chi^2 = 12.645$, $P = 0.013$; day 62: d.f. = 2, $\chi^2 = 16.129$, $P = 0.0003$ and day 139: d.f. = 2, $\chi^2 = 16.129$, $P = 0.0003$).

Violacein

Survival of frogs was strongly associated with presence of violacein, an anti-Bd metabolite produced by *J. lividum* (Figure 1d). The five frogs that died in the Bd treatment had no detectable violacein concentration on their skin. The frog that survived in the Bd treatment did not have detectable quantities of violacein (< 5 μM); however, its weight loss, zoospore load and lethargic behavior indicated serious Bd infection. The mean violacein concentrations were much higher on frog skins in the Bacteria and the Bacteria + Bd treatments than in the Bd treatment (Figure 1d; Kruskal–Wallis test: d.f. = 2, $\chi^2 = 8.727$, $P = 0.018$). The three juveniles that were not exposed to *J. lividum* or Bd had no detectable violacein concentration on their skins.

Discussion

We found that morbidity and mortality caused by Bd were prevented by bioaugmentation of frog skins with *J. lividum* and conclude that ecological interactions between resident microbes and colonizing pathogens are an important component of their innate immunity. There is an increasing awareness

that in many species microbial community composition of a host individual can determine disease outcome once a pathogen colonizes (Belden and Harris, 2007; Dethlefsen *et al.*, 2007; Ostfeld *et al.*, 2008). In addition, microbial interactions in disease vectors can affect pathogen prevalence and disease (Clay *et al.*, 2006). It is becoming clear that to understand disease dynamics, the interactions between the host, its resident microbes and colonizing pathogens need to be characterized.

In this experiment, we exposed juvenile frogs to a low concentration of Bd zoospores relative to most other investigators (Rachowicz *et al.*, 2006; Retallick and Miera, 2007; Woodhams *et al.*, 2007a). However, the concentration of zoospores we used was higher than that estimated from pond water in nature (Kirshtein *et al.*, 2007; Walker *et al.*, 2007). For example, Kirshtein *et al.*, 2007 and Walker *et al.*, 2007 estimated that zoospore density in ponds varied from 0.5 to 454 zoospores per liter, whereas our exposure protocol was 20 000 zoospores per liter for 24 h. A sampling of recent experimental papers reveals that individual amphibians were exposed to Bd densities in their experimental containers that ranged from 25 000 zoospores per liter (Rachowicz and Vredenburg, 2004) to 10^5–10^6 zoospores per liter (Rachowicz *et al.*, 2006; Retallick and Miera, 2007; Woodhams *et al.*, 2007a). Further experimentation that varies level and duration of exposure to Bd zoospores in relation to population densities of anti-Bd bacteria is needed to determine under what conditions the cutaneous microbiota are protective.

Our experiment tested whether bacterial residents from one species can effectively colonize another species. We used *J. lividum* isolated from a salamander species to inoculate juvenile frogs used in this experiment. We have found *J. lividum* on *R. muscosa*, suggesting that *J. lividum* is a resident microbe and that *J. lividum* from another amphibian species can be used to augment its density (Woodhams *et al.*, 2007b). In addition, we had completed a study of anti-Bd metabolites produced by this strain, so the reduction in morbidity and mortality caused by Bd can be linked to its metabolites (Brucker *et al.*, 2008b). Our results suggest that we will have the potential to target the broader susceptible amphibian community if bioaugmentation as a way to control Bd moves from the laboratory to field environments.

The metabolite violacein inhibits Bd at a low concentration *in vitro* (Brucker *et al.*, 2008b) and is produced by *J. lividum* and not by amphibians. This metabolite is produced by other bacterial species as well and has a protective function (Yang *et al.*, 2007; Matz *et al.*, 2008). Analysis of violacein on wild caught salamanders has shown concentrations on skins high enough to inhibit Bd (Brucker *et al.*, 2008b). Because bacteria generally do not produce antifungal secondary metabolites such as violacein until population densities are high, we suggest that in our experiment *J. lividum* was present at high population densities on frog skins. Amphibians may be able to regulate microbial community structure on their skins by their secretion of antimicrobial peptides, which are produced in granular glands (Rollins-Smith and Conlon, 2005). It is a question for future research to determine if this regulation occurs and if it acts in a way to increase selectively the growth of skin bacterial strains that produce antibiotic metabolites when the amphibian is exposed to skin pathogens.

Our results suggest that bioaugmentation with suitable anti-Bd bacteria can be used to prevent Bd infections in survival assurance or captive breeding colonies of amphibian species. These colonies are an important component of conservation efforts, especially if individuals can be re-introduced into the wild. A barrier to re-introduction is that invariably one or more amphibian species in their natural habitats are resistant to Bd and serve as reservoirs (Mendelson *et al.*, 2006; Young *et al.*, 2007). Treatment of susceptible amphibian species with antifungal skin bacteria may allow re-introduced individuals to coexist with Bd in native habitats. In this context, it is important to estimate how long bacterial treatments work after release of treated amphibians into the wild. We found violacein on frogs at the end of the experiment 20 weeks after bacterial inoculation, which suggests that the effects of bioaugmentation last at least that long.

Amphibians as a group are facing large population declines and extinctions due to chytridiomycosis, which makes research into management and prevention a high priority for amphibian conservation biologists. Our results demonstrate that an understanding of the community ecology of amphibian skins is a key component of attempts to control this emerging infectious disease. A simple manipulation of amphibians' cutaneous microbial community is a promising management tool to treat infected individuals. In addition, previous work has shown that populations of *R. muscosa* that coexist with Bd had a higher proportion of individuals with at least one anti-Bd isolate of skin bacteria (Woodhams *et al.*, 2007b; unpublished data). Treatment of individual frogs may allow populations to achieve a higher proportion of individuals with protective bacteria and therefore allow coexistence with the pathogen.

There are a number of questions for future research that should be addressed before bioaugmentation is considered in natural settings. One important question is whether bioaugmentation is effective without first reducing frogs' cutaneous microbiota. We have shown in another laboratory study that bioaugmentation of salamander skins without first reducing resident microbiota did ameliorate the symptoms of chytridiomycosis, which is encouraging (Harris *et al.*, 2009). A bioaugmentation protocol in nature has the potential to be self-disseminating after initial treatment. However, it is critical to understand any effects bioaugmentation might have on nontarget

species. One way to minimize such effects is to add skin bacteria already found on at least some frogs in the population to increase the proportion of individuals with anti-Bd bacteria. Indeed, we have found *J. lividum* on *R. muscosa* (Woodhams *et al.*, 2007b). We note that bacterial additions in agricultural contexts are effective in controlling disease and do not negatively affect nontarget species (Berg *et al.*, 2007; Scherwinski *et al.*, 2008), which is encouraging. Whether to adopt a bioaugmentation strategy to control chytridiomycosis in nature presents a challenging ecological dilemma. The decision-making process can benefit from the framework of ecological ethics (Minteer and Collins, 2008), which balances the duties and risks to various entities including amphibians, ecosystems and public welfare. In sum, additional research on the relationship of amphibians' skin microbes and Bd is urgently needed to assess the efficacy of bioaugmentation as a control strategy for Bd.

Acknowledgements

We thank G Wyngaard and J Kastendiek for valuable comments on the paper, J Wood for PCR analysis and M Toothman for technical support. Permits to collect were provided by the California Department of Fish and Game and the National Park Service. Animal care protocols were approved by UC Berkeley's and JMU's Animal Care and Use Committee. This work was supported by a Research Corporation Cottrell College Science Award (KPCM), the Thomas F Jeffress and Kate Miller Jeffress Memorial Trust (KPCM), a National Science Foundation grant 0640373 (RNH) and James Madison University.

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