



## Short communication

Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*Brianna A. Lam<sup>a,\*</sup>, Jenifer B. Walke<sup>b</sup>, Vance T. Vredenburg<sup>c</sup>, Reid N. Harris<sup>a</sup><sup>a</sup> Department of Biology, James Madison University, MSC 7801, Harrisonburg, VA 22807, USA<sup>b</sup> Department of Biological Sciences, Virginia Tech, MC 0406, Blacksburg, VA 24061, USA<sup>c</sup> Department of Biological Sciences, San Francisco State University, 1600 Holloway Avenue, San Francisco, CA 94132, USA

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## ABSTRACT

The emergence of the infectious disease chytridiomycosis is a major factor responsible for amphibian extinctions in pristine habitats. However, some populations coexist with *Batrachochytrium dendrobatidis* (Bd), the agent of chytridiomycosis, and others go extinct when Bd arrives. Variation of pathogen severity among populations may be explained by differences of antimicrobial skin peptides and anti-Bd skin bacteria. Previous work showed that a population of the frog *Rana sierrae* had a high proportion of individuals with at least one species of cultured anti-Bd skin bacteria and was coexisting with Bd for more than 6 years. A population of the closely related sister species *Rana muscosa* had a significantly lower proportion of individuals with anti-Bd bacteria, and the following year, it went extinct due to Bd. We extended previous work to include another sampling of the *R. sierrae* population coexisting with Bd and found that, although the anti-Bd bacterial community somewhat differed, both populations had a high proportion of individuals with antifungal bacteria. We also included a population of *R. muscosa* that was naïve of Bd and predicted to go extinct once Bd emerged since that was the fate of neighboring populations. However, the naïve *R. muscosa* population had a high proportion of antifungal bacteria, which suggested that the population might survive if Bd emerged. Two years later, the population was surviving with Bd endemic. Variation in the proportion of individuals with anti-Bd skin bacteria appears to explain why some populations persist when Bd emerges in a population.

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## 1. Introduction

Global amphibian declines are a part of the current biodiversity crisis, and although there are many explanations for amphibian declines, the emergence of the infectious disease chytridiomycosis is the major cause of enigmatic declines in areas unaffected by habitat loss (Skerratt et al., 2007). These areas are often federally protected land and are in remote locations where human activity is generally limited (Skerratt et al., 2007). The cause of chytridiomycosis is a chytrid fungus, *Batrachochytrium dendrobatidis* (Bd) (Longcore et al., 1999). Interestingly, some species of amphibians coexist with Bd whereas others succumb to disease (Davidson et al., 2003; Hanselmann et al., 2004; Retallick et al., 2004). Even within species, some populations can coexist with Bd whereas others decline to extinction (Briggs et al., 2005). Amphibians' immune defenses contribute to disease resistance and coexistence with Bd. These defenses may include adaptive and innate responses (Richmond et al., 2009). Innate defenses include antimicrobial peptides and symbiotic skin bacteria, both of which have been found to in-

hibit Bd growth *in vitro* (Rollins-Smith et al., 2006; Harris et al., 2006; Woodhams et al., 2006a,b). In addition the skin bacterial species, *Janthinobacterium lividum*, was shown to protect the frog *Rana muscosa* from the lethal disease if added to frog skins prior to Bd exposure (Harris et al., 2009).

The mountain yellow-legged frog (*R. muscosa*) is native to the Sierra Nevada mountains of California. Recent population declines have been attributed to Bd (Briggs et al., 2005), however, populations of *Rana sierrae* (*R. muscosa*'s sister species, Vredenburg et al., 2007) have been found to persist and coexist with Bd (Woodhams et al., 2007). Field work performed in 2005 (Woodhams et al., 2007) surveyed two frog populations: one population of *R. sierrae* (referred to as *R. muscosa* in that paper because Vredenburg's taxonomic revision was not completed) that was coexisting with Bd, referred to as "Conness Pond," and a population of *R. muscosa* that was declining due to the recent emergence of Bd, referred to as "Sixty Lake". They found that the population coexisting at Conness had a significantly higher proportion of individuals with at least one cultured anti-Bd bacteria species than the population declining at Sixty Lake. The following year the population at Sixty Lake went extinct.

We extend the Woodhams et al. (2007) study with a microbiological survey of a Bd-naïve population of *R. muscosa* from Sixty

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Lake Basin, referred to as “Lake 52”, which was predicted to decline because virtually all populations in that area did so once Bd emerged. We also re-surveyed the coexisting population of *R. sierrae* at Conness Pond to test the hypothesis that the population at Conness continues to be characterized by a higher proportion of individuals with anti-Bd skin bacteria.

## 2. Methods

### 2.1. Study species and site

We sampled adult frogs from two populations in 2006. These populations included a population of *R. muscosa* at Lake 52 in Sixty Lakes Basin, Kings Canyon National Park, California and a population of *R. sierrae* at Conness in Yosemite National Park, California. Populations were in similar high alpine habitats and presumably experienced similar environmental conditions. Using a hand net, we sampled 19 adults from Lake 52 in fall 2006 and 20 adults from Conness Pond also in fall 2006. At the time of sampling, Conness had been coexisting with Bd for longer than 6 years. At the time of sampling, the Lake 52 was Bd free, but the emergence of Bd was predicted to occur the following year based on the pattern of spread of the disease in the Sixty Lake Basin (unpublished data). The frog population was expected to decline because large population declines occurred in other populations in this area once Bd arrived. In 2007, Bd emerged at Lake 52.

### 2.2. Culture and identification of bacteria from frog skin

After capture, frogs were rinsed twice in sterile water to remove transient bacteria (Lauer et al., 2007). Collection and isolation of bacteria from frog skins was performed as previously published (Woodhams et al., 2007). The morphology and color of each isolate was described and each isolate was tested for ability to inhibit the growth of Bd. Bd-challenge assays were performed as previously published (Harris et al., 2006; Woodhams et al., 2007). Bacteria that produced a clear zone with no fungal growth around the bacterial streak were considered inhibitory. Clear zones were also examined with a compound microscope at 100× magnification to verify death of Bd zoospores. DNA from pure cultures of anti-Bd isolates was extracted with a MoBio Ultraclean Microbial DNA Isolation Kit (Carlsbad, CA) and amplified using universal bacterial 8F and 1492 rRNA primers (Lane, 1991). Products were sent to Agencourt Bioscience Corporation (Beverly, MA) for DNA sequencing and identified according to similarity with NCBI GenBank entries (<http://www.ncbi.nlm.nih.gov>) (Harris et al., 2006). The average number of cultured anti-Bd bacterial species in each population was compared using a Student's *t*-test. Averages are presented with standard deviations. The proportion of frogs from each population with at least one species of Bd-inhibitory bacteria was compared with a two-tailed Fisher's exact test. The microbial species diversity of cultured, inhibitory bacteria was compared between two populations with a Shannon Weaver index.

## 3. Results

The average number of cultured anti-Bd bacterial species on individual frogs between populations was not significantly different. The average number of anti-Bd bacterial species isolated was  $1.684 \pm 1.416$  species per frog at Lake 52 and  $1.35 \pm 1.071$  species at Conness (*t*-test: *t* = 0.8299; *df* = 37; *P* > 0.05). The total number of anti-Bd bacterial species differed between populations: frogs at Lake 52 had 21 anti-Bd species, and frogs at Conness had 15. The taxonomic distribution of anti-Bd species was different at both populations (Table 1). Lake 52 had anti-Bd species from the phyla

Firmicutes and Bacteroidetes that were absent at the Conness population. Conness had an anti-Bd species from the class Bacilli that was absent from Lake 52. The most commonly identified anti-Bd species from both Lake 52 and Conness populations came from the phylum Proteobacterium in the genus *Pseudomonas*. Cultured anti-Bd bacterial species diversity was approximately similar within the two populations (Shannon Weaver Index, Conness = 3.159; Lake 52 = 3.644).

The proportion of individual frogs that possessed at least one anti-Bd bacterial species was similar in both populations. Approximately 79% (15/19) of individuals from the Lake 52 population had at least one anti-Bd species and about 80% (16/20) of individuals from the Conness population had an anti-Bd bacterial species. This result was surprising, given past results, and suggested that Lake 52 would persist with Bd, despite the declines of *R. muscosa* in most other populations in the Sixty Lake Basin.

**Table 1**

The microbial species composition isolated from frogs' skins at two sites, Conness and Sixty Lake.

Bacterial taxa	Number <sup>a</sup>
Sixty Lake	
Firmicutes	
Actinobacteria	
<i>Arthrobacter</i> sp. TSBY-24 (DQ150099)	1
Proteobacteria	
β-Proteobacteria	
<i>Delftia acidovorans</i> (AM180725)	1
Uncultured Clone ANTVLTV-G07 – Beta Proteo (AJ964894)	1
γ-Proteobacteria	
<i>P. fluorescens</i> (AB266613)	2
<i>P. fluorescens</i> strain CCM (DQ207731)	7
<i>P. sp.</i> Nj-63 (AM491466)	2
<i>P. sp.</i> Nj-55 (AM409368)	7
<i>P. sp.</i> NZ099 (AF388207)	1
<i>P. sp.</i> Yged98 (EF419337)	1
<i>P. sp.</i> 12A_10 (AY689075)	1
<i>P. sp.</i> Cam-1 (DQ777728)	1
<i>P. sp.</i> 33/2 (DQ310485)	1
<i>P. sp.</i> 3C_6 (AY689033)	1
<i>P. syringae</i> strain PNA29 (AY574913)	1
<i>P. oreintalis</i> (AF064457)	1
<i>P. brennerii</i> (AF268968)	1
<i>Lysobacter</i> sp. YC5194 (EF488749)	1
<i>Yersinia</i> sp. ARCTIC-P11 (AY573038)	1
Bacteroidetes	
Sphingobacteria	
<i>Pedobacter</i> sp. Tb4-9-11 (AY599663)	1
Flavobacter	
<i>Chryseobacterium</i> sp. (DQ37556)	1
<i>Flavobacterium</i> sp. (AM177618)	1
Conness	
Proteobacteria	
β-Proteobacteria	
Bacterium H2-Iodabacter (AY345552)	1
Bacterium H15-Beta Proteo (AY34557)	1
<i>Janthinobacterium</i> sp. An8 (AJ551147)	1
γ-Proteobacteria	
<i>P. fluorescens</i> (AB266613)	1
<i>P. fluorescens</i> strain CCM (DQ207731)	11
<i>P. sp.</i> NZ099 (AF388207)	2
<i>P. sp.</i> BIHB (DQ53652)	2
<i>P. sp.</i> Cam-1 (DQ777728)	1
<i>P. sp.</i> LPPA (AJ970167)	1
<i>P. syringae</i> strain PNA29 (AY574913)	1
<i>P. borealis</i> (AJ012712)	3
<i>P. oreintalis</i> (AF064457)	2
Uncultured Bacterium Clone rRNA040 (AY958813)	1
<i>Rahnella</i> sp. 254 (AM403660)	1
Bacilli	
<i>Bacillus</i> sp. MHS002 (DQ993324)	1

GenBank accession numbers are in parenthesis.

<sup>a</sup> Number of frogs that host a microbe for which the indicated species is the closest match.

A year after populations were surveyed for skin microbes (2007), Bd emerged in the previously naïve population at Lake 52. The following year (2008), Bd was still present at Lake 52 and although the frog population had declined, it appeared to be persisting and coexisting with sublethal infectious.

#### 4. Discussion

Woodhams et al. (2007) have shown that a population of *R. sierrae* that had been coexisting with Bd for more than 6 years had a significantly larger proportion of cultured anti-Bd bacterial species than did a population of *R. muscosa* that went extinct a year after Bd arrived. When surveyed the following year, the frog population at Conness was found to have approximately the same proportion of cultured anti-Bd bacterial species although bacterial species composition was not identical, and Bd remained endemic (Woodhams et al., 2007). To our surprise, the population at Lake 52 had a high proportion of individuals with anti-Bd bacteria, which was similar to that found at Conness. At the time of the survey the population was still Bd-naïve, and we then predicted that the frog population at Lake 52 would either not decline or decline less drastically than other populations in that area. The year following the survey (2007), Bd emerged in the naïve population and, a year later (2008), the population was found to be coexisting and persisting with Bd.

Anti-Bd bacteria from the genus *Pseudomonas* were common residents of *R. muscosa* and *R. sierrae*. Although *Pseudomonas* species can be associated with secondary infections, they can also provide a protective effect in amphibians and in other systems, such as crop plants (Das et al., 2008). It is likely that many species of bacteria will fall along a continuum from mutualistic to pathogenic depending on factors such as host regulation of its microbes and environmental variables.

The association of proportion of individuals with anti-Bd bacteria and coexistence with Bd suggests that the mechanism of protection may be analogous to herd immunity. The concept of herd immunity is that only a proportion of individuals in a population need to be vaccinated in order for the disease to die out (Gonclaves, 2008). We suggest that a proportion of individuals need to have anti-Bd bacteria in order to protect the population and allow coexistence. This study and a preliminary analysis of Panamanian frogs coexisting with Bd suggest that if 80% of individuals have an anti-Bd bacterial species, coexistence with Bd occurs (Woodhams, unpublished data). If the protective bacteria on frog skins limit Bd growth on individuals, the  $R_0$  of Bd is less than 5, and 80% of individuals have protective bacteria, then an epidemic of Bd will be prevented (Edelstein-Keshet, 1988; p. 255). A population with a high proportion of individuals with protective bacteria may limit Bd survival and reproduction to the extent that epidemic outbreak is prevented.

An alternative to herd immunity as an explanation for our results is the possibility that individuals with protective bacteria have a higher survival rate than those that lack protective bacteria. Mortality of individuals without protective bacteria can increase the chances of long-term population persistence by providing more resources for surviving individuals. Our two-year survey at Conness Pond showed a consistent 80% of individuals with protective bacteria, which suggests that mortality of individuals without protective bacteria may not be high. However, detailed mark-recapture data are necessary to associate bacterial composition with survival.

Although we found an association between a high proportion of individuals with anti-Bd bacteria and population persistence, we are hesitant to associate causation due to many factors that could not be controlled during a field survey. In addition, Lake 52 was used as a source of frogs moved into other ponds, however the den-

sity of frogs remained within the range typically found in Lake 52 (Vredenburg, unpublished data). Variation in proportion of individuals with protective bacteria remains a viable hypothesis for explaining whether population coexistence with Bd occurs. Our results also suggest that a variety of bacterial species, rather than a few specialists, provide protection. Together with recent experimental evidence that shows the efficacy of anti-Bd bacteria in preventing morbidity and mortality in *R. muscosa* due to chytridiomycosis (Harris et al., 2009), our results suggest that inoculating individuals with anti-Bd bacteria prior to Bd exposure may be a feasible management strategy to control the disease.

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