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Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*

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ARTICLE INFO

Article history:
Received 5 February 2007
Accepted 8 May 2007
Available online 28 June 2007

Keywords:
Antimicrobial peptide
Batrachochytrium dendrobatidis
Herd immunity
Innate immune defense
MALDI mass spectrometry
Rana muscosa
Symbiotic bacteria

ABSTRACT

Symbiotic microorganisms influence health and disease and may contribute to the innate immune defenses of amphibians. The mountain yellow-legged frog, *Rana muscosa*, is currently undergoing unprecedented population declines. One cause of recent declines is the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*). Skin swabs for detection of *Bd*, skin peptide secretions, and symbiotic skin bacteria were collected from 70 adult *R. muscosa* from two populations designated "Sixty Lake" and "Conness" in 2004–2005. The Conness population has persisted with the presence of *Bd* for at least 6 years whereas the Sixty Lake population is newly infected and declining. Of the frogs sampled at Conness, 67.5% were infected; whereas 96.7% of the Sixty Lake frogs were infected. Sixty Lake frogs were also more intensely infected than frogs at Conness. We isolated symbiotic bacteria that may contribute to immune defense. A significantly greater proportion of individuals with at least one anti-*Bd* bacterial species present were found at Conness (85%) than at Sixty Lake (62%). We observed no apparent differences in total skin peptides recovered; however, peptide mixtures from frogs at Sixty Lake showed better growth inhibitory activity against *Bd* than peptides from frogs at Conness. By MALDI-TOF MS analysis, there were no differences between the two populations in the previously described antimicrobial peptides (ranatuerin-2Ma, ranatuerin-2Mb, and temporin-1M). Antimicrobial skin peptides are only one factor in the resistance of *R. muscosa* to *Bd* infection. We suggest that symbiotic bacteria with the ability to persist in the presence of mucosal peptides may inhibit infection and colonization of the skin by *Bd* and increase the effectiveness of innate defense mechanisms in the skin.

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0006-3207/$ - see front matter
doi:10.1016/j.biocon.2007.05.004

1. Introduction

The mountain yellow-legged frog, *Rana muscosa*, is native to cold and remote lakes in the California Sierra Nevada mountains. This once very abundant species is currently undergoing unprecedented population declines (Bradford, 1991; Drost and Fellers, 1996; Sparling et al., 2001; Vredenburg et al., 2005). Although previous declines were attributed to the introduction of non-native trout (Bradford, 1989; Bradford et al., 1993; Knapp and Matthews, 2000; Vredenburg, 2004) or to pesticides (Davidson, 2004; Fellers et al., 2004), recent declines have continued even in apparently unpolluted fishless lakes. One cause of recent declines is the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*) (Briggs et al., 2005; Rachowicz et al., 2006). This fungus is associated with amphibian population declines on several continents (Berger et al., 1998; Waldman et al., 2001; Weldon et al., 2004; Garner et al., 2005; Lips et al., 2006). The factors that lead to extinction or to persistence of amphibian populations with *Bd* are currently under examination (Briggs et al., 2005; Bosch and Martinez-Solano, 2006; Woodhams et al., 2006a,b).

One factor that may contribute to disease resistance is innate immune defense. Innate skin defenses including antimicrobial peptides and symbiotic microbial barriers may be crucial for defending amphibians against the skin-invasive fungus, *Bd* (Harris et al., 2006; Woodhams et al., 2006a,b; Lauer et al., in press). Granular glands in the skin of *R. muscosa* secrete a mixture of peptides including antimicrobial peptides (ranatuerin-2Ma, 2Mb, and temporin-1M). These peptides inhibit the growth of *Bd in vitro* (Rollins-Smith et al., 2006). Symbiotic bacteria of amphibians are not well-known and have not been previously described in *R. muscosa*. However, the salamanders *Plethodon cinereus* and *Hemidactylium scutatum* host beneficial bacteria that inhibit growth of *Bd* in vitro and may be important for resisting *Bd* colonization of skin (Harris et al., 2006; Lauer et al., in press). Here we describe the microbiota of *R. muscosa* that contribute to resistance against *Bd*, and suggest that populations which host beneficial bacteria may be more likely to persist with *Bd*.

2. Methods

2.1. Study species and sites

Adult *R. muscosa* were sampled from two populations in August, 2005. These populations were Lake 11 (elevation 3390 m, depth 3.5 m) in Sixty Lake Basin, Kings Canyon National Park, California (36.82°N, 118.43°W) and Conness (elevation 3175 m, depth 3.9 m) in Yosemite National Park, California (37.97°N, 119.34°W). Populations were in similar high alpine habitats and presumably experienced similar environmental conditions. Using a hand net, we sampled 30 adults from the Sixty Lake site on August 11–12, 2005 and 40 adults from the Conness site on August 15, 2005. Although the Sixty Lake population was thought to be healthy at the time of sampling, a die-off occurred after our sampling between fall 2005 and spring 2006. During subsequent surveys in 2006, no living frogs were found at this site. Fig. 1 illustrates the mass mortality seen in a nearby lake in Sixty Lake Basin in August, 2006. No decline was found at Conness.

2.2. Assessment of Bd infection status

To assess whether frogs were infected with *Bd*, the skin was swabbed as described by Kriger et al. (2006), and real-time Taqman PCR was used to determine the intensity of infection (or the number of zoospore DNA equivalents) (Boyle et al., 2004). Because frogs in each population inhabited the same body of water and were caught in the same net, it is possible that low intensity positive results represent contamination of the swab rather than actual infections. However, precautions were taken to minimize contamination by rinsing the net in lake water between use, and changing gloves before handling a new frog. Frequency of infection (infection prevalence) was compared between populations with Fisher's exact test. Intensity of infection was compared between populations by Mann–Whitney *U*-test.

2.3. Culture and identification of bacteria from frog skin

After capture, frogs were rinsed twice with sterile water to remove transient bacteria (Lauer et al., in press). Symbiotic bacteria from frog skin were sampled using sterile swabs and cultured onto low nutrient Difco R2A media (Becton, Dickinson and Co., Sparks, MD) in the field and quickly transported to James Madison University for isolation. The growth form and color of each isolate was described and each isolate was tested for ability to inhibit the growth of *Bd*. Zoospores were washed from *Bd* culture plates and applied to new 1% tryptone agar plates and allowed to dry in a sterile hood. *Bd*-challenge assays involved adding a streak of an unknown bacterial isolate to one side of the culture plate and a streak of a known bacteria that does not inhibit *Bd* to the other side as a negative control. Bacteria that produced a clear zone with no fungal growth around the streak were considered inhibitory. DNA from these bacteria was then extracted from pure cultures with a MoBio Ultraclean Microbial DNA Isolation Kit (Carlsbad, CA), and then amplified by PCR using universal bacterial 8F and 1492R rRNA primers (Lane, 1991). Products were sent to the Brigham Young University DNA Sequencing Center for sequencing and identified according to similarity with NCBI GenBank entries (http://www.ncbi.nlm.nih.gov) (Harris et al., 2006). 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. All culturable anti-*Bd* symbionts at each site were identified. The microbial species composition was compared between the two populations.

2.4. Collection and quantification of skin peptides

Skin peptides were collected after subcutaneous injection of 10 nmole per gram body weight norepinephrine (bitartrate salt, Sigma, St. Louis, Missouri) by two methods. For a direct survey of all skin peptides, they were absorbed onto 80 μm carbon-imbedded conductive polyethylene film (Goodfellow Cambridge Ltd., Cambridge, England). For collection of larger quantities, the peptides were rinsed from frogs after a 10 min water bath (Chaurand et al., 1999; Rollins-Smith et al., 2002; Woodhams et al., 2006b). For each frog from the Conness population, small pieces (1 × 2 cm) of film were activated by soaking in methanol for 5 s, allowed to air-dry, and

Fig. 1 – Mass mortality of a *Rana muscosa* population at Sixty Lake Basin, Kings Canyon National Park, California in August, 2006.

applied to the dorsal surface for 5–10 s with forceps. For both Connness and Sixty Lake frogs, peptide mixtures rinsed from the skin were partially purified by passing over a C-18 Sep-Pak (Waters Corp., Milford, MA) and quantified by Micro BCA assay (Pierce, Rockford, Illinois) using bradykinin (FW 1060.2, Sigma) as a small peptide standard. The mean quantities of peptides recovered (μg/gbw) are not normally distributed and were therefore compared between the populations with a nonparametric Mann–Whitney *U*-test. In addition, the peptide quantities were compared between groups of infected and uninfected frogs in each population by Mann–Whitney *U*-test, and tested for correlation with infection intensity by Spearman's rank correlation.

2.5. *Bd growth inhibition assay*

The growth inhibition assay to measure activity against *Bd* was previously described (Rollins-Smith et al., 2002; Woodhams et al., 2006a,b). Briefly, peptide mixtures were lyophilized and re-hydrated in HPLC-grade water to a concentration of 100 μg/ml. Five replicates of 50 μl peptide mixtures were then added to 96-well microtiter plates (Costar, Corning, New York) containing an equal volume of 5×10^4 *Bd* zoospores in 1% tryptone broth. The optical density at 490 nm was measured with an MRX Microplate Reader (Dynex Technologies, Inc., Chantilly, VA) after 7 days of growth and compared to positive controls (100% growth, water and no peptides added) and negative controls (0% growth, heat-killed *Bd*). The percentage of *Bd* growth inhibition was determined for each peptide sample and populations were compared by Mann–Whitney *U*-test. In addition, *Bd* growth inhibition was compared between groups of infected and uninfected frogs in each population by Mann–Whitney *U*-test, and tested for correlation with infection intensity by Spearman's rank correlation.

2.6. *Analysis of skin peptides by mass spectrometry*

Skin peptide mixtures were analyzed by matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS). For direct examination of peptides absorbed onto film, the film was attached to a sample plate with electrically conductive adhesive transfer tape (3M, St. Paul, MN) and spotted with 0.6 μl α-Cyano-4-hydroxycinnamic acid matrix solution (Sigma). Washed peptides were spotted onto the sample plate at 1 mg/ml before adding an equal volume of matrix. An Applied Biosystems Voyager DE-STR spectrometer was operated in reflector, delayed extraction and positive ion mode. For external calibration a series of peptide and protein standards (Sigma–Aldrich) were applied. Mass spectra were acquired across the range of *m*/*z* (mass to charge ratio) 600–10,000 and analyzed by Vanderbilt University's High Dimensional Data Analysis Center (HDDAC).

The sequence of analysis of MALDI MS profiles with Wavespec software included baseline subtraction, de-noising (smoothing), normalizing (by total ion current), and recalibration based on pre-selected peptides already identified from the mixture (Rollins-Smith et al., 2006). Conness and Sixty Lake MS profiles were compared using methods of functional data analysis (FDA, Ramsay and Silverman, 1997). Heuristically, this analysis approach uses a *t*-test at each *m*/*z* value to test for differences in the distribution means. The actual tests conducted use ranked normalized intensities, and thus correspond to a

Wilcoxon test. Because tests were conducted at multiple *m/z* values, pointwise p-values of 1×10^{-6} were judged significant. Previous simulation studies (unpublished) indicated that this threshold provides better than 0.05 spectrum-wide significance. This method was used to compare males and females within each population, and to compare the direct sampling of peptides on polyethylene film to peptide washes from individuals in the Conness population. Peptide profiles obtained from washes were compared between the two populations. The intensity of infection (number of *Bd* zoospore equivalents detected per frog) was tested for correlation with skin peptides using Spearman's rank correlation.

3. Results

3.1. Assessment of Bd infection status

The prevalence and intensity of infection with *Bd* differed between the two populations. Of the Conness frogs, 67.5% were infected, whereas 96.7% of the Sixty Lake frogs were infected (Fisher's exact test, $p = 0.0024$). Infections of frogs at Sixty Lake (median = 6723.9 zoospore equivalents) were significantly more intense than infections at Conness (median = 9.5 zoospore equivalents) (Mann–Whitney *U*-test, $Z = 6.583$, $p < 0.0001$). Of the infected frogs only, the infection intensity of the Sixty Lake population (median = 7179.2 zoospore equivalents) was significantly greater than that of the Conness population (median = 37.1 zoospore equivalents) (Mann–Whitney *U*-test, $Z = -6.190$, $p < 0.0001$).

3.2. Culture and identification of bacteria from frog skin

Based on morphology, the number of bacterial isolates obtained per frog at Sixty Lake (mean = 8.03) did not vary significantly from that at Conness (mean = 8.62) (Independent samples *t*-test, $p > 0.05$). Fig. 2 illustrates a lawn of *Bd* co-cultured with streaks of skin bacteria; an inhibitory zone is apparent. Based on our sequencing analysis, the number of anti-*Bd* bacterial species was similar at both ponds: Sixty Lake had 21 anti-*Bd* species, and Conness had 19 (Table 1). The taxonomic distribution of species is similar as well, with most culturable anti-*Bd* species coming from the γ-proteobacteria group. Interestingly, at the Conness population, 29.4% of individuals had one anti-*Bd* species related to *Chromobacterium*. No anti-*Bd* species were found at anything approaching this frequency at Sixty Lake (Table 1). The proportion of frogs hosting at least one species of bacteria that caused growth inhibition of *Bd* in co-culture assays was significantly different between the populations by Fisher's exact test ($p = 0.045$). About 85% (29/34) of individuals from the Conness population had at least one anti-*Bd* bacterial species present whereas about 62% (18/29) of individuals from the Sixty Lake population possessed an anti-*Bd* bacterial species (Fig. 3).

3.3. Quantification of skin peptides and growth inhibitory activity against Bd

The total quantity of skin peptides collected per gram body weight did not differ significantly between the two populations (Mann–Whitney *U*-test, $Z = -0.097$, $p = 0.923$) and the mean ± SD was 617.5 ± 416.0 μg/gbw. The growth of *Bd* was inhibited by skin peptide mixtures in vitro. When a standardized amount of 50 μg/ml peptide was added to *Bd* zoospores, the peptides from Conness frogs ($N = 38$) inhibited growth by 21.9 ± 3.9 (% ± SE). Peptides from Sixty Lake frogs ($N = 24$) inhibited growth by 39.6 ± 6.4%. The higher inhibitory activity found in peptides from frogs at Sixty Lake was significant by Mann–Whitney *U*-test ($Z = -2.436$, $p = 0.015$; Fig. 3). When the quantity of peptides recovered (μg/gbw) is multiplied by the activity of peptides (% inhibition), this gives a measure of skin peptide effectiveness for each frog (Woodhams et al., 2006b). Frogs in the Sixty Lake population had greater skin peptide effectiveness against *Bd* than frogs in the Conness population (Mann–Whitney *U*-test, $Z = -2.002$, $p = 0.045$).

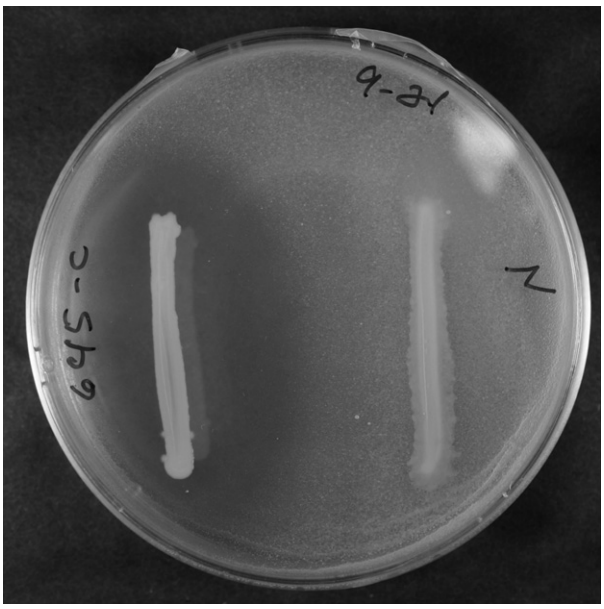

Fig. 2 – Co-culture assay testing the ability of *Pedobacter* sp. (left streak) to inhibit the growth of *Batrachochytrium dendrobatidis* (zoospores plated as lawn). The control streak on the right side of the plate is a non-inhibitory bacterium. Microscopic analysis reveals the presence of zoosporangia and living (motile) zoospores. First the zoosporangia disappear, then the zoospores disappear, as proximity to the inhibitory bacteria increases. Photo credit: B.A. Lam.

Only the Conness population contained frogs of variable infection status. In that population, groups of infected and uninfected frogs did not differ significantly in the quantity of peptides recovered (Mann–Whitney *U*-test, $Z = -0.699$, $p = 0.499$), or the activity of the peptides against *Bd* growth (Mann–Whitney *U*-test, $Z = -0.047$, $p = 0.963$). At Conness and Sixty Lake, infection intensity was not correlated with peptide quantity or inhibition of *Bd* (Spearman rank correlation, $p > 0.05$).

3.4. Analysis of skin peptides by mass spectrometry

The skin peptide profiles obtained by MALDI-TOF MS analysis showed no differences between Conness and Sixty Lake populations in the three previously described antimicrobial peptides: Ranatuerin-2Ma (*m/z* 3273), Ranatuerin-2Mb (*m/z*

Table 1 – The microbial species composition isolated from the skin of Rana muscosa at two sites, Conness and Sixty Lake

Bacterial taxa	Number[a]
Conness	
Firmicutes	
Bacilli	
Bacillus weihenstephanensis (AM062685)	1
Proteobacteria	
β-Proteobacteria	
Uncultured beta proteobacterium, clone:OS1L-24 (AB07687)	10
Bacterium H12 (AY345556)	1
Uncultured bacterium clone LOP-89 (DQ241394)	1
Chromobacterium sp. 71 (AY117572)	1
γ-Proteobacteria	
Serratia sp. 9A_5 (AY689057)	1
Bacterium H16 (AY345555)	3
Stenotrophomonas maltophilia (AY040357)	1
γ-Proteobacterium BT-P-1 (AY539822)	1
Uncultured *Pseudomonas* sp. clone TM14_3 (DQ279329)	1
Pseudomonas sp. NZ099 (AF388207)	5
Pseudomonas lini (AY035996)	1
Pseudomonas fluorescens (DQ207731)	4
Pseudomonas fluorescens strain BOH3 (AY947533)	2
Pseudomonas orientalis (AF064457)	2
Pseudomonas sp. 3A_7 (AY689026)	1
Pseudomonas sp. BE1dil (AY263471)	1
Pseudomonas sp. WG7#1 – *P. migulae* (AY263469)	1
Bacteroidetes	
Sphingobacteria	
Pedobacter cryoconitis (AJ438170)	2
Sixty Lake	
Firmicutes	
Bacilli	
Bacillus weihenstephanensis (AM062685)	1
Proteobacteria	
β-Proteobacteria	
Bacterium H2 – *Iodobacter* (AY345552)	3
Glacier bacterium – *Iodobacter* (AY315171)	2
Iodobacter sp. (DQ226069)	1
Uncultured bacterium clone – *Janthinobacterium* (AY959146)	2
Bacterium H15 (AY345557)	1
γ-Proteobacteria	
γ-Proteobacterium BT-P-1 (AY539822)	2
Pseudomonas sp. NZ007 – *P. tolaasii* (AY014801)	1
Pseudomonas sp. DhA-91 – *P. tolaasii* (AF177916)	
Pseudomonas sp. WG7#1 – *P. migulae* (AY263469)	2
Pseudomonas sp. 3A_7 – *P. orientalis* (AY689026)	1
Pseudomonas sp. Fa2 (AY747590)	1
Pseudomonas fluorescens (DQ207731)	2
Pseudomonas fluorescens PfO-1 (CP000094)	1
Pseudomonas borealis (AJ012712)	1
Pseudomonas syringae pv. Coryli (AJ889841)	1
Pseudomonas syringae strain PNA29.1a (AY574913)	1
Pseudomonas costantinii (AF374472)	1
Bacteroidetes	
Sphingobacteria	
Pedobacter cryoconitis (AJ438170)	1
Pedobacter sp. TB4-9-II (AY599663)	1
Flavobacteria	
Bacterium H_2O – *Chyrseobacterium* (AY345551)	1

GenBank accession numbers are shown in parentheses.
a Number of frogs that host a microbe for which the indicated species is the closest match.

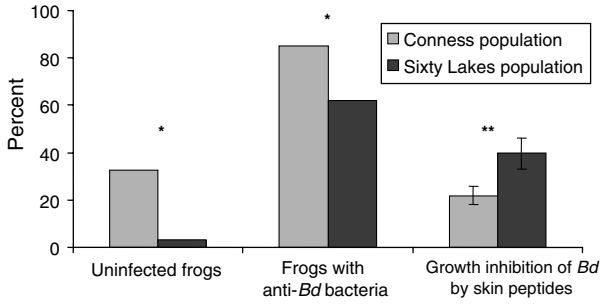

Fig. 3 – A summary of comparisons between two populations of *Rana muscosa* including prevalence of infection with *Batrachochytrium dendrobatidis* (*Bd*), proportion of frogs hosting bacteria that can inhibit *Bd* in co-culture assays, and the percentage of growth inhibition of *Bd* by secreted skin peptides (mean ± SE). * Fisher's exact test, $p < 0.05$. ** Mann–Whitney *U*-test ($Z = -2.436$, $p = 0.015$).

2929), and Temporin-1M (*m*/z 1368), all of which showed activity against *Bd* (Rollins-Smith et al., 2006). Four other skin peptides were significantly different between the two populations including *m*/z: 1842 (better signal at Sixty Lake), 1351, 1376, and 3345 (better signals at Conness). Comparisons between the populations of peptides at *m*/z 1842 and 3345 are shown in Fig. 4. Peptides corresponding to these signals have not yet been sequenced. Detection of an additional seven peptide signals, though minor, were significantly different between sampling techniques. One signal was detected with film but not washes (*m*/z 2881) and six signals were better with washes (1071, 1085, 1255, 1403, 2941, 3283). Comparisons of detection method of peptides at *m*/z 1071 and 2881 are shown in Fig. 4. Males and females did not significantly differ in skin peptide profiles.

Infection intensity was correlated with skin peptide profiles (Table 2). Three peptides were positively correlated and two were negatively correlated with infection intensity (number of zoospores).

4. Discussion

4.1. *Batrachochytrium dendrobatidis* and population declines of *R. muscosa*

The emerging infectious disease, chytridiomycosis, is linked to population declines and extinctions of *R. muscosa* (Briggs et al., 2005). The prevalence of *Bd* infection varies among sites; frogs can persist with the fungus at some sites but not others.

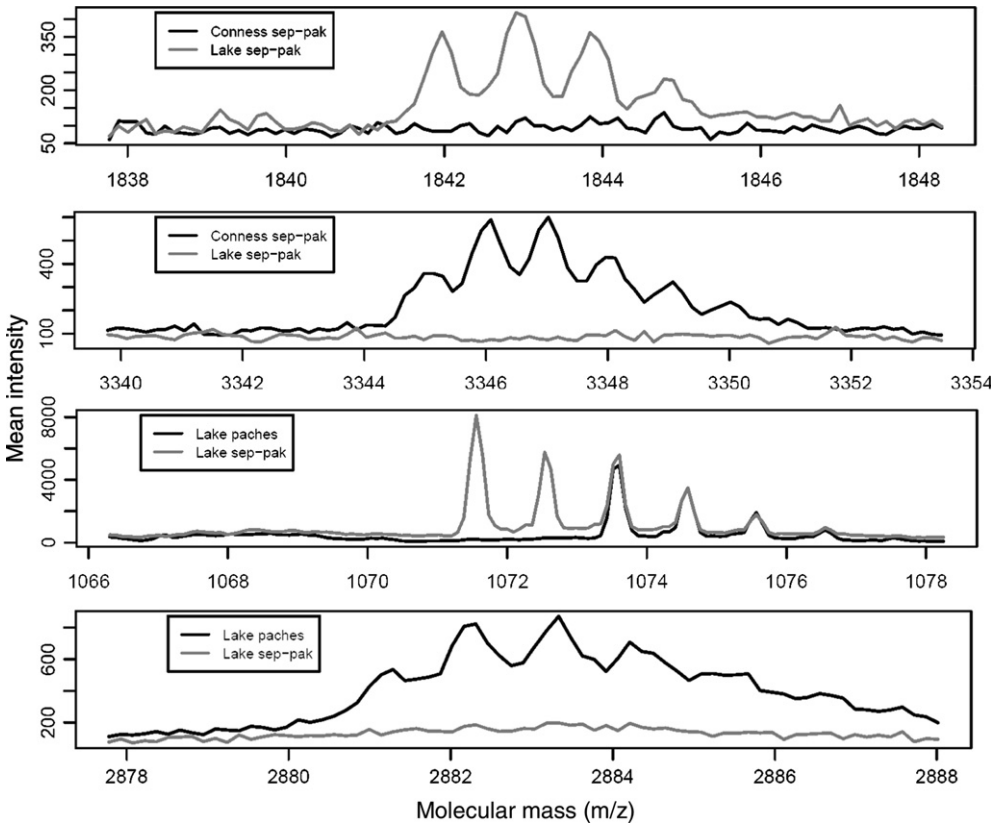

Fig. 4 – Mean MALDI mass spectra of selected skin peptides from *Rana muscosa*. Top two panels show comparisons between Conness and Sixty Lake of peptides identified at mass to *m*/z (mass to charge ratio) 1842 and 3345. Bottom two panels show comparisons between the efficiency of the sampling method at identifying peptides at *m*/z 1071 and 2881. "Sep-Pak" indicates the method of washing the peptides from the skin and subsequent concentration and partial purification over carbon-activated Sep-Paks. "Patches" indicates the method of directly blotting the skin with polyethylene film. Monoisotopic mass is indicated by the first peak in the series.

Table 2 – The molecular mass to charge (*m/z*) values of peptides detected on host *Rana muscosa* skin and their correlation with intensity of infection (number of *B. dendrobatidis* zoospore equivalents detected per frog)

m/z	Spearman	
	Correlation	*p*-Value
1858	0.58	<0.0001
1843	0.54	<0.0001
3347	−0.52	<0.0001
3359	−0.48	<0.0001
1448	0.44	0.0002

For example, the disease history of Sixty Lake and Conness populations with respect to chytridiomycosis is quite different. The Conness population has persisted in the presence of *Bd* for about 6 years whereas individuals from the Sixty Lake population only became infected beginning in 2005 (Briggs unpublished). There are morphological, behavioral and genetic differences between the frogs at the two populations. Based on these factors, a recent study has delineated new taxomony that recognizes two distinct species of *R. muscosa sensu lato* in the Sierra Nevada (Vredenburg et al., 2007). The Sixty Lake and Conness populations are thus recognized as being members of distinct, but closely related species. Thus, it seemed possible that we might observe differences in the level of infection, the profiles of antimicrobial skin peptides, or the symbiotic bacteria associated with frogs in each population.

Infection prevalence and intensity was higher at the recently infected site at Sixty Lake where mass mortality occurred over the winter following our sampling (Fig. 1). This indicates that the initial effects of *Bd* on a naïve population of *R. muscosa* are severe. Long-term monitoring programs already in place will assess whether the arrival of chytridiomycosis in Sixty Lake Basin will lead to population extinction of the entire metapopulation (19 associated populations occur in the basin, Vredenburg, 2004) or whether frogs will persist at lower densities. The persistence of frogs at Conness and coexistence with *Bd* is intriguing given that many other populations in the Sierra Nevada have become extinct in association with disease (Rachowicz et al., 2006).

4.2. *Innate skin peptide defenses of R. muscosa*

The quantities of skin peptides recovered from frogs at Conness and Sixty Lake in 2005 were not significantly different (~617 μg/gbw). In a previous study, peptides were collected from the same population at Sixty Lake in 2004, before the appearance of *Bd* (Rollins-Smith et al., 2006). In that study, greater than twice the amount of peptides was recovered per gram body weight of frog (~1437 μg/gbw). Peptides were collected by a similar method in both studies, except that in 2004, frogs were bathed for 15 min instead of 10 min after skin secretions were induced. An alternative explanation for this discrepancy is that *Bd* infection may chronically stimulate peptide secretion and gradually deplete skin peptide defenses. This hypothesis is not supported here because neither the quantity of peptides recovered (μg/gbw) nor the inhibitory activity of the peptides against *Bd* were correlated with infection intensity. These measures of skin peptide defense also did not differ significantly between groups of infected and uninfected frogs at Conness when given the same stimulus. If infection of the skin by *Bd* does affect skin peptide defenses, it is not by depleting stores of skin peptides. However, infection may disrupt skin structure and block granular gland discharge, or regulate the synthesis of individual peptides. The correlation of some skin peptides with infection intensity (Table 2) supports the hypothesis that skin peptide expression can be regulated by *Bd* infection. The expression or suppression of some skin peptides may alter survival of *Bd* or other microbial symbionts. This also suggests that the differences in peptide signals noted between the two populations (Fig. 4) may be related to infection status or history rather than genetic or environmental factors.

The expression of the three skin peptides described as antimicrobials (Rollins-Smith et al., 2006) were not correlated with infection intensity. These antimicrobial peptides also did not differ between populations, although variation between populations was found in other skin peptides. Frogs at Conness had lower infection prevalence and intensity than those at Sixty Lake; yet the activity of their skin peptide mixtures against *Bd* was also lower. This indicates that skin peptides alone do not fully explain immune defense against chytridiomycosis.

4.3. *Symbiotic bacteria may contribute to innate immune defense*

Symbiotic bacteria may contribute to innate immune defenses against *Bd* in populations of *R. muscosa*. Table one lists the bacteria found on *R. muscosa* skin which are capable of inhibiting *Bd* in vitro. The cutaneous bacteria sampled in the Conness population (*Bd* infected, but populations are persisting) and the Sixty Lake population (newly infected and beginning to decline) showed some differences. A significantly higher proportion of frogs at Conness hosted at least one anti-*Bd* bacterial isolate compared to frogs at Sixty Lake. It is surprising that at Sixty Lake, a population that died-off, 62% of frogs hosted anti-*Bd* bacteria. However, a critical fraction of the population may need a protective microflora in order to prevent epidemic spread of the pathogen, thus leading to herd immunity (Anderson and May, 1990; Ferrari et al., 2006). In addition, the abundance of these bacteria on the skin of frogs is not known. A better understanding of the relative abundance or dominance of the various microbiota is needed in order to predict whether immunity from the pathogen is achieved (Harris et al., 2006). Because many microbes cannot be easily cultured it is likely that other anti-*Bd* isolates inhabit the skin of *R. muscosa*.

We believe it is possible that the Conness population was once naïve and declined dramatically when *Bd* arrived. Infected and persisting populations, such as Conness, would likely have undergone intensive natural selection such that the survivor population would have different gene frequencies and associated biological attributes than did the pre-infection population. In addition, skin microflora would also have undergone natural selection. While there are many possibilities, surviving individuals might have survived on the

basis of a protective skin microflora and these bacterial species would then be present in a higher frequency in the survivor population. There is also the potential that during mating, or during communal basking or at retreat sites, skin bacteria are horizontally transmitted from frog to frog. The incorporation of culture-independent methods of bacterial identification, such as denaturing gradient gel electrophoresis of 16S rRNA gene fragments, will help in testing the hypothesis that the skin microflora differs before and after large population declines due to *Bd*.

This study includes two *R. muscosa* populations and indicates that symbiotic microbiota may contribute to disease defense. Future studies including a greater number of sites that range in infection prevalence will provide a better understanding of the correlation between the occurrence of symbiotic skin bacteria and population dynamics. Also needed are experiments to test whether certain symbiotic skin bacteria actually benefit frogs by inhibiting disease in vivo. If so, it may be possible to inoculate frogs with naturally occurring anti-*Bd* skin bacteria from other *R. muscosa* individuals to achieve a herd immunity effect in populations and to prevent further dramatic declines.

Acknowledgements

The authors thank J. Morgan, M. Stice, and T. Tunstall for field assistance and B.A. Lam for assistance in the laboratory. L.A. Rollins-Smith was funded by an Integrated Research Challenges in Environmental Biology (IRCEB) grant IBN-9977063 (J.P. Collins, P.I.) and grant IOB-0520847 from the U.S. National Science Foundation. R.N. Harris was funded by NSF grant 0413981, and C.J. Briggs was funded by the NIH/NSF Ecology of Infectious Disease Program grant R01 ES12067 from the National Institute of Environmental Health Sciences. D. Woodhams was partially supported by an NHBLI Immunology of Blood and Vascular Systems Training Grant 5T32 HL069765-05 (J. Hawiger, P.I.).

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