

Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses

D. C. Woodhams^{1,2}, K. Ardipradja³, R. A. Alford¹, G. Marantelli³, L. K. Reinert² & L. A. Rollins-Smith^{2,4}

¹ School of Marine and Tropical Biology, James Cook University, Townsville, Qld, Australia

² Department of Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, TN, USA

³ Amphibian Research Centre, Western Treatment Plant, Werribee, Vic., Australia

⁴ Department of Pediatrics, Vanderbilt University Medical Center, Nashville, TN, USA

Keywords

amphibian; antimicrobial peptide; *Batrachochytrium dendrobatidis*; chytridiomycosis; innate immunity; skin; susceptibility.

Correspondence

Douglas C. Woodhams, Department of Microbiology and Immunology, A-5301 Medical Center North, Vanderbilt University Medical Center, Nashville, TN 37232, USA.
Tel: + 1 615 343 9449;
Fax: + 1 615 343 7392
Email: dwoodhams@gmail.com

Received 3 June 2007; accepted 11 June 2007

doi:10.1111/j.1469-1795.2007.00130.x

Abstract

Innate immune mechanisms of defense are especially important to ectothermic vertebrates in which adaptive immune responses may be slow to develop. One innate defense in amphibian skin is the release of abundant quantities of antimicrobial peptides. Chytridiomycosis is an emerging infectious disease of amphibians caused by the skin fungus, *Batrachochytrium dendrobatidis*. Susceptibility to chytridiomycosis varies among species, and mechanisms of disease resistance are not well understood. Previously, we have shown that Australian and Panamanian amphibian species that possess skin peptides that effectively inhibit the growth of *B. dendrobatidis in vitro* tend to survive better in the wild or are predicted to survive the first encounter with this lethal pathogen. For most species, it has been difficult to experimentally infect individuals with *B. dendrobatidis* and directly evaluate both survival and antimicrobial peptide defenses. Here, we demonstrate differences in susceptibility to chytridiomycosis among four Australian species (*Litoria caerulea*, *Litoria chloris*, *Mixophyes fasciolatus* and *Limnodynastes tasmaniensis*) after experimental infection with *B. dendrobatidis*, and show that the survival rate increases with the *in vitro* effectiveness of the skin peptides. We also observed that circulating granulocyte, but not lymphocyte, counts differed between infected and uninfected *Lit. chloris*. This suggests that innate granulocyte defenses may be activated by pathogen exposure. Taken together, our data suggest that multiple innate defense mechanisms are involved in resistance to chytridiomycosis, and the efficacy of these defenses varies by amphibian species.

Introduction

Research on declining amphibian populations has recently focused on emerging diseases (Berger *et al.*, 1998; Daszak *et al.*, 1999; McCallum, 2005). Many amphibian declines have been linked to the emergence of chytridiomycosis, an infectious skin disease of amphibians caused by a chytrid fungus, *Batrachochytrium dendrobatidis* (Berger *et al.*, 1998; Longcore, Pessier & Nichols, 1999; Stuart *et al.*, 2004; Lips *et al.*, 2006). Disease-associated population declines might be caused by the appearance of a novel pathogen or by environmental changes altering the balance of host–pathogen interactions (Rachowicz *et al.*, 2005; Lips *et al.*, 2006; Pounds *et al.*, 2006). Patterns of amphibian population declines in many localities worldwide show that some species decline while other sympatric species do not (Alford & Richards, 1999; Lips, 1999; McDonald & Alford, 1999; Parker *et al.*, 2002; Stuart *et al.*, 2004; Kriger & Hero, 2006). Understanding the basis for disease resistance of these species may promote conservation of more susceptible species.

It is likely that multiple factors underlie disease resistance. The joint effects of environmental conditions, pathogen biology and host biology may regulate the timing and severity of chytridiomycosis. One aspect of host biology that may contribute to disease resistance is the immune defense system. Amphibians have a well-developed immunity featuring both adaptive and innate responses (Carey, Cohen & Rollins-Smith, 1999; Apponyi *et al.*, 2004). Amphibian skin is a primary defensive barrier against infection by environmental pathogens; it is also the site of infection of *B. dendrobatidis* in post-metamorphic amphibians. Limited lymphocytic infiltration in the skin of frogs susceptible to severe chytridiomycosis suggests that these animals develop poor adaptive immune responses against *B. dendrobatidis* (Berger *et al.*, 1998; Pessier *et al.*, 1999; Berger, Speare & Skerratt, 2005b). However, very little is known about the possible role of circulating lymphocyte and granulocyte populations in the response to chytridiomycosis. Here, we investigated differences in populations of circulating lymphocytes, monocytes, neutrophils, eosinophils and

basophils in infected and uninfected control *Litoria chloris* to determine whether changes in any of these populations would reflect an ongoing immune response to *B. dendrobatidis*. Because the adaptive immune response may be ineffective against *B. dendrobatidis*, innate defenses such as epithelial barriers, competitive microbiota (Harris *et al.*, 2006), phagocytic cells and antimicrobial peptides may be central to effective protection.

Amphibian skin produces large quantities of host-defensive peptides (Apponyi *et al.*, 2004; Pukala *et al.*, 2006). Although alkaloids and amines can also be present in amphibian skin secretions, many of the secreted peptides have antimicrobial activities. In addition, antimicrobial peptides within naturally secreted mixtures can act together in synergy (Westerhoff *et al.*, 1995; Rollins-Smith *et al.*, 2002; Patrzykat & Douglas, 2005). *In vitro* experiments show that many isolated skin peptides and natural peptide mixtures have activity against *B. dendrobatidis* (Rollins-Smith & Conlon, 2005; Woodhams *et al.*, 2006a). In Australian rainforest amphibians, population trends associated with chytridiomycosis were correlated with skin peptide defenses (Woodhams *et al.*, 2006a). In a study of amphibians at an upland rainforest at El Copé, Panama, the resistance of some species to emerging chytridiomycosis was predicted based on skin peptide defenses (Woodhams *et al.*, 2006b). Here, we examine the susceptibility of four Australian species to experimental *B. dendrobatidis* infection and correlate susceptibility or resistance with antimicrobial peptide defenses *in vitro*. We suggest that species variation in these defenses partially accounts for the disparity in disease susceptibility among species.

Materials and methods

Animal husbandry

We bred four species of wild-caught adult frogs at the Amphibian Research Centre in Melbourne, Victoria, Australia. From a single egg clutch of each species, we raised full siblings through metamorphosis under conditions restricting the potential for exposure to *B. dendrobatidis*. We randomly allocated 40 juvenile frogs of each species to exposure and control treatments. These species overlap in range in south-eastern Australia, and were chosen for their variability in size under the hypothesis that development of lethal chytridiomycosis would take longer in species with a larger skin surface area. Species included (mean snout–vent length at 2 months of age \pm SD in brackets): orange-eyed treefrogs, *Litoria chloris* (17.40 \pm 1.18 mm), green tree frogs, *Lit. caerulea* (40.24 \pm 3.80 mm), spotted marsh frogs, *Limnodynastes tasmaniensis* (23.21 \pm 2.15 mm) and great barred frogs, *Mixophyes fasciolatus* (40.08 \pm 2.57 mm). We housed frogs individually in 1.6 L transparent plastic enclosures (18 \times 11 \times 12 cm) with ventilated lids (Critter KeeperTM, Rolf Hagen & Co. Inc: Mansfield, MA, USA). The bottoms of containers were covered by a 2–3 cm layer of aquarium gravel, with a depression at one end that allowed access to standing water. Drain holes 2 cm from the bottom of

containers maintained water levels. Water was renewed through low-volume inlets for 15 min daily. We maintained all enclosures on a 12L:12D photoperiod and placed them on shelving in 20 blocks, each block containing all treatments. We fed frogs every other evening with crickets dusted in a vitamin supplement and occasionally with houseflies. Enclosures had separate drain pipes, minimizing the opportunity for disease transmission.

Survival following exposure to *B. dendrobatidis*

In order to compare patterns of survival among juvenile frogs of four species following standardized exposure to a pathogen, we exposed 20 individuals of each species to *B. dendrobatidis* zoospores and 20 to culture broth lacking zoospores (sham-infected controls) in May 2001. *Batrachochytrium dendrobatidis* (isolate GibboRiver-Llesueuri-00-LB-1) culture was obtained from CSIRO in Geelong, Victoria. To expose the frogs, we placed them in 100 mL specimen jars containing 2 mL of water and either 150 μ L of *B. dendrobatidis* culture broth, diluted to contain *c.* 5000 zoospores, or 150 μ L of sterile broth. We then placed the jars horizontally and rolled them every 15 min for 15 h to thoroughly expose frogs to the zoospores, and then placed the frogs into their enclosures. Because the tree frogs were prone to climbing out of the solution, this method insured exposure to the pathogen. This level of exposure is likely more severe than occurs in nature but was used to ensure infection. We monitored the survival of all frogs for 108 days after *B. dendrobatidis* exposure. Once the animals became fully symptomatic for chytridiomycosis, they were euthanized in 0.2% tricaine methane sulfonate (TMS). Clinical signs of severe disease include erythema of ventral skin, lethargy and delayed righting reflex. These signs are indicative of mortality within hours (Berger, Speare & Hyatt, 1999; Berger *et al.*, 2004, 2005b). Surviving frogs including controls were maintained at the Amphibian Research Centre following the experiment. We analyzed survival data with a logrank test on censored survival data in StatXact 4.0 (CYTEL Software Corporation).

Infection status of frogs exposed to *B. dendrobatidis*

To examine the status and intensity of infection, we removed one toe from each frog before exposure, 10 days after exposure and at death or 108 days following experimental exposure for surviving frogs. Following the protocol of Berger, Speare & Kent (1999), we diagnosed infection status from histological sections without reference to treatment. We assessed the intensity of infection as follows: we determined the density of *B. dendrobatidis* sporangia on the toes of deceased frogs by dividing the average number of sporangia per cross section by the average perimeter of sections from that species. We estimated perimeters from a sample of five sections per species measured by superimposing a section on a hemocytometer with a 0.0025 mm² grid. Using

a Kruskal–Wallis test in SPSS v.12 (SPSS Inc.), we compared the infection intensities among species.

Effectiveness of natural mixtures of skin peptides against *B. dendrobatidis*

In order to test for a correlation between the survival rate of infected frogs and skin peptide defenses against *B. dendrobatidis*, we shipped five *Lit. chloris* [mean weight (g) \pm SD: 2.43 ± 0.32], six *Lit. caerulea* (21.17 ± 3.85), five *Lim. tasmaniensis* (3.56 ± 0.34) and five *M. fasciolatus* (29.88 ± 2.58) of the control frogs of the four species from the Amphibian Research Centre, Melbourne, Victoria, to James Cook University (JCU), Townsville, Queensland, in October 2002. Because sampling of skin peptides may interfere with measurement of survival time, we only sampled peptides from the control animals after completion of the infection experiment. At JCU, we housed frogs individually at room temperature (*c.* 24 °C) with a 12L:12D photoperiod and regular feeding schedule with vitamin-dusted crickets. After a 3-week acclimation, we collected skin peptide secretions by bathing frogs in collection buffer containing 100 μ M nor-epinephrine–bitartrate salt (Sigma–Aldrich, St. Louis, MO, USA) for 15 min (Woodhams *et al.*, 2006a). We passed the skin secretions over C-18 Sep-Pak cartridges (Waters Corporation, Milford, MA, USA) to enrich for cationic and hydrophobic peptides and shipped the Sep-Paks to Vanderbilt University in Nashville, Tennessee. We eluted the peptides bound to the Sep-Paks with 70% acetonitrile, 29.9% water, 0.1% trifluoroacetic acid (v/v/v) and concentrated them to dryness by centrifugation under vacuum. To determine total skin peptides recovered after Sep-Pak purification, we followed the manufacturer's instructions for Micro BCA Assay (Pierce, Rockford, IL, USA), except that bradykinin (RPPGFSPFR) (Sigma) was used to establish a standard curve (Woodhams *et al.*, 2006a).

We diluted peptide mixtures to concentrations between 1.6 and 500 μ g mL⁻¹ and incubated them with *B. dendrobatidis* zoospores to determine whether the peptides could inhibit growth after 7 days of incubation at 23 °C. We determined the minimal inhibitory concentration (MIC in μ g mL⁻¹) for each species (Rollins-Smith *et al.*, 2002). MIC is defined as the lowest concentration of added peptide at which no significant growth of *B. dendrobatidis* was observed. We calculated the mean MIC equivalents for each species (Woodhams *et al.*, 2006b). An MIC equivalent is the total amount of peptides (μ g) recovered from each frog per 1 cm² surface area, divided by the experimentally determined MIC (μ g mL⁻¹) for each species. Surface area was calculated using the standard equation from McClanahan & Baldwin (1969): surface area (cm²) = 9.90 (weight in grams)^{0.56}.

We used a Jonckheere–Terpstra trend test carried out in StatXact 4.0 to test for differences among species in the distribution of MIC equivalents along the ordered gradient formed by per cent survival. We compared the MIC levels of the three groups of species with differing survival rates (*Lit. caerulea* and *M. fasciolatus*, 5%, *Lit. chloris*, 35%, and *Lim. tasmaniensis*, 100%).

Response of leukocyte populations to infection in *Lit. chloris*

This experiment was designed to determine how leukocyte populations change in response to environmental temperature or infection with *B. dendrobatidis*. *Litoria chloris* were chosen because they showed a non-constant survival curve and intermediate susceptibility to chytridiomycosis compared with the other species tested here (Fig. 1) and were therefore most likely to display an immune response such as changes in leukocyte populations after exposure to *B. dendrobatidis*. At the Amphibian Research Centre, 72 captive-bred frogs were raised for 3 months post-metamorphosis to *c.* 20 mm snout–vent length. We then randomly allocated them to treatments. The experiment had a factorial design, with two levels of infection (yes or no) crossed with three levels of a thermal regime (constant 20 °C, naturally fluctuating temperatures ranging from 14 to 23 °C \pm 1.8 °C SD, and naturally fluctuating temperatures with two nights of cooling down to 8 °C 12 days after exposure to *B. dendrobatidis*). Because temperature can affect amphibian immunity and mortality due to chytridiomycosis (Woodhams, Alford & Marantelli, 2003; Berger *et al.*, 2004; Pounds *et al.*, 2006; Raffel *et al.*, 2006), three thermal regimes were chosen to represent constant laboratory conditions, natural temperature variation and a cold-shock scenario. There were 12 frogs in each treatment, housed in two enclosures each containing six individuals. Samples were collected from four individuals in each group, and the data for individual frogs were used as replicates in analyses. We inoculated frogs as described above with 15 000 zoospores or sterile broth.

On day 27 post-infection, four frogs from each group enclosure were anesthetized with 0.01% TMS. We collected blood for differential leukocyte counts in heparinized microhematocrit tubes from a cut in the abdominal vein. We then euthanized the frogs in 0.02% TMS, and verified their

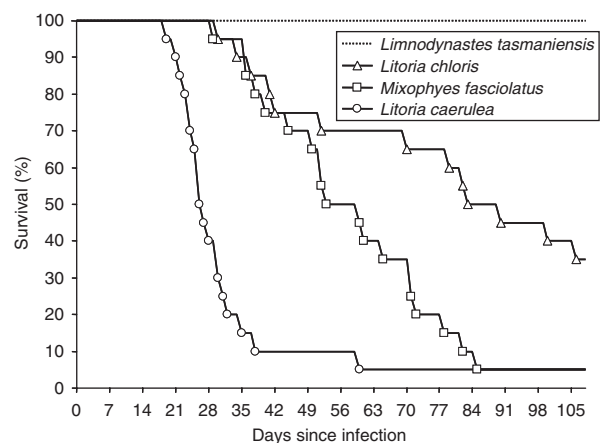


Figure 1 Patterns of survival over 108 days of four Australian anuran species exposed on day 0–5000 zoospores of *Batrachochytrium dendrobatidis*. Symbols represent individual frog mortalities. Control frogs ($n=20$ per species) were sham-infected with sterile medium and are omitted from the figure. Logrank test on censored survival data, $P<0.001$.

Table 1 Infection status and intensity of infection of four species of Australian anurans diagnosed by histology of skin sections 10 days post-infection, after death or euthanasia, or after surviving 108 days post-exposure to *Batrachochytrium dendrobatidis*.

Species	Per cent positive 10 days post-infection (<i>n</i>)	Per cent positive post-mortem (<i>n</i>) ^a	Per cent positive surviving 108 days post-exposure (<i>n</i>)	Post-mortem infection intensity ^b
<i>Litoria caerulea</i>	60 (20)	100 (19)	0 (1)	58.9 ± 14.6
<i>Mixophyes fasciolatus</i>	20 (20)	100 (19)	0 (1)	131.3 ± 53.9
<i>Litoria chloris</i>	0 (20)	100 (13)	0 (7)	48.3 ± 31.7
<i>Limnodynastes tasmaniensis</i>	0 (18 ^c)	0 (0)	0 (18)	N/A

^aAll dead or euthanized frogs exhibiting symptoms of severe chytridiomycosis were confirmed positive for infection with *B. dendrobatidis* by post-mortem histology.

^bNumber of sporangia per linear mm skin surface ± sd.

^cTwo additional *L. tasmaniensis* were removed from the experiment because their captive-rearing enclosure was contaminated.

infection status by histology of the skin. Insufficient volume was available from these juvenile frogs for total white and red blood cell counts, but relative counts were made from blood smears. We stained blood smears with Diff-Quik (Dade Behring Inc: Newark, DE, USA) for identification of lymphocytes, monocytes, neutrophilic, eosinophilic and basophilic leukocytes. We calculated the ratio of leukocytes to erythrocytes by counting the number of leukocytes among at least 300 erythrocytes.

To test for overall differences among temperature treatments and between infected and uninfected frogs, we used multivariate oneway randomization tests [R. A. Alford, RAMAN v 1.73, based on Manly (1991)]. When these were significant, we then used Mann–Whitney *U*-tests to determine which blood parameters differed significantly.

Results

Survival following experimental exposure to *B. dendrobatidis*

Survival following exposure to *B. dendrobatidis* zoospores varied among species (logrank test on censored survival data, $P < 0.001$, Fig. 1). All (18/18) of the exposed *Lim. tasmaniensis* survived to the termination of the experiment at day 108, whereas only 5% of exposed *Lit. caerulea* (1/20) and *M. fasciolatus* (1/20) survived. Survival of chytrid-exposed *Lit. chloris* was intermediate, with 35% of the animals (7/20) surviving to the end of the experiment (Fig. 1). The survival times of individual frogs of each species also reflected their comparative sensitivity to lethal infection. The median survival time of *Lit. caerulea* was only 8.9 days (mean ± se: 28.8 ± 2.1), whereas the median survival time of *M. fasciolatus* and *Lit. chloris* was 21.5 days (56.6 ± 3.9) and 26.8 days (65.1 ± 7.4), respectively. All of the *Lim. tasmaniensis* survived longer than the 108-day experimental period (Fig. 1).

Infection status of exposed frogs

No exposed animals were positive for infection by *B. dendrobatidis* on the toe removed before chytrid exposure. The level of infection detectable on the toes at 10 days post infection varied with species. Sixty per cent (12/20) of the *Lit. caerulea* and 20% (4/20) of *M. fasciolatus* were positive for *B. dendrobatidis*, whereas none of the *Lit. chloris*

(0/20) or *Lim. tasmaniensis* (0/18) were positive at 10 days post infection (Table 1). All of the dead animals including those euthanized with severe clinical chytridiomycosis were heavily infected as determined by the histology of the toe clips, but infection was not detected in surviving frogs 108 days after experimental exposure (Table 1).

The intensity of infection at the time of death differed significantly among *Lit. caerulea*, *Lit. chloris* and *M. fasciolatus* (Kruskal–Wallis test, $\chi^2_2 = 20.248$, $P < 0.001$; Table 1). *Batrachochytrium dendrobatidis* sporangia were the most dense on the toes of *M. fasciolatus*. Histological sections of the toes of *Lim. tasmaniensis* toes often revealed missing or sloughing stratum corneum and were all negative for infection. Therefore, infection intensity was not determined for this species.

Effectiveness of natural mixtures of skin peptides against *B. dendrobatidis*

Natural mixtures of skin peptides from all four species were capable of inhibiting the growth of *B. dendrobatidis* *in vitro*. Significant dose-dependent inhibition was detected for skin peptides from individuals of *Lit. caerulea* (mean MIC = 271 µg mL⁻¹), *Lit. chloris* (mean MIC = 267 µg mL⁻¹) and *Lim. tasmaniensis* (mean MIC = 133 µg mL⁻¹). Individual *M. fasciolatus* did not provide sufficient peptides to test. However, peptides pooled from groups of three individuals were tested. These mixtures of peptides inhibited *B. dendrobatidis* growth, and the mean MIC was estimated at 272 µg mL⁻¹. Representative experiments showing growth inhibition curves and MICs of individual frogs are shown (Fig. 2). MIC equivalents cm⁻² differed among species (Kruskal–Wallis test, $\chi^2_3 = 10.475$, $P = 0.0149$). The pattern of MIC equivalents mirrors the pattern of survival in these species; species differ in their effectiveness of skin peptide defenses per surface area of skin along the ordered gradient of per cent survival after *B. dendrobatidis* exposure. A significant trend is indicated (Jonckheere–Terpstra statistic = 178, exact two-tailed $P = 0.001$).

Response of leukocyte populations to infection in *Lit. chloris*

Leukocyte populations in the blood of *Lit. chloris* did not differ significantly among the three temperature treatments

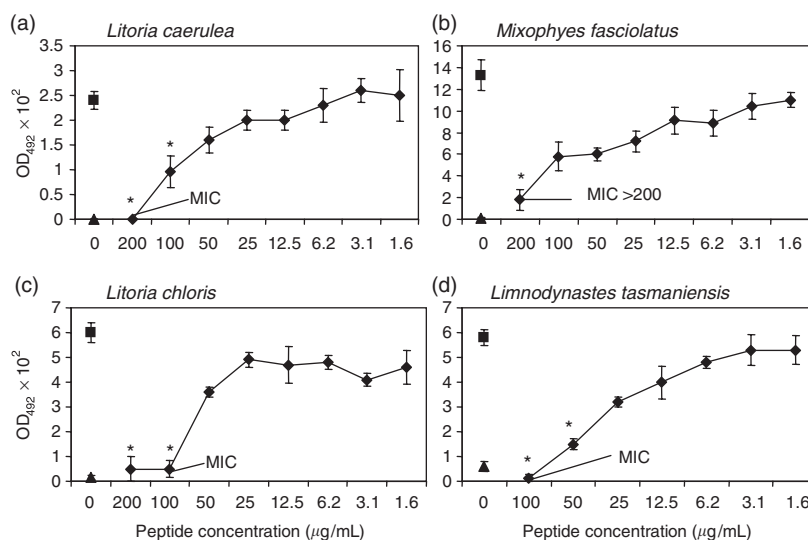


Figure 2 Growth inhibition of *Batrachochytrium dendrobatidis* zoospores by natural mixtures of peptides from representative (a) *Litoria caerulea*, (b) *Mixophyes fasciolatus*, (c) *Litoria chloris* and (d) *Limnodynastes tasmaniensis*. Growth was measured on day 7 (peak of growth) as optical density at 492 nm (OD_{492}). Each data point represents the mean \pm SE of five replicate cultures. *Significantly less than positive control for growth by Student's *t*-test, $P \leq 0.05$. Triangles represent the negative control, zoospores cultured with 0.4% paraformaldehyde; squares represent the positive control, live cells with no added peptides. Note that axes differ among panels because assays vary in terms of maximal positive control growth. Minimal inhibitory concentration (MIC) is defined as the lowest concentration of added peptide at which no significant growth was observed.

Table 2 Leukocyte populations of juvenile *Litoria chloris*

Treatment	Uninfected	Infected
<i>n</i>	24	19
Mean \pm SE		
Neutrophils	13.93 \pm 1.34	7.72 \pm 1.46
Eosinophils	0.53 \pm 0.12	0.22 \pm 0.09
Basophils	3.69 \pm 0.41	6.26 \pm 1.20
Lymphocytes	75.36 \pm 2.25	71.2 \pm 5.41
Monocytes	6.5 \pm 1.14	6.22 \pm 0.70
Ratio of leukocytes to 1000 erythrocytes	2.7 \pm 0.31	2.15 \pm 0.52

Mean count out of 100 leukocytes for five cell types and the ratio of leukocytes to 1000 erythrocytes. Numbers in bold indicate significant differences, $P < 0.05$.

(multivariate randomization test, $P = 0.595$). They did differ between infected and uninfected frogs across the temperature treatments (multivariate randomization test, $P = 0.011$, Table 2). Neutrophilic and eosinophilic granulocyte counts were lower, and basophilic granulocyte counts were higher in the blood of infected frogs (Mann–Whitney *U*-tests, $P = 0.006$, 0.046 and 0.026, respectively).

Discussion

Varied survival following experimental infection

The emergence of amphibian chytridiomycosis has devastated some species while leaving others almost untouched.

Here, we show that when environmental conditions and pathogen strain are held constant, the outcome of infection by *B. dendrobatidis* varies among species. *Lit. caerulea* and *M. fasciolatus* were highly susceptible to chytridiomycosis and experienced 95% mortality. *Lit. chloris* was less susceptible with 65% mortality, and *Lim. tasmaniensis* exhibited resistance to infection. Only *Lit. chloris* showed a non-constant survival curve, reaching a temporary plateau between 50 and 70 days post-exposure (Fig. 1), indicating a potential adaptive immune response. Apponyi *et al.* (2004) found that *Limnodynastes* spp. produced large volumes of secretions containing small inactive peptides. Here, we show that *Lim. tasmaniensis* secretions are active against *B. dendrobatidis* and this species survives infection better than three other species. Future studies should investigate the active components of *Lim. tasmaniensis* skin secretions. Survival, compared among these four species, tended to be higher in species with more effective skin peptides. None of these species are known to be in population decline and are of the least conservation concern (Global Amphibian Assessment, 2006, www.globalamphibians.org). Although *Lit. caerulea* and *M. fasciolatus* are highly susceptible to chytridiomycosis in the laboratory, perhaps environmental or behavioral characteristics provide some protection in the field. Experimental infection experiments in field enclosures may be ethically problematic.

At least some of the variation among species in patterns of survival under controlled environmental conditions appears to be caused by differences in skin peptides. Figure 3 shows that species reaching a level (roughly 0.02 MIC equivalents cm^{-2}) of peptide effectiveness may be protected

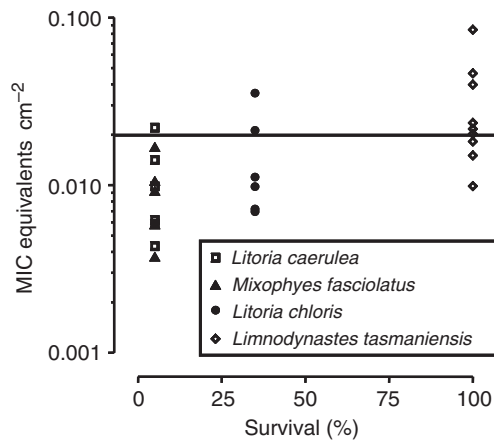


Figure 3 Concentrations of skin peptides, standardized to minimal inhibitory concentration (MIC) equivalents, of each individual frog tested, plotted against proportion of that species surviving 108 days post-exposure. MIC equivalents are defined in terms of the total amount of peptides (μg) recovered from each frog per 1 cm^2 surface area, divided by the experimentally determined minimal inhibitory concentration (MIC; $\mu\text{g mL}^{-1}$) for each species. A Jonckheere–Terpstra trend test comparing the MIC levels of the three groups of species with differing survival rates indicated that they differed significantly in peptide concentrations (Jonckheere–Terpstra statistic = 178, exact two-tailed $P = 0.001$). Examining the data suggests that there may be a level of peptide effectiveness of c. $0.02\text{ MIC equivalents cm}^{-2}$ needed for survival that is relatively constant across species, as indicated by the horizontal line.

from chytridiomycosis. This is consistent with a previous study of Australian rainforest species demonstrating population trends associated with peptide effectiveness against *B. dendrobatidis* in which species of conservation concern tended to have $\text{MIC equivalents cm}^{-2} < 0.02$ (Woodhams *et al.*, 2006b). It should be pointed out that the x -axis of Fig. 4 of that study should be labeled 0–0.08, and was erroneously labeled in publication.

In our experiments, species with better skin peptide defenses had lower infection intensity and higher survival rates (Table 1, Fig. 3). In *Lit. caerulea* that died of chytridiomycosis, we observed an infection intensity within the range previously reported by others (mean = $94.3\text{ sporangia mm}^{-1}$, Berger *et al.*, 2005b). There may be a species-specific threshold of infection intensity that leads to mortality and thus determines the length of time survived after exposure to *B. dendrobatidis* (Carey *et al.*, 2006). *Lim. tasmaniensis* may also reduce infection loads by shedding infected skin layers more efficiently than other species; this may explain the lack of stratum corneum on many histological sections of the toes of this species. This result is not unexpected, given that intermolt period varies among amphibian species (Duellman & Trueb, 1986; Weldon & du Preez, 2006), and infection intensity can be reduced after molt (Berger *et al.*, 2005b). Excessive skin shedding is a common clinical sign of chytridiomycosis (Nichols *et al.*, 2001; Parker *et al.*, 2002; Berger *et al.*, 2005a,b) and may be a

host mechanism to reduce infection loads or a manipulation of the host to increase keratinized substrate for the pathogen. In future studies, a comparison of the rates of shedding and growth rate between infected and uninfected individuals and across species could help determine the effects of shedding on the outcome of infection.

Other potential sources of variation among species include the distribution of granular glands. Some species have granular glands evenly distributed on all skin surfaces while others may have areas of concentrated skin glands (Duellman & Trueb, 1986; Bevins & Zasloff, 1990; Lacombe *et al.*, 2000; Warburg *et al.*, 2000; Apponyi *et al.*, 2004). Antimicrobial peptides may spread across the skin surface within the mucous layer. However, the protective mucous layer may be thin or missing from some skin surfaces. The ventral surfaces of the toes of *Lit. caerulea* lack granular glands (Berger *et al.*, 2005). Both the toes and inguinal region of anurans may be particularly vulnerable to water-borne infectious zoospores of *B. dendrobatidis* that can be found on moist substrates (Berger *et al.*, 2005; Lips *et al.*, 2006; Puschendorf & Bolaños, 2006; Weldon & du Preez, 2006). Amphibian species vary in other aspects of skin physiology including structure (Schwinger, Zanger & Greven, 2001) and the presence of lipid glands (Warburg *et al.*, 2000). It is unknown whether species vary in their ability to release defensive peptides onto the skin surface in response to stressors including norepinephrine or infectious agents.

Changes in circulating leukocytes following infection with *B. dendrobatidis*

Amphibians have well-developed immune systems including both adaptive and innate immune responses (Carey *et al.*, 1999; Rollins-Smith, 2001). Adaptive immunity to *B. dendrobatidis* has not been reported in the literature. Innate immune function in amphibians is not thoroughly understood (Boman, 1995). For example, little is known about leukocyte population responses to infection in amphibians (Wright & Whitaker, 2001). The typical amphibian response to stressors includes increased neutrophil counts and decreased lymphocyte counts (Bennet & Daigle, 1983; Cooper *et al.*, 1992; Carey, Maniero & Stinn, 1996; Maniero & Carey, 1997). However, *Lit. chloris* did not show this response in this study and the response did not vary with the thermal regime including a 2 days cold shock. Frogs infected with the amphibian chytrid exhibited relatively fewer circulating neutrophils and eosinophils, and more basophils, than uninfected frogs. This response is similar to the one found in frogs with red leg disease attributed to *Pseudomonas hemophila* (Kaplan, 1952), which showed decreases in neutrophils and eosinophils and an increase in lymphocytes. Insufficient blood volume precluded the total cell counts, but one explanation of these proportional changes in circulating granulocytes may be that cells were moving toward infected areas. Weldon & du Preez (2006) suggest that the tubercles on the feet and toes of some frogs that are heavily keratinized and likely to contact infectious zoospores are often subjected to abrasion. Recruitment of leukocytes to

these areas and inflammatory responses around microscopic ulcerations and lesions common with chytridiomycosis may be important for an adaptive immune defense (Nichols *et al.*, 2001; Parker *et al.*, 2002; Berger *et al.*, 2005). Some innate defense peptides including amphibian temporin may be involved in chemoattraction of leukocytes (Chen *et al.*, 2004; Kurosaka *et al.*, 2005). The bradykinin-like peptides secreted from amphibian skin (Pukala *et al.*, 2006; Suzuki *et al.*, 2007) may act to increase vascular permeability, but this has not been tested in amphibians. These peptide defense mechanisms may be particularly important for skin wounds, although further study is needed to uncover whether amphibian skin peptides can signal an effective adaptive immune response against *B. dendrobatidis* at areas of skin abrasion.

It is clear from our study that innate immune defenses are an important component of resistance to chytridiomycosis and may partially account for variation in susceptibility among species. Skin peptides may be more essential in immune defense than was previously considered, although little is known about their mode of operation *in vivo* or their role in the microbial environment of the skin. We suggest that species-specific innate immune defenses are important determinants of infection intensity and subsequent morbidity or resistance to chytridiomycosis.

Acknowledgements

The authors are grateful for the assistance of T. Langkilde, S. Bell, B. Mott, S. Reilly, L. Reilly, B. Cullen, J. Whittier and D. Cochrane. Thanks are due to volunteers and friends of the Amphibian Research Centre, including C. Steele, R. Hobbs, S. Barber, D. Taylor, T. Dornum, N. Nolas, M. Velik-Lord, A. Crigo, N. Evangelou, P. Oliver, A. Marantelli and P. Marantelli. The authors thank the CSIRO amphibian disease research team, including L. Berger, A. Hyatt, V. Olsen and D. Boyle. Thanks are due to R. Potter and K. Hoek for helpful comments on a previous version of this manuscript. Ethical approval for all experiments was granted from James Cook University (A451 and A699_01) and the Amphibian Research Centre. Subcontracts to R.A.A. and L.R.S. from Integrated Research Challenges in Environmental Biology (IRCEB) grant IBN-9977063 (J.P. Collins, P.I.) and grant IOB-0520847 (L.R.S., P.I.) from the US National Science Foundation funded this research. D.C.W. 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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