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# Skin peptide defences of New Zealand frogs against chytridiomycosis

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*Batrachochytrium dendrobatidis*; skin peptides; *Leiopelma*; *Litoria*; inhibition assay; conservation management.

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## Abstract

Over the past few decades, amphibian populations have undergone drastic declines on a global scale. Declines in many anuran populations have been linked to the emergent skin-invasive amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). Antimicrobial peptides in the skin are thought to act as important components of the innate immune system that may protect some species from infectious diseases. The four archaic species of *Leiopelma* in New Zealand are of great conservation concern and a severe population crash of *Leiopelma archeyi* between 1996 and 2001 has been tentatively linked with the outbreak of *Bd*. Here, we investigated the *in vitro* activity of skin secretions of six frog species in New Zealand against *Bd* zoospore growth. The activity of skin secretions produced by frogs in the wild varied significantly between species, with those of *Le. archeyi* being the most active. The skin secretions of native Leiopelmatid species showed greater *Bd* zoospore inhibition (31.0–71.9%) than the naturalized *Litoria* species (17.4–18.2%). *Leiopelma archeyi* has the most active peptides, even though it is the only native species with known susceptibility to *Bd* infections.

## Introduction

Amphibian populations have undergone drastic global declines over the past few decades. Suspected agents of decline include habitat destruction, overexploitation, exotic species and infectious diseases (Stuart *et al*., 2004; Mendelson *et al*., 2006; Rohr *et al*., 2008; Wake & Vredenburg, 2008). Chytridiomycosis is an emerging infectious disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), which has been linked to the decline or extinction of more than 200 amphibian species (Berger *et al*., 1998; Collins & Storfer, 2003; Stuart *et al*., 2004; Skerratt *et al*., 2007). The presence of *Bd* in New Zealand was first confirmed in 1999 (Waldman *et al*., 2001) and population declines associated with this disease have been detected in the three naturalized *Litoria* species and in one out of four native *Leiopelma* species (Bishop, 1999; Bell *et al*., 2004; Sadic & Waldman, 2004). Examining variations in disease resistance is critical for efficient conservation management, especially in small isolated populations with low genetic diversity, which often display low resistance to infectious diseases (Jones *et al*., 2007).

Both innate (e.g. skin peptides and phagocytic cells such as leucocytes, macrophages and dendritic cells) and acquired (production of antibodies in response to foreign antigens) immune responses play important roles in combating fungal pathogens (Romani, 2004). Factors contributing to resistance against infectious diseases can include the skin itself as a physical barrier, phagocytic cells, symbiotic microbes and antimicrobial peptides in skin secretions (Carey, Cohen & Rollins-Smith, 1999; Richmond *et al*., 2009; Rollins-Smith, 2009; Rollins-Smith *et al*., 2009). Skin secretions produced in the granular glands of amphibians may act as a part of their innate immune defence system against disease-causing agents such as *Bd* (Clarke, 1997; Duda, Vanhoye & Nicolas, 2002; Daly, Spande & Garraffo, 2005). Among the compounds of interest are bioactive peptides from a multitude of amphibian species, which have been shown to inhibit the growth of several different microorganisms *in vitro* (Nicolas & Mor, 1995; Chinchar *et al*., 2001; Rinaldi, 2002; Rollins-Smith *et al*., 2002*a*,*c*; Apponyi *et al*., 2004; Conlon *et al*., 2007; Davidson *et al*., 2007; Woodhams *et al*., 2007*a*). Several species with strong skin peptide defences tend to have better survival rates after an experimental infection with *Bd* (Woodhams *et al*., 2007*a*). The speed and severity of *Bd*-related declines and extinctions highlight the necessity for conservation actions that can be implemented quickly when *Bd* emerges in a population (Lips *et al*., 2008). Identifying species that are at a higher risk to *Bd* infection is of great importance when limited resources are available. For many critically endangered species with extremely small populations, it is vital to assess the risk that *Bd* poses before it is actually detected in the wild, allowing for the prioritization of captive breeding programmes. In New Zealand, all *Leiopelma* species are threatened and *Leiopelma archeyi* is now recognized as the most evolutionarily distinct and globally endangered amphibian in the world (Isaac *et al*., 2007; IUCN, 2008; Edge, 2009). Recently, *Bd* has been detected in both natural populations of *Le. archeyi*, one of which crashed by 88% within 5 years (Bell *et al*., 2004; Potter & Norman, 2006). It is therefore of paramount importance to assess the risk of imminent

  

extinction by chytridiomycosis of the other three *Leiopelma* species, which primarily exist in small or isolated populations.

*In vitro* growth inhibition assays have been widely used to determine the effectiveness of skin secretions and isolated peptides against a range of different microorganisms (Rollins-Smith *et al*., 2002*a*; Urban *et al*., 2007; Mangoni *et al*., 2008; Sheafor *et al*., 2008). The skin secretions of Leiopelmatid species contain a variety of novel peptides and compounds with anti-chytrid properties, which are in the process of being identified (S. Melzer, unpubl. data). The peptides present in the skin secretions of *Litoria aurea*, *Litoria ewingii* and *Litoria raniformis* have been fully sequenced (Steinborner *et al*., 1997*a*; Rozek *et al*., 2000*a*,*b*).

While the isolated peptides Aurein 2.1 (present in *Li. aurea* and *Li. raniformis*) and Caerin 1.1 (present in *Li. ewingii*) have been tested for their anti-chytrid activity (Woodhams *et al*., 2006*a*), the activity of the natural mixtures of skin peptides of these species remain to be quantified. A minimum of 50 $\mu$M of Caerin 1.1 is needed to completely inhibit the growth of both zoosporangia and zoospores of *Bd* isolate JEL 197 (Woodhams *et al*., 2006*a*). *Litoria aurea* and *Li. raniformis* produce between 16 and 17 aurein peptides, 10 of which are found in both species. Aurein 2.1 has a minimum inhibitory concentration of 200 $\mu$M for both mature cells and zoospores of *Bd* isolate JEL 197 (Woodhams *et al*., 2006*a*).

Variations between studies using isolated peptides and natural mixtures of skin secretions are expected to occur because peptides have been shown to act synergistically (Westerhoff *et al*., 1995; Matsuzaki *et al*., 1998; Rollins-Smith *et al*., 2002*b*; Patrzykat & Douglas, 2005).

In this study, we establish the relative effectiveness of natural skin peptide mixtures of New Zealand frogs in inhibiting *Bd* zoospore growth.

## Methods

### Skin secretion collection

Skin secretions containing peptides were sampled in January and February 2007 from adult/subadult individuals of six species of anurans from the North Island and Maud Island of New Zealand under Department of Conservation permits (WK-20068-RES, NM-19892-RES) and in accordance with procedures approved by the Otago University Animal Ethics Committee (AEC no. 63/06). The species sampled included three naturalized *Litoria* species from Australia, *Li. aurea* ($n = 16$), *Li. ewingii* ($n = 7$) and *Li. raniformis* ($n = 9$) as well as three native *Leiopelma* species, *Le. archeyi* ($n = 17$), *Leiopelma hochstetteri* ($n = 19$) and *Leiopelma pakeka* ($n = 9$). All frogs were dry swabbed (Medical Wire & Equipment Co. MW 100-100, Corsham, Wiltshire, UK) as described by Hyatt *et al*. (2007). An in-house SYBR green qPCR assay (S. Herbert *et al*., unpubl. data), which has been validated against the Taqman qPCR assay (Bishop *et al*., 2009), was used to diagnose *Bd* infection. Skin secretions were collected by the non-invasive method of mild transdermal electric stimulation, which has been widely used since 1992 and has no adverse effects on frogs (Tyler, Stone & Bowie, 1992; Apponyi *et al*., 2004; Smith *et al*., 2004). Each frog was captured by hand, held individually in new plastic bags for no longer than 20 min and weighed to the nearest 0.1 g before collecting skin secretions. All frogs were handled using fresh latex gloves for each individual and strict hygiene protocols were adhered to at all times to prevent the potential spread of *Bd*. To collect skin secretions, individual frogs were held by their back legs, their skin moistened with ultra-purified, deionized water (hereafter referred to as MQW; Millipore, Molsheim, France) and a bipolar platinum electrode was gently applied to the dorsal glands. Stimulus strength was adapted to the size of the frogs, ranging from 1 to 1.4 V (AC), and was administered three times for 10 s each. Skin secretions were washed into a clean polypropylene collection beaker with a total of 12 mL MQW per frog and acidified with 0.1% glacial acetic acid to inactivate endogenous peptidases, if present (Resnick *et al*., 1991; Steinborner *et al*., 1997*b*). The acidified collection solution was kept at 4 °C and transported to the lab. Each sample was passed over C-18 Sep-Pak cartridges (Waters Corporation, Milford, MA, USA) and eluted with 70% acetonitrile, 29.9% water and 0.1% trifluoroacetic acid (v/v/v). The volume of the elute was recorded and 1 mL removed for subsequent calculation of peptide concentration. A micro BCA assay (Pierce, Rockford, IL, USA) was performed to determine the total amount of peptides present in each sample using bradykinin (RPPGFSPFR; Sigma Chemical Co., St Louis, MO, USA) to establish a standard curve (Rollins-Smith *et al*., 2002*c*). The remaining sample was then centrifuged to dryness, reconstituted with MQW to a standard concentration of 100 $\mu g\,mL^{-1}$ and sterilized using 0.2-$\mu$m syringe filters (Corning Inc., Corning, NY, USA) to be used for *in vitro* growth inhibition assays.

### Culture and maintenance of *Bd*

The *Bd* type isolate JEL 197, which was isolated from *Dendrobates azureus* from the National Zoological Park, Washington, DC (Longcore, Pessier & Nichols, 1999) was obtained from a cryo-archive (Boyle *et al*., 2003) held by R. Poulter (University of Otago). The fungus was maintained using standard methods (Rollins-Smith *et al*., 2002*b*,*c*) on T-agar plates (1% tryptone), sub-cultured every 7 days by streaking and incubated at 23 °C.

### *Bd* growth inhibition assays

Zoospores were harvested by flooding plates with 3 mL of sterile T-broth for 20 min and gently tipping the plate to collect the liquid containing the motile zoospores, without displacing the mature zoosporangia. The total number of viable cells per millilitre was estimated by counting dilutions of zoospores in Lugol's solution (5% iodine, 10% potassium iodide, 85% MQW) using a haemocytometer.

We tested the ability of natural skin secretion mixtures to inhibit *Bd* zoospore growth as described previously (Rollins-Smith *et al*., 2002*b*,*c*; Woodhams *et al*., 2006*b*). For growth

inhibition of zoospores, $5 \times 10^4$ zoospores in 50 $\mu$L of T-broth were plated in replicates of five in a 96-well microtitre plate (Costar 3596, Corning Inc., Corning, NY, USA). Fifty microlitres of each skin secretion sample was added at a standard concentration of 100 $\mu g\,mL^{-1}$ at a pH of 6.5–7.0 to give a final concentration of 50 $\mu g\,mL^{-1}$. Positive control wells (50 $\mu$L of live zoospores/50 $\mu$L of sterile MQW), negative control wells (50 $\mu$L of heat-killed zoospores/50 $\mu$L of sterile MQW) and blank wells (50 $\mu$L of T-broth per 50 $\mu$L of sterile MQW) were included in each plate in replicates of five. Zoospores were heat-killed at 65 °C for 10 min in a water bath (Johnson *et al.*, 2003). Plates were covered, wrapped in thin plastic film (GLAD® wrap, Auckland, New Zealand) to limit moisture loss, and incubated at 23 °C. Optical density was measured daily for a week at 492 nm using a Fluostar Omega spectrophotometer (Alphatech systems, Auckland, New Zealand). The relative effectiveness of skin secretions was defined as the quantity of peptides produced and their ability to inhibit *Bd* zoospore growth. This was calculated by multiplying the per cent growth inhibition at 50 $\mu g\,mL^{-1}$ by the peptide concentration in $\mu g\,g^{-1}$ bw produced by each frog (Woodhams *et al.*, 2006*b*; Tennessen *et al.*, 2009).

### Statistical analyses

To explore if there were differences in *Bd* growth inhibition, the amount of peptides produced (in $\mu$g per gram body weight) and the relative effectiveness of peptides across the six different species, Kruskal–Wallis tests and subsequent *post hoc* tests (Wilcoxon signed rank tests using a Bonferoni-adjusted $\alpha$ value of $P = 0.01$) were used. All statistics were performed using SPSS version 17 (SPSS Inc., Chicago, IL, USA, 1999).

## Results

### Peptide yield and activity against *Bd*

There was a significant difference between the six New Zealand species in the ability of skin secretions to inhibit *Bd* zoospore growth at 50 $\mu g\,mL^{-1}$ ($\chi^2_5 = 33.45$; $P < 0.001$; Table 1) and in the concentration of skin secretions produced per gram body weight ($\chi^2_5 = 33.47$; $P < 0.001$; Table 1). *Leiopelma archeyi* secreted significantly larger quantities of skin peptides than *Le. hochstetteri* ($U = 38$, $z = -3.9$, $P < 0.001$), *Le. pakeka* ($U = 19$, $z = -3.1$, $P = 0.002$) and *Li. aurea* ($U = 30$, $z = -43.8$, $P < 0.001$). There was no significant difference in the quantities of peptides secreted between either *Le. archeyi* and *Li. ewingii* ($U = 42$, $z = -1.1$, $P = 2.88$) or *Li. raniformis* ($U = 61$, $z = -8.35$, $P = 4.26$). Comparisons of *Bd* inhibition showed that *Le. archeyi* produced skin secretions that were significantly more active against zoospores (71.9% growth inhibition) than *Le. hochstetteri* ($U = 45.5$, $z = -3.7$, $P < 0.001$), *Li. aurea* ($U = 18.5$, $z = -4.2$, $P < 0.001$), *Li. ewingii* ($U = 9.5$, $z = -3.2$, $P = 0.001$) and *Li. raniformis* ($U = 11$, $z = -3.5$, $P < 0.001$). No significant difference between *Le. archeyi* and *Le. pakeka* ($U = 39$, $z = -2.02$, $P > 0.01$) was detected. Despite producing the largest quantities of peptides, *Li. ewingii* possess the least active secretions of the New Zealand species (Table 1). Overall, the skin secretions of native *Leiopelma* species (31–71.9% growth inhibition) were more effective at inhibiting zoospore growth than the naturalized *Litoria* species (17.4–18.2% growth inhibition).

### Relative effectiveness of peptide defences against *Bd*

There was a statistically significant difference in relative peptide defences (skin peptide quantity produced multiplied by their % growth inhibition) among the species tested ($\chi^2_5 = 37.39$; $P < 0.001$; Fig. 1). *Leiopelma archeyi* and *Li. ewingii* have strong peptide defences based on the quantity and quality of their skin secretions (Fig. 1). While all three *Litoria* species are generally susceptible to chytridiomycosis in New Zealand, Australia and Tasmania (Berger, 2001; Waldman *et al.*, 2001; Carver, 2004; Sadic & Waldman, 2004; Obendorf & Dalton, 2006), individuals of *Le. archeyi* in the wild are susceptible to *Bd* infection but able to eliminate the fungus when held in captivity (Bishop *et al.*, 2009). It remains to be determined if *Le. pakeka* and *Le. hochstetteri* are susceptible to *Bd* infection or develop symptoms of chytridiomycosis. The analysis of the skin

**Table 1** Summary of skin secretion defences of New Zealand species

| | Peptides recovered ($\mu g\,g^{-1}$ body weight) | | | | % growth inhibition of *Batrachochytrium dendrobatidis* at 50 $\mu g\,mL^{-1}$ | | | |
|---|---|---|---|---|---|---|---|---|
| Species | Mean | SE | Median | *n* | Mean | SE | Median | *n* |
| *Leiopelma archeyi*[a] | 163.2 | 48.3 | 59.1 | 17 | 71.9 | 6.9 | 84.0 | 17 |
| *Leiopelma hochstetteri*[a] | 21.3 | 3.4 | 15.2 | 19 | 31.0 | 4.5 | 32.0 | 19 |
| *Leiopelma pakeka*[a] | 24.9 | 27.9 | 13.1 | 9 | 43.7 | 24.9 | 41.0 | 9 |
| *Litoria aurea*[b] | 21.2 | 34.4 | 9.9 | 16 | 18.2 | 10.4 | 15.5 | 16 |
| *Litoria ewingii*[b] | 181.8 | 151.4 | 108.8 | 7 | 17.4 | 7.3 | 21.0 | 7 |
| *Litoria raniformis*[b] | 72.2 | 23.2 | 51.4 | 9 | 18.2 | 2.8 | 16.0 | 9 |

Individuals were induced to secrete by mild electric stimulation.

[a]Native species.

[b]Naturalized species from Australia.

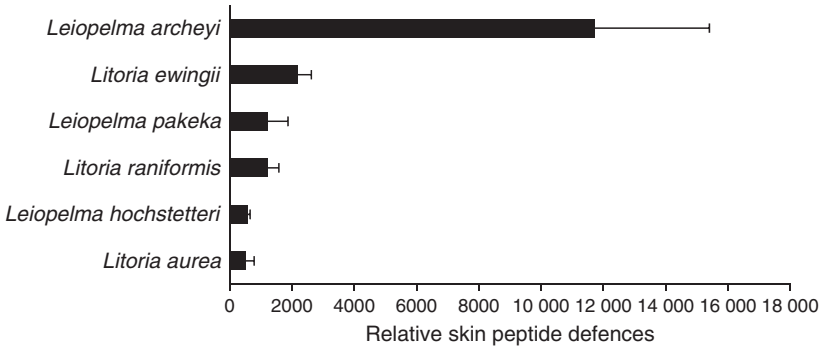


**Figure 1** Relative peptide defences (± SE) of New Zealand frog species as calculated by multiplying the % growth inhibition of *Bd* zoospores at concentrations of 50 $\mu g\,mL^{-1}$ by the total amount of peptides produced per gram body weight.

swabs showed *Bd* zoospores were present on one *Li. aurea* (65.2 zoospore equivalents) and the *Bd* presence of one *Li. raniformis* was equivocal.

## Discussion

### Activity of skin secretions against *Bd* growth

The present study has provided evidence that the skin secretions of both native and naturalized anuran species in New Zealand can inhibit *Bd* zoospore growth *in vitro*. *Litoria ewingii* and *Le. archeyi* produce the largest amount of peptides of all six New Zealand species. When comparing the efficacy of skin secretions (at a concentration of 50 $\mu g\,g^{-1}$ bw) to inhibit zoospore growth, the skin peptides of native New Zealand species are among the most active (see Table 1). In addition, *Le. archeyi* showed great individual variability in both peptide yield and efficacy at inhibiting *Bd* zoospores *in vitro*; a pattern that was also found in *Centrolene prosoblepon* (Woodhams *et al*., 2006*b*). However, it is important to note that the total yield of peptides collected may vary dependent on the method of collection. Both mild electric stimulation and Norepinephrine injection cause the contraction of smooth muscle surrounding the granular skin glands and subsequent discharge of secretions, but the effect on peptide yield remains to be quantified. While *Le. hochstetteri* and *Le. pakeka* produce lower quantities of peptides, their inhibitory activity is comparatively high. This shows that producing higher quantities of skin peptide mixtures does not necessarily equate to increased inhibition of zoospores growth.

### Relative effectiveness of skin secretions and chytridiomycosis

Of all New Zealand species, *Le. archeyi* and *Li. ewingii* have the highest potential to reduce zoospore colonization of the skin or infection intensity based on the amount of skin secretions produced ($\mu g\,g^{-1}$) and their ability to inhibit *Bd* zoospore growth *in vitro* (Fig. 1). However, *Bd* infections of *Le. archeyi* have been detected in the Coromandel region, where populations suffered major declines (Bell *et al*., 2004). In 2006, *Le. archeyi* from a second population in the Whareorino, were also diagnosed with *Bd* infections (Smale, 2006), even though no population declines were recorded for the area. Although *Le. archeyi* is susceptible to infection and reinfection with *Bd* (Bishop *et al*., 2009; S. Shaw *et al*., unpubl. data), no clinical signs of chytridiomycosis have ever been reported for this species and individuals are able to self-cure within 2–6 weeks. Similarly, *Litoria wilcoxi* can eliminate *Bd* in the wild (Kriger & Hero, 2006) and *Taudactylus eungellensis* persists with stable *Bd* infections in Australia (Retallick, McCallum & Speare, 2004). It is possible that the *Le. archeyi* population in the Whareorino are in an endemic phase of chytridiomycosis and declines in the epidemic phase remained undetected when no monitoring occurred. Alternatively, this species may not be very susceptible to *Bd* and the population crash in the Coromandel may not have been related to the amphibian chytrid. We suggest that skin peptides might control *Bd* growth as a first line of defence when initially encountering *Bd*, until further immunological responses can be mounted. Although all of our study animals, except one *Li. aurea* and one *Li. raniformis*, tested negative for *Bd*, we cannot assume they were naive to the pathogen given that individuals can apparently clear themselves of *Bd* infections.

The relative peptide defences of the three *Litoria* species are variable (Fig. 1) but all are susceptible to *Bd* infections and show clinical signs of chytridiomycosis in the wild and laboratory, both in Australia and New Zealand (Bell *et al*., 2004; Carver, 2004; Sadic & Waldman, 2004; Speare *et al*., 2005;R. Poulter *et al*., unpubl. data). Interestingly, *Le. hochstetteri* produces only small amounts of a relatively effective peptide mixture. This semi-aquatic species is sympatric and syntopic with the fully terrestrial *Le. archeyi*, and therefore likely to have also been exposed to the pathogen. However, a comprehensive New Zealand-wide *Bd* survey of 420 *Le. hochstetteri* has failed to detect any symptoms of chytridiomycosis or *Bd* infection using qPCR (Thurley & Haigh, 2008), despite studies showing that species breeding in permanent water bodies are often more affected by the waterborne amphibian chytrid than terrestrial species (Kriger & Hero, 2007). While the skin peptides of *Le. pakeka* are among the most effective at inhibiting zoospore growth, the peptide yield of this species is relatively low (Table 1). As a result, the overall peptide defences of *Le. pakeka* are comparatively low and might not reduce zoospore growth significantly.

*Leiopelma pakeka* might be seriously affected if the fungus was introduced into these populations. Although, due to its restriction to several off-shore islands with highly controlled access, the chance of an encounter with *Bd* is relatively low.

Recently, it has been suggested that the role of anuran skin peptides in protecting species from *Bd* infections in the wild is not clear-cut (Conlon, Iwamuro & King, 2009). For example, Woodhams *et al.* (2006*b*) provided correlative evidence suggesting that peptide defences are linked to the persistence of *Xenopus laevis* and *Rana pipiens* in the wild, while *Bufo boreas*, which has weak peptide defences, is endangered. However, species with highly active skin peptides, such as *Rana tharumarae* or *Xenopus tropicalis* can still suffer from *Bd*-related declines (Ali *et al.*, 2001; Parker *et al.*, 2002; Rollins-Smith *et al.*, 2002*c*). Additionally, *Litoria caerulea* is highly susceptible to *Bd* infections in the laboratory (Pessier *et al.*, 1999; Berger, Speare & Skerratt, 2005; Woodhams *et al.*, 2007*a*) and in the wild (Speare & Berger, 2005), despite the fact that it produces moderately effective skin secretions that can inhibit *Bd* zoospore growth at concentrations of 271 $\mu g\,mL^{-1}$ (Woodhams *et al.*, 2006*a*). The skin peptides of *Litoria lesueuri*, *Litoria genimaculata*, *Litoria nannotis*, *Nyctimystes dayi* and *Litoria rheocola* vary in their effectiveness to inhibit *Bd in vitro* (Woodhams *et al.*, 2006*a*) and all of them have been found with *Bd* infections in the wild (Speare & Berger, 2005). *Centrolene prosoblepon* and *Hylomantis lemur* were predicted to be comparatively resistant to chytridiomycosis based on the quality and quantity of their skin peptides, but are suffering *Bd*-related declines in highland sites while populations in lowland sites seem stable (Lips *et al.*, 2006; Woodhams *et al.*, 2006*b*). Furthermore, there are several species of anurans that do not produce cytolytic peptides in their skin secretions (Conlon *et al.*, 2009; C. Shaw, pers. comm.) and it would be of interest to determine their susceptibility to chytridiomycosis.

### Factors influencing infection outcome

The level of virulence a pathogen expresses in its host is the result of complex interactions between host immunity, pathogen and environmental context and is not always easily explained by one factor alone (Poulin & Combes, 1999; Wolinska & King, 2009). Many factors are likely to influence the susceptibility to *Bd*, including developmental stage (Smith *et al.*, 2005), behaviour (Parris, Reese & Storfer, 2006) and environmental conditions (Rohr *et al.*, 2008). Both host and pathogen are highly affected by temperature and moisture; thus changes in these factors can have direct impacts on disease dynamics (Lips *et al.*, 2008). Temperature severely impacts growth rates and infectivity of *Bd* (Piotrowski, Annis & Longcore, 2004; Woodhams *et al.*, 2008), as well as causing physiological stress to anurans (Reading, 2006). In addition, peptide synthesis and secretion is dependent on a variety of factors such as bacterial flora present on the frog's skin, temperature and exposure to pollutants (Matutte *et al.*, 2000; Harris *et al.*, 2006; Davidson *et al.*, 2007). Symbiotic bacteria on the skin of frogs and salamanders alone can inhibit growth of pathogenic fungi (Harris *et al.*, 2006; Woodhams *et al.*, 2007*b*; Banning *et al.*, 2008; Lauer *et al.*, 2008). Certain bacteria resident on amphibian skin produce anti-*Bd* metabolites, which can reduce morbidity and mortality of infected frogs (Harris *et al.*, 2009). Thus, there seems to be an interaction between the presence of microorganisms and the immune response. The induction of defensive peptide production is altered by the presence of bacteria, with peptide production completely inhibited in a sterile environment (Mangoni *et al.*, 2001). Furthermore, there are indications that peptide synthesis can in turn be impacted by *Bd* infection (Woodhams *et al.*, 2007*b*). The complex interactions between temperature, bacteria and antimicrobial peptide production in amphibians could explain why we see *Bd*-associated declines of species with skin peptides that showed high *in vitro* efficacy. We show that all three native Leiopelmatid species produce skin secretions that are effective in inhibiting *Bd* zoospore growth *in vitro* but only small amounts are secreted onto the skin when induced by mild electric stimulation. Based on our results, we therefore recommend to continue the intense conservation management of all three native species.

Future work on the interactions of peptide defences and chytridiomycosis could include studies where susceptibility to *Bd* of frogs with full skin glands is compared with frogs with emptied glands. Additional studies are needed to determine the quantity of peptides released onto the skin in undisturbed individuals and in response to a *Bd* infection and finally, to document the complex interactions between peptide production, symbiotic skin bacteria and temperature.

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