Variable Susceptibility to an Emerging Infectious Disease, Chytridiomycosis, in Anurans

Picture of *Nyctimystes dayi* by N. Kenyon

Thesis submitted by

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STATEMENT OF SOURCES DECLARATION

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I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given. All research reported in this thesis received the approval of the ethics committees and the QPW.

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Signature Date

STATEMENT OF CONTRIBUTION OF OTHERS

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Two publications have been submitted from Chapter 2. Dr. Andrea Phillott provided input on the manuscripts and generously provided her frog monitoring population to compare the true identity of the frogs via toe-tipping and photographic identification method. Professor Ross Alford gave valuable advice on statistical analysis and writing the manuscript.

Chapter 5 is in the process of being submitted to Journal of Zoology with the help of Professor Ross Alford. The design of this experiment was an ongoing development and Ross provided valuable input during that time, including the analysis.

Chapter 6 has been submitted to Journal of Herpetology with the help of Sara Bell and Professor Ross Alford. Sara Bell offered advice on culturing *Bd* and writing the manuscript. Professor Ross Alford gave valuable advice on the experimental design, statistical analysis and writing the manuscript, which is currently in revision.

The submission of several chapters to different journals required the use of different English styles and reference formats which were retained in this thesis.

Declaration of ethics

Animals were obtained and all data collected under Animal Ethics Approval A960 granted by James Cook University Animal Ethics Committee, and Scientific Purposes Permits WITK01932505 and WISP01764304 granted by Queensland Parks and Wildlife Service.

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ABSTRACT

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Chytridiomycosis is an emerging infectious amphibian disease, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), and has caused numerous amphibian declines around the globe. Chytridiomycosis can be lethal in many amphibian species but not in others, leading to three different responses to exposure, 1) the amphibian becomes infected with *Bd* and dies, 2) the amphibian becomes infected with *Bd* and survives and 3) the amphibian does not become infected even though it occurs in a habitat where *Bd* exists. My project aimed to increase our understanding of the causes of these interspecific differences. I investigated the hypotheses that they could be caused by innate immune defences (antimicrobial peptides) against *Bd*, innate or adaptive responses of individuals through microenvironment selection, or behavioural avoidance of infective water.

I found evidence for all three mechanisms. *In vitro*, antimicrobial peptides (AMPs) of *Litoria genimaculata* (vulnerable to infection with *Bd* with highland population declines followed by recovery) and *L. rheocola* (vulnerable to infection with *Bd* with severe declines at higher elevations with little or slow recolonisation) can completely inhibit *Bd* growth. I also found large seasonal variation in antimicrobial peptide defences in both species. This may result from physiological shifts driven by temperature, or may reflect adaptation to seasonal fluctuations in the risk of infection. The proportion of *L. genimaculata* from high elevation populations, which have experienced strong viability selection pressure imposed by chytridiomycosis outbreaks, that produced AMPs that effectively inhibited *Bd in vitro,* was significantly higher than in low elevation populations, which have been protected from chytridiomycosis by environmental factors. There was also evidence that high elevation populations produced AMPs that differed slightly in chemical composition from those produced by low elevation populations. However, when individuals of either frog species produced AMPs that inhibited the growth of *Bd*, the effectiveness of AMPs from high and low elevation populations did not differ significantly. This suggests that any responses to selection may have occurred through an increase in the proportion of individuals producing effective AMPs, with no change in the types of AMPs produced. Antimicrobial peptide defences did not differ significantly between high and low elevation population of *L. rheocola*, suggesting that this species may have recolonised

ABSTRACT

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upland areas. On the other hand, *L. rheocola* had more effective antimicrobial peptide defences against *Bd* than *L. genimaculata* and may have experienced stronger selection pressure after the appearance of chytridiomycosis.

Thermal microenvironments selected in the laboratory corresponded to those expected from decline patterns observed in the wild. *Litoria caerulea* (vulnerable to infection with *Bd* but no population declines due to chytridiomycosis have been detected) selected warm and hot environments significantly more often than *L. genimaculata*. Additionally, although not significant, there was a trend that intensity of *Bd* infection in all three species was more likely to decrease over time in individuals that had a choice of hydric and thermal microenvironments than in frogs that were housed under standard environmental conditions. There was also evidence of disease avoidance behaviour; some *L. caerulea* and *L. genimaculata* chose uncontaminated water significantly more often than water that contained *Bd* zoospores. None of the frog species were able to completely avoid water containing *Bd* zoospores, possibly in part because their pond selection was also influenced by side fidelity.

My study demonstrates the complexity of host-pathogen interactions and that multiple factors, including innate immune defence, microenvironment selection and disease avoidance behaviour, can influence the progress of chytridiomycosis and should be considered when establishing species specific management plans.

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Diseases

Introduction

A disease can be defined as a reduction in health or loss of vital processes that is caused by a pathogen (Hale et al., 1995; Begon et al., 1996). Pathogens and diseases have co-existed with the world's flora and fauna throughout the history of life, and have probably strongly influenced biodiversity throughout evolutionary time. In evolutionary terms, it is not to the benefit of a pathogen to kill its host, as it then loses its own environment (Woodroffe, 1999), however, many factors, such as environmental conditions (Gubbins and Gilligan, 1997) or migration (Hosseini et al., 2004), can influence and change the dynamic interaction between a host and pathogen and may result in higher mortality rates of the host (Elliot et al., 2002; Berger et al., 2004; Hosseini et al., 2004; Ibelings et al., 2004). For example, drought in Asia forced rats to move from rice fields into human populated houses and their fleas transferred the bacterium *Yersinia pestis*, better known as "the plague", to the "naive" human population. This disease reduced the European human population by 25-33 percent in the fourteenth century (Stolley and Lasky, 1995). Today, infectious diseases such as acquired immune deficiency syndrome (AIDS), malaria, severe acute respiratory syndrome (SARS), ebola virus, tuberculosis and more recently the avian influenza virus H5N1, are responsible for around 19% of all recorded human deaths (Morens et al., 2004; World Health Organization, 2004; Oner et al., 2006).

Emerging Infectious Diseases (EIDs)

All the above-mentioned diseases were at some time categorised as "emerging infectious diseases" (EIDs). Epidemiologists define an emerging infectious disease as one that has recently increased either or both of its geographical range or the range of host species it infects (Morse, 1995; Daszak et al., 2001; Dobson and Foufopoulos, 2001). In the past, research has focused mainly on EIDs that affect humans directly or result in economic losses, such as those affecting domestic and farmed fauna, including hoofed animals, crocodiles and fishes (Blake, 1968; Huchzermeyer et al., 1994; Cleaveland et al., 2001; Cheatwood et al., 2003; Caldow et al., 2004). Some wildlife diseases, such as chronic wasting disease (CWD) (Miller et al., 2006) and rinderpest

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(Rossifer et al., 1987; Mariner et al., 2003; Gabre-Madhin and Haggblade, 2004), originated from farmed animals and consequently have received substantial attention. Only a few emerging infectious diseases of wildlife, such as scabies in Europe, have received similar levels of attention (Fuchs et al., 2000; Rossi et al., 2007).

It has been emphasised that wildlife are likely to be important reservoirs for many pathogens that affect humans or their livestock, and that closer and more frequent interactions between humans and wildlife may increase the risk that EIDs of wildlife pose for human health (Bosch and Martinez-Solano, 2006; Colizza et al., 2006; Cunningham, 2007). Additionally, the economic value of wildlife has increased as tourism has become more popular (Colizza et al., 2006; Daszak, 2006). As a consequence, EIDs of wildlife have lately received more attention (Berger and Speare, 1998; Daszak and Cunningham, 1999; de Castro and Bolker, 2005). In particular, commonly used conservation tools, such as re-introduction and translocation, have been investigated to understand their effects on spread and outbreaks of diseases (Tompkins et al., 2003; Walker et al., 2004; de Castro and Bolker, 2005).

Recognition of the importance of wildlife EIDs may have come too late; in the last few decades a number of EIDs have already caused large-scale declines in wildlife (Table 1.1). These include the rinderpest that devastated Africa's wildlife (Rossifer et al., 1987; Mariner et al., 2003; Gabre-Madhin and Haggblade, 2004); devil facial tumour disease (DFTD) in Tasmania, Australia (Hawkins et al., 2006; McCallum and Jones, 2006); and chytridiomycosis worldwide (Berger et al., 1998; Bosch and Martinez-Solano, 2006; Pounds et al., 2006).

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Table 1.1 Emerging infectious diseases (EIDs) and their impact on wildlife.

Disease	Area	First Reported Outbreak	Mortality Rate	Hosts	Possible Source of Outbreaks	Reference
HPAI avian influenza virus (includes the fowl plague)	World wide	1878, 18 outbreaks since 1959	150 million birds in Asia	Birds (Order Anseriformes and Charadriiformes)	Wild bird contact with poultry	Alexander, 2000
Devil facial tumour disease (DFTD)	Tasmania, Australia	1996 to present	Up to 80% decline in areas of outbreak	Tasmanian devil *Sarcophilus harrisii*	Unknown	Hawkins et al., 2006; McCallum and Jones, 2006
Avian malaria (*Plasmodium* sp), transmitted by mosquito	World-wide	Early 1900's	Extinction of 9 avian species	Birds	Introduction of intermediate host (mosquito)	Warner, 1968; van Riper et al., 1986
Crayfish plague 2, caused by fungus *Aphanomyces astaci*	Europe	1860s	Declines of all native freshwater crayfish	Freshwater crayfish	Introduction of infected North American crayfish in 1860s	Edgerton et al., 2004; Gherardi, 2006
Scabies transmitted by mites (*Sarcoptes rupicaprae*)	Europe (mainly Austria, Italy and Slovenia)	1952	Declines between 49-77% of chamois	Alpine chamois (*Rupicapra rupicapra*) and other sympatric ruminants	Unclear, bad weather, infestation of endo-parasites,	Fuchs et al., 2000; Rossi et al., 2007
Rinderpest caused by virus (*Rinderpest virus*)	Africa	1979	Unknown	Ruminants	Unknown	Rossifer et al., 1987

Wilcove et al. (1998) investigated the impact of EIDs on wildlife species in the USA, using the IUCN database. This database is not comprehensive and as the authors correctly pointed out, in many cases the extent of the impact of any disease is unknown. Nevertheless, 37 percent of listed avian species and 11 percent of all vertebrate species have been classed as vulnerable or endangered due to EIDs (Wilcove et al., 1998). Repeating the investigation with vertebrate species on a worldwide scale resulted as well in 11 percent being classed as vulnerable or endangered due to EIDs. To avoid or reduce future extinction events due to EIDs we need to identify risks and gather more information on host-parasite dynamics (Woodroffe, 1999; Cleaveland et al., 2001).

EIDs in ectotherms

We have substantial knowledge of host-parasite dynamics in humans and similar endothermic animals but this understanding may not be transferable to the host-parasite dynamic of terrestrial ectotherms, which appear to differ substantially (Delajara, 1991; Waldmann, 2003). For example, water balance - especially in amphibians as their skin is highly permeable - and body temperature experience dramatic changes as ectotherms modulate their internal environment to conform to physiological needs through microhabitat selection and the use of external sources of heat (Lillywhite et al., 1998; Hillyard, 1999; Wolcott and Wolcott, 2001; Seebacher and Alford, 2002; Beck and Jennings, 2003). Body temperature can change by at least 15°C within one hour (Tattersall et al., 2006). Physical performance, innate and acquired immune defences, and digestive function, as well as other systems, may fluctuate as a result (Huey and Stevenson, 1979; Huey and Kingsolver, 1989; Blanford and Thomas, 1999; Freidenburg and Skelly, 2004).

Consequently, the use of external sources of heat and microhabitat selection can greatly affect the host-parasite interactions of ectotherms. Carruthers et al. (1992) showed that the clearwinged grasshopper (*Camnula pellucida*) eliminated infection by a fungal pathogen (*Entomophaga grylli*) by exploiting external sources of heat to increase its internal body temperature. It is not clear if *C. pellucida* increased its body temperature to higher than normal or if its natural temperature range caused the mortality of the

pathogen. Nevertheless, behavioural fever as a response to a microbial infection has been observed in several orders of terrestrial ectotherms (Myhre et al., 1977; Lefcort and Blaustein, 1995; Blanford et al., 1998; do Amaral et al., 2002; Elliot et al., 2002).

Amphibians

Introduction

Amphibia (caecilians, frogs and salamanders) is a diverse class that consists of 5,948 recognized species from which around 5,067 described species belong to the Order Anura (frogs and toads) (Frost et al., 2006; Alford et al., 2007). The earliest frog fossil indicates that they have been on earth for more than 100 million years (Alford et al., 2007). In the early 1980s several herpetologists reported a dramatic series of amphibian declines from many parts of the world (Hayes and Jennings, 1986; Heyer et al., 1988; Barinaga, 1990; Blaustein and Wake, 1990) but mainly in protected, pristine high elevation areas, such as the montane rainforests of Australia, Central America and South America (Bradford, 1991; Hays et al., 1996; Berger and Speare, 1998; Berger et al., 1998; Lips, 1998; Daszak et al., 1999; Green et al., 2002). After years of debate it has been generally accepted that these declines occurred or are occurring at a greater rate than the overall global biodiversity crisis (Blaustein and Wake, 1990; Pechmann et al., 1991; Wake, 1991; Blaustein, 1994; Lips, 1998; Meyer et al., 1998; Stallard, 2001; Daszak et al., 2005). Today, it is recognised that more than 30 percent of amphibian species are declining, and these species make up a third of all critically endangered animals (IUCN, 2006; Smith et al., 2006).

Obvious potential causal factors for the amphibian declines, such as habitat loss and change (Adams, 1999), agricultural practices, harvesting of wild populations, effects of pesticides (Sparling et al., 2001; Blaustein et al., 2003; Davidson and Knapp, 2007), introductions of predators/competitors (Fellers and Drost, 1993; Hecnar and McLoskey, 1997) and climate change, including increased levels of UV-B radiation (Blaustein and Wake, 1995; Lips, 1998; Pessier, 2002; Blaustein et al., 2003; Reading, 2007; Van Uitregt et al., 2007; Wake, 2007; Whitfield et al., 2007), have been investigated and may have contributed to some declines. However, in the last decade it has become clear

that the disease chytridiomycosis has contributed to many amphibian declines (Berger et al., 1998; Bosch et al., 2001; Bonaccorso et al., 2003; Muths et al., 2003; Burrowes et al., 2004; Bosch and Martinez-Solano, 2006; Lips et al., 2006; Skerratt et al., 2007).

Chytridiomycosis

The fungus *Batrachochytrium dendrobatidis* (*Bd*) (Phylum Chytridiomycota) is the causative agent of the amphibian disease chytridiomycosis (Berger et al., 1998; Longcore et al., 1999). Berger et al. (1998) were the first scientists to demonstrate that *Bd*, in a controlled environment, is fatal to frogs. It is the first chytrid fungus known to cause mortality in vertebrates (Berger and Speare, 1998).

The asexual life cycle of *Bd* consists of dispersal, growth, amplification and infectious phases (Berger et al., 2005a). During dispersal, flagellated, motile zoospores may either re-enter the infected amphibian host or the aquatic environment (Figure 1.1). The longevity of zoospores and potential migration distance in natural aquatic environment, such as a fast flowing rainforest creek, is unknown. When the zoospore reaches a suitable host it encysts in the keratinised epidermis, which includes the mouthparts of tadpoles (Blaustein et al., 2005; Drake et al., 2007). Once encysted, it develops rhizoids (hair/root-like structures) in several directions and can develop into one or colonial (with separate divisions) sessile zoosporangia within several days (Longcore et al., 1999; Berger et al., 2005a). The amplification phase begins at this time. Between four and ten zoospores develop within the zoosporangium (Berger et al., 2000). The zoosporangium forms one discharge tubule that opens to the surface of the epidermis to allow the zoospores to be released (Longcore et al., 1999; Berger et al., 2000). A sexual life cycle has been observed in other Chytridiomycetes (Ibelings et al., 2004) and evidence suggests that there may also be sexual reproduction in *Bd* (Morgan et al., 2007), but further studies are needed to demonstrate this.

The mechanism by which *Bd* causes pathogenesis is unclear. As the colonisation of the skin by zoospores progresses, the described effects are similar among anuran species. These include loss of energy, appetite and reflexes, tetany, macroscopic lesions,

excessive shedding of the epidermis and in tadpoles, loss of teeth on oral discs (Berger et al., 1999; Fellers et al., 2001; Nichols et al., 2001; Bradley et al., 2002; Pessier, 2002; Mazzoni et al., 2003). It has been suggested that the presence of zoosporangia on the ventral epidermis of the frog may reduce water uptake (Berger et al., 1998). Personal observation in the laboratory has shown that infected frogs tend to sit in water before death, possibly to maximise water uptake. Voyles et al. (2007) compared blood samples from healthy and diseased *Litoria caerulea* and suggested a loss of electrolytes due to reduced circulation rather than loss of water uptake. However, the exact mechanism by which chytridiomycosis causes death is yet to be identified.

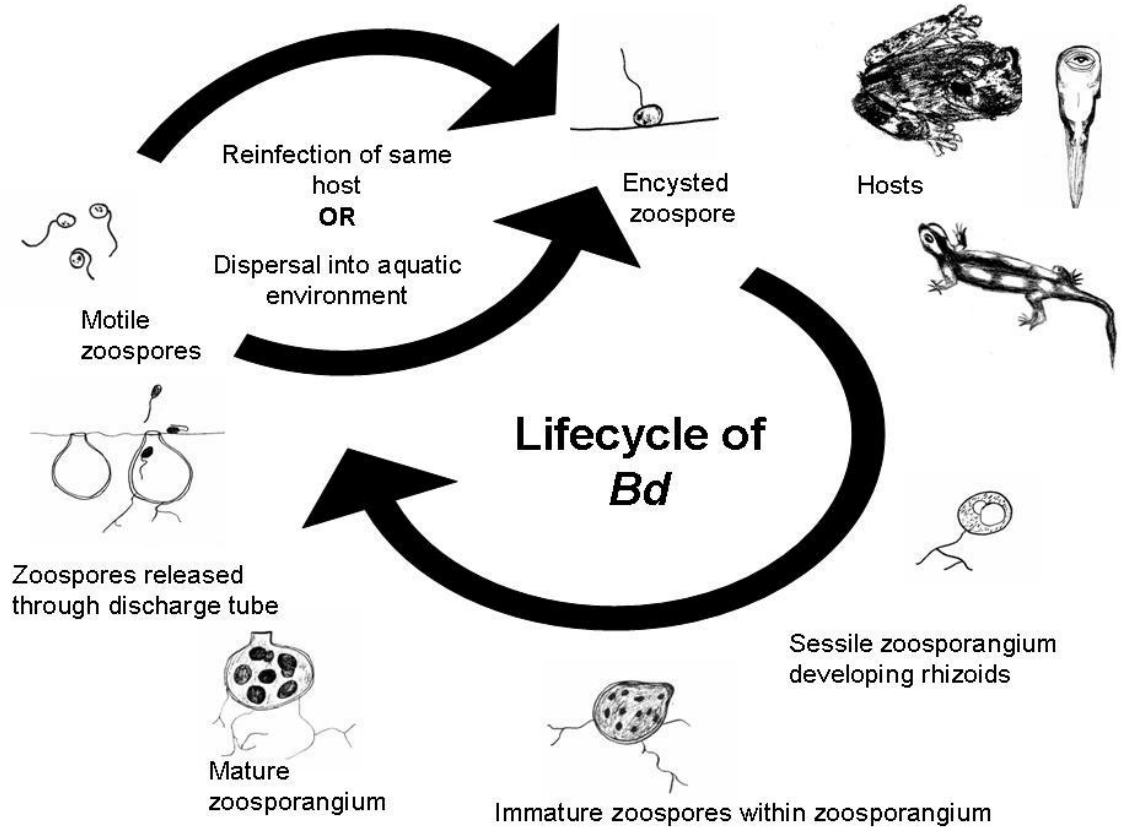

Figure 1.1. Life cycle of *Batrachochytrium dendrobatidis* (*Bd*), a fungus that causes chytridiomycosis in amphibians. Adapted from Woodhams (2003).

The combination of *Bd* being a) low in host specificity and able to infect multiple amphibian species in each geographical area (Berger et al., 1998), b) able to survive if water is present in the absence of its vertebrate host (Daszak et al., 1999; Daszak et al., 2003; Lips et al., 2006; Walker et al., 2007) and c) able to infect but not kill some hosts (Retallick et al., 2004; Kriger and Herman, 2006), may have contributed to its global distribution. To date, 233 wild amphibians worldwide have been found infected with *Bd* (Figure 1.2; Appendix A). In Australia, 57 wild anuran species have been diagnosed positive for *Bd*; 14 of these appear to have suffered declines (McDonald and Alford, 1999). Chytridiomycosis has been listed since 2002 as a key threatening process under the Australian Environment Protection and Biodiversity Conservation Act (EPBC).

The worldwide occurrence of *Bd* has raised the question of its origin (Daszak et al., 2003; Weldon et al., 2004; Rachowicz et al., 2005; Fisher and Garner, 2007; Skerratt et al., 2007). To date, the earliest record of an amphibian diagnosed with *Bd* is from the african clawed frog (*Xenopus laevis*) from the western cape coastal lowland in 1938 (Weldon et al., 2004). This species was used for pregnancy tests and exported world wide between the 1930's and 1960's (Weldon et al., 2004). The lack of historical evidence of *Bd* in amphibians favours the novel or spreading pathogen hypothesis (Rachowicz et al., 2005), which states that *Bd* has a single local point of origin and spread globally over a short time period. If this is so, why was there not an outbreak in the 1960's? Why 20 to 30 years later? In order to reject the hypothesis of a novel or spreading pathogen, researchers have tried to trace the emergence of *Bd* by analysing tissue samples from museum specimens. To date, no study has found *Bd* in museum specimens from other areas than western cape Africa before the export of *X. laevis* (Burrowes et al., 2004; Ouellet et al., 2005). However, amphibians decompose quickly and hence the probability that an infected amphibian will be collected and preserved by a museum may be very low. The additional low likelihood of actually finding infected specimens within museum collections makes the complete resolution of the history of *Bd* almost infeasible.

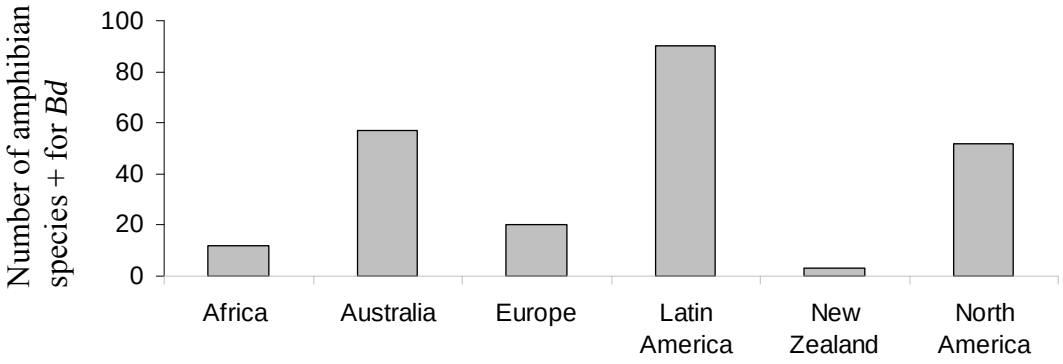

Figure 1.2. The global distribution of amphibian species known to be infected with *Batrachochytrium dendrobatidis* as of 30 June 2008.

The endemic pathogen hypothesis differs from the spreading pathogen hypothesis in that it implies that *Bd* has been in the environment for a longer period and other abiotic factors have negatively influenced the host-pathogen interaction and increased its virulence (Morehouse et al., 2003; Rachowicz et al., 2005; Pounds et al., 2006; Skerratt et al., 2007; Wake, 2007; Whitfield et al., 2007). These hypotheses may not be mutually exclusive in that the pathogen may have originated from *X. laevis* but additional changes in abiotic factors may have contributed to the later observed outbreaks. Molecular evidence supports both hypotheses (Fisher and Garner, 2007). Low genetic diversity, suggesting a recent global spread, was found on a global scale by comparing 35 *Bd* strains from Africa, Australia, North and Central America (Bosch and Martinez-Solano, 2006). Alternatively, Morgan et al. (2007) found, by comparing nine local *Bd* isolates (Sierra Nevada, California) with four *Bd* isolates of frog species from Africa, Australia, North and South America, greater genetic divergence on a local than global scale. It is important that current research investigates abiotic factors that may influence the prevalence and hence host-pathogen dynamics after an initial outbreak.

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Climate

One of the abiotic factors that has been intensively investigated to determine its potential influence on chytridiomycosis is climate. Laboratory study has shown that *Bd* is more virulent in conditions of high humidity (Woodhams, 2003) as *L. chloris* developed chytridiomycosis more slowly in dry environmental conditions than in wetter conditions. However, several field studies have detected a higher prevalence of *Bd* in Australian anuran species, including *L. genimaculata*, *L. rheocola* and *Nyctimystes dayi,* in winter (dry season) rather than summer (wet season) (Bradley et al., 2002; Berger et al., 2004; McDonald et al., 2005; Woodhams and Alford, 2005; Kriger and Herman, 2006). This suggests that temperature may have a greater influence on prevalence of *Bd* than does humidity.

The strong effects of temperature have been documented in several other studies. Woodhams et al. (2003) found that holding *L. chloris* at 37°C for less than 16 hours cured them of *Bd* infection. Additionally, Drew et al. (2006), who used field observations from 56 sites in Australia, found a significant relationship between *Bd* and temperature only, not elevation or rainfall. They concluded that *Bd* is more likely to occur at sites where the average summer maximum temperature is below 30°C.

While climate, especially temperature, has an impact on host-pathogen dynamics, there are large differences in susceptibility among anuran species living in the same geographical area, and it is not clear why this is so (McDonald and Alford, 1999; Martinez-Solano et al., 2003; Bell et al., 2004; Hale et al., 2005; La Marca et al., 2005; McDonald et al., 2005; Garcia et al., 2006).

Variation in susceptibility to chytridiomycosis

Studies have shown that chytridiomycosis can be lethal in many amphibian species but not in others (Retallick et al., 2004). There are distinct intra- and inter-specific patterns in susceptibility (McDonald and Alford, 1999; Blaustein et al., 2005; La Marca et al., 2005; Lips et al., 2006) suggesting three different responses to exposure:

1) The amphibian becomes infected with *Bd* and dies

2) The amphibian becomes infected with *Bd* and survives

3) The amphibian does not become infected even though it occurs in a habitat where *Bd* is present.

Life history, biology and ecology of anurans with varying susceptibility to chytridiomycosis have been compared in an effort to understand what traits make species more or less susceptible. Richards et al. (1993) first noted that declining amphibian species in the Australian Wet Tropics were associated with streams, and several studies since have suggested that stream-breeding frogs may experience higher rates of exposure to *Bd* than terrestrial species (McDonald and Alford, 1999; Lips et al., 2003; Kriger and Hero, 2007a). However, there is substantial variability even among stream-breeding species. Some of this may be due to differences in transmission rates caused by effects of innate immune responses and/or behaviour on exposure risk, including microenvironment selection, water selection and conspecific interactions.

Innate immune system defences

Innate immune defences include any mechanism that successfully limits initial microbial invasion (Hoffman et al., 1999). One mechanism known from all living organisms is the production of anti-microbial peptides (AMPs), short-chain proteins (Papagianni, 2003; Rolff and Siva-Jothy, 2003). In anurans, AMPs can exist internally or in the epidermal layer within the secretory granular glands (Bevins and Zasloff, 1990; Boman, 1995; Nicolas and Mor, 1995; Nicolas et al., 2003; Boland and Separovic, 2006).

AMPs of anurans became of particular interest to Csordas and Michl (1970) as they suspected some sort of anti-microbial activity on the skin of the European yellow-bellied toad *Bombina variegate*, since most individuals did not suffer any infection after being operated under, and housed in, non-sterile conditions. They described the first AMP in anurans but were unaware of its broad spectrum inhibitory effect. Ten years later, Zasloff (1987) discovered that *X. laevis* possessed AMPs that successfully inhibited growth of bacteria and fungal species. Today, more than 300 AMPs have

been described from nearly 100 anuran species (Chia et al., 1999; Rozek et al., 2000; Ali et al., 2003; Brinkworth and Bowie, 2003; Apponyi et al., 2004; Chen et al., 2006; Rollins-Smith et al., 2006; Chen et al., 2007). Unfortunately, no complete database of anuran AMPs exists (see online database http://www.bbcm.univ.trieste.it).

AMPs of anurans are all cationic (positively charged) with an alpha-helical structure that has both hydrophobic and hydrophilic segments (i.e., they are amphipathic). Being amphipathic allows the AMPs to penetrate the phospholipid membrane of an invading cell and disrupt its osmotic balance, thus killing it (Nicolas and Mor, 1995; Zasloff, 2002; Chan et al., 2006). However, different types of AMPs, depending on amino acid sequence and overall molecular charge, can vary in inhibitory function, being either broad spectrum, encompassing a variety of bacteria, protozoa and fungi, or narrow spectrum, affecting only one small group of microbiota (Simmaco et al., 1998; Batista et al., 1999; Bowie et al., 1999; Kim et al., 2001)

Considering the vast variety of AMPs, including their varying levels and specificity of function against micro-organisms, and that each frog species can produce up to at least 20 different types of AMPs, it has become clear that species may differ widely in their innate immune response (Vanhoye et al., 2003). This may contribute to the variable susceptibility of anurans to *Bd* (Rollins-Smith et al., 2003; Woodhams et al., 2005; Woodhams et al., 2006). For example, Woodhams et al. (2005) compared the AMPs of five different Australian frog species that have suffered from chytridiomycosis-related declines to very different extents. These included *L. lesueuri* (no detectable ill effects), *L. genimaculata* (experienced severe declines at higher elevations but recovered), *L. nannotis*, *L. rheocola* and *N. dayi* (severe declines at higher elevations with little and very slow recovery). Their results underlined what has been seen in the wild. *Nyctimystes dayi* and *L. rheocola* secreted smaller amounts of peptides per gram of bodyweight that was also less effective in inhibiting *Bd* growth than *L. lesueuri* and *L. genimaculata*. *Litoria nannotis* produced a larger amount of peptides; however greater concentrations were needed to inhibit *Bd* growth. Unfortunately, sample sizes

were fairly small and did not consider possible variation within species, which could have distorted their results.

Woodhams et al. (2006) compared the quantity and quality of skin peptides from twelve frog species from Panama that had not yet been exposed to *Bd* and used these results to predict likelihood of *Bd* associated declines. However, as the authors correctly pointed out, other factors, such as microenvironment selection or differences in exposure time, could also influence the progress of chytridiomycosis.

Microenvironment selection

As described previously, several studies (Woodhams et al., 2003; Drew et al., 2006; Woodhams et al., 2008) have demonstrated that environmental temperature can affect the progress of chytridiomycosis. Microenvironment selection by frogs could expose them to a range of environmental temperatures, even when species share a common macroenvironment.

Behavioural fever, which occurs when an organism increases its body temperature above normal by means of seeking external heat sources, has been observed in anurans at all life stages (Lefcort and Blaustein, 1995; Cabanac and Cabanac, 2004). For example, *Bufo marinus* and *Bombina bombina* increased their body temperature when injected with a bacterium (*Pseudomonas aeruginosa*) by seeking the heat lamps provided, clearly demonstrating behavioural fever (Cabanac and Cabanac, 2004). The authors failed to describe whether this behaviour cured the toads but did demonstrate that the rise in body temperature was solely due to seeking warmer microenvironments and not due to handling stress, which can influence and increase body temperatures in terrestrial ectotherms (Cabanac and Cabanac, 2000).

What is known so far is that in non-fluctuating, controlled environments that are optimal for *Bd* growth, chytridiomycosis becomes fatal to many frog species including *L. caerulea*, *L. chloris*, *Mixophyes faxciolatus* and *Xenopus tropicalis* (Berger et al., 1998; Parker et al., 2002; Bosch and Martinez-Solano, 2006). However, those species

have not experienced major population declines, even though wild animals have been found positive for *Bd* (Berger et al., 1998; Kriger et al., 2007a). It is possible that these species either generally use microenvironments that are not optimal for the progress of chytridiomycosis, or may induce behavioural fever where warmer microenvironments are chosen to raise body temperature and clear infection.

Rowley (2007) compared the microenvironment selection and body temperature of three anuran species, *L. lesueuri*, *L. genimaculata* and *L. nannotis,* that have suffered from chytridiomycosis-related declines to different extents. *Litoria lesueuri*, which has suffered the least decline in numbers, reached higher body temperatures and hence provided less favourable conditions for *Bd* growth compared with *L. genimaculata* and *L. nannotis.* She found no evidence that infected individuals elevated their body temperature, but did find that individuals within all three species that experienced higher body temperatures were less likely to be positive for *Bd*. Rowley's (2007) study underlines the importance of microenvironment selection and its impact on the host pathogen dynamics.

Difference in exposure

As discussed previously, variation among anuran species in their susceptibility to declines caused by chytridiomycosis can be related to their innate immune defences (Rollins-Smith, 2001; Rollins-Smith et al., 2002a; Rollins-Smith and Conlon, 2005; Bosch and Martinez-Solano, 2006; Woodhams et al., 2006) and/or their microenvironment selection (Lips et al., 2003; Woodhams et al., 2003; Rowley, 2007; Rowley and Alford, 2007a). Less attention has been given to another potential source of variation; differences in rate of exposure to *Bd*.

A variety of mechanisms may affect the exposure of frogs to *Bd* zoospores and thus their risk of infection. These include general levels of interaction with other frogs and possible avoidance of contact with infected frogs or contaminated water bodies. Rowley and Alford (2007a) observed interactions with conspecifics in three frog species, *L. nannotis*, *L. genimaculata* and *L. lesueuri*, and found the level of interaction

correlated with the species' decline history. However, no study has yet clearly demonstrated frog to frog transmission, only transmission through contaminated water (Berger, 2001; Davidson et al., 2003), or from tadpole to tadpole (Rachowicz and Vredenburg, 2004).

If infection is often via contact with contaminated water bodies, it is possible that anurans could reduce the probability of acquiring an infection by detecting and avoiding water bodies containing *Bd* zoospores. Parasite avoidance behaviour has been observed in several mammal species (Lozano, 1991; Gilbert, 1997; Hutchings et al., 2001a; Hutchings et al., 2001b), mainly by separating feeding areas from faeces disposal areas. Even though faeces and parasite avoidance are not mutually exclusive, Hutchings et al. (1998) observed an increased faeces/parasite avoidance behaviour in parasitised animals versus "naïve" animals, suggesting that it is a parasite avoidance strategy.

Aims of this study

Studies of the amphibian disease chytridiomycosis provide an opportunity to gain insight into factors influencing the dynamic between an ectothermic host and its pathogen, as many anuran species have suffered from chytridiomycosis-related declines to very different extents, allowing comparative studies. I concentrated on factors that may alter the fate of an infected organism (such as innate immune defence and microenvironment selection) or affect the degree of exposure to infection by *Bd* (such as disease avoidance behaviour). To conduct some of these studies I first needed to trial a technique to recognise individual frogs in the field to prevent multiple sampling of individuals (Chapter 2). In detail, my four aims were to determine:

1) Whether the amount of peptides produced and the degree to which they inhibit *Bd* differed seasonally and geographically between two sympatric anuran species *L. rheocola* and *L. genimaculata*. Both species declined at high elevation sites during *Bd* outbreaks but *L. genimaculata* subsequently showed greater recovery (Chapter 3),

2) Whether antimicrobial peptide profiles differ amongst geographically different populations of *L. genimaculata* (Chapter 4),
3) Whether frogs of three species (*L. caerulea*, *L. wilcoxii* and *L. genimaculata*) select different microenvironments and how this selection may alter the progress of chytridiomycosis (Chapter 5),
4) Whether frogs of three species (*L. caerulea, L. wilcoxii* and *L. genimaculata*), which have suffered from chytridiomycosis-related declines to very different extents, select water bodies for rehydration based on whether they contain *Bd* zoospores (Chapter 6).

Host-pathogen dynamics in terrestrial ectotherms are complex and many factors may contribute to the progress of the disease. The findings of these studies will aid in understanding to what extent the innate immune system, microhabitat selection and differences in exposure influence the relationship between host and pathogen. It will give further insight into the three possible causes of differences in susceptibility (frog does not become infected when exposed to *Bd*, frog becomes infected after exposure to *Bd* and either survives or dies) and may help in future conservation decisions, such as captive breeding, translocation or re-introduction of amphibian species.

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Photographic identification method (PIM) as a means of recognising individual adult and juvenile green-eyed tree frogs, *Litoria genimaculata* (Anura: Hylidae)

Modified version of:
The Photographic Identification Method (PIM) as a tool to identify adult *Litoria genimaculata* (Anura: Hylidae). N. Kenyon, A.D. Phillott and R.A. Alford. Submitted to *Wildlife Research.*
Temporal variation in dorsal patterns of juvenile green-eyed tree frogs, *Litoria genimaculata* (Anura: Hylidae). N. Kenyon, A.D. Phillott and R.A. Alford. Submitted to *Herpetological Conservation and Biology*.

Abstract

Marking anurans by invasive methods has been strongly debated on grounds of ethics, public opinion, possible infection and potential impact on survival of anurans. Photographic identification of natural markings on amphibians is a non-invasive method of identifying individuals, but is only applicable to species that have persistent, distinguishable patterns. I evaluated whether the photographic identification method (PIM) can be used to identify juvenile and adult *Litoria genimaculata* individuals based on their dorsal pattern with sufficient consistency to be useful in field sampling. In the laboratory, I photographed 20 juvenile *Litoria genimaculata* for nine weeks after metamorphosis to determine whether individual frogs can be distinguished using dorsal pattern, and whether dorsal pattern changes over time. In the field I compared PIM with traditional toe-tipping of adult *L. genimaculata.* Each individual frog (juvenile or adult) had distinguishable dorsal markings, so identification through PIM was possible. In juvenile frogs I found no detectable change in the dorsal pattern during the nine-week period, although the colour of the pattern and the background did change. However, I observed a change in dorsal pattern of an adult frog after a two month period and conclude that PIM is unsuitable for longer term mark-recapture studies on this species. During short-term studies, 96 percent of new captures were correctly identified. Extra care has to be taken to prevent multiple sampling of individuals as 38.5 percent of recaptured frogs were misidentified as new captures.

CHAPTER 2 PHOTOGRAPHIC IDENTIFICATION METHOD

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Introduction

Studies involving mark-recapture in amphibians use techniques that allow the identification of individuals, including toe-tipping and -clipping (Clarke, 1972; Waichman, 1992; Richards and Alford, 2005; Simoncelli et al., 2005), fluorescent marking (Anholt et al., 1998; Schlaepfer, 1998), and passive integrated transponder (PIT) tagging (Brown, 1997). All of these techniques are invasive, but some can provide additional valuable information on animal age and population genetics (Halliday, 1995; Funk et al., 2005) and researchers have to balance the possible effects of the marking procedure on individuals and populations against cost, time efficiency, and the value of information collected (Phillott et al., 2007).

To date, the photographic identification method (PIM), which uses variation in integument patterns to distinguish among individuals, is the only non-invasive technique for individual recognition of amphibians (Hagstrom, 1973; Bradfield, 2004). One of the limitations of this procedure is that its suitability is restricted to amphibians with individually recognizable natural features or markings (Bradfield, 2004). Even in those species, studies are needed to determine if their patterns are permanent or show temporal variation, in both juveniles and adults.

Litoria genimaculata is a stream dwelling rainforest frog that occurs between Townsville and Cooktown, Queensland, Australia. Adult dorsal colouration and pattern vary among individuals, usually consisting of a broad russet/brown pattern, and a bi- or tri-lobed band with irregularly dispersed patches of green and copper (Barker et al., 1995). This distinctive and variable patterning suggests that PIM may be successfully applied to adults. Juvenile *L. genimaculata* also possess a mottled dorsal pattern (Barker et al., 1995) but its persistence through ontogeny has not been examined. Ontogenetic pattern changes could render PIM unsuitable for studies of juveniles and sub-adults.

This study was necessary to determine whether PIM could be applied to *L. genimaculata* in the field to distinguish among individuals during my peptide surveys

(see Chapter 3 and 4). Consequently, the aims were to determine whether PIM could be used to recognise individual juvenile and adult *L. genimaculata* based on their dorsal pattern, whether individuals remained identifiable over a substantial time period, and to compare the accuracy and time requirements of the procedure with traditional toe-tipping.

Materials and Methods

Juvenile frogs

Litoria genimaculata were raised in the laboratory from eggs from one clutch that were collected at Birthday Creek, Paluma Range National Park, Queensland, Australia (S18°58′54″ E146°10′02″). Each individual juvenile frog (n=20) was digitally photographed using a Pentax Optio 33WR camera against a 0.5cm^2 grid to allow measuring snout-vent length (SVL). The camera was placed on a fixed stage (Figure 2.1) to standardise focal distance. Initial photographs were taken within two weeks of metamorphosis and then weekly for nine weeks so that I could examine the dorsal pattern through ontogeny. Dorsal patterns were categorised into a series of binary state variables as illustrated in Figure 2.2: A) the shape of the posterior line of the dorsal "hourglass" marking (categorized as either an "S" or "V" shape), (B) greatest length of dorsal hourglass, (C) narrowest width of dorsal hourglass, (D) the presence or absence of dots between the eyes, (E) the presence or absence of a straight line of the dorsal hourglass, and (F) the presence or absence of dorsal dark lined circles. Characteristics A and D-F are naturally binary. So that we could conduct an ordination of all characters on a common scale, we used measurements B and C to create three additional binary variables, one which had a value of 1 if the ratio C/B was less than or equal to 0.25, and 0 otherwise, one which was 1 if C/B was between 0.25 and 0.30, 0 otherwise, and one which was 1 if C/B was greater than or equal to 0.30, 0 otherwise. Examination of the data set showed that the features of each individual remained constant throughout the period of the experiment. To illustrate differences among individuals I ordinated the Euclidean distances among them using a non-metric multidimensional scaling analysis performed using the PROXSCAL procedure in SPSS version 14. I used Euclidean distances since shared zeros are informative. In addition to measuring quantitative

characters, each week I categorised the background colour of the dorsal pattern as either predominantly green or predominantly brown. To determine whether changes in this colour were correlated among frogs across time, I estimated mean rates of transition from the data and used these to calculate expected numbers of individuals with each background colour in each week from four to nine. I calculated an overall chi-squared statistic across weeks that compared observed numbers to expected numbers with each colour pattern in each week.

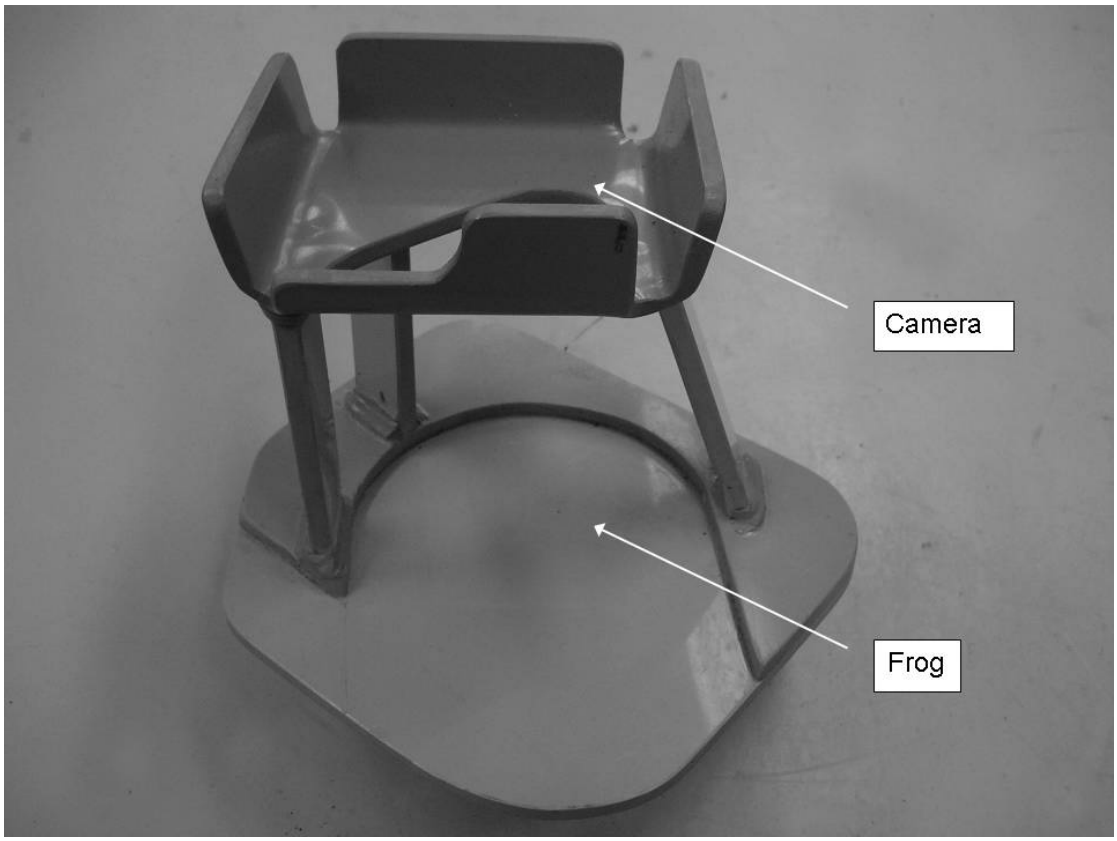


Figure 2.1 Stage, allowing constant focal distance, used to take digital images of *Litoria genimaculata*.

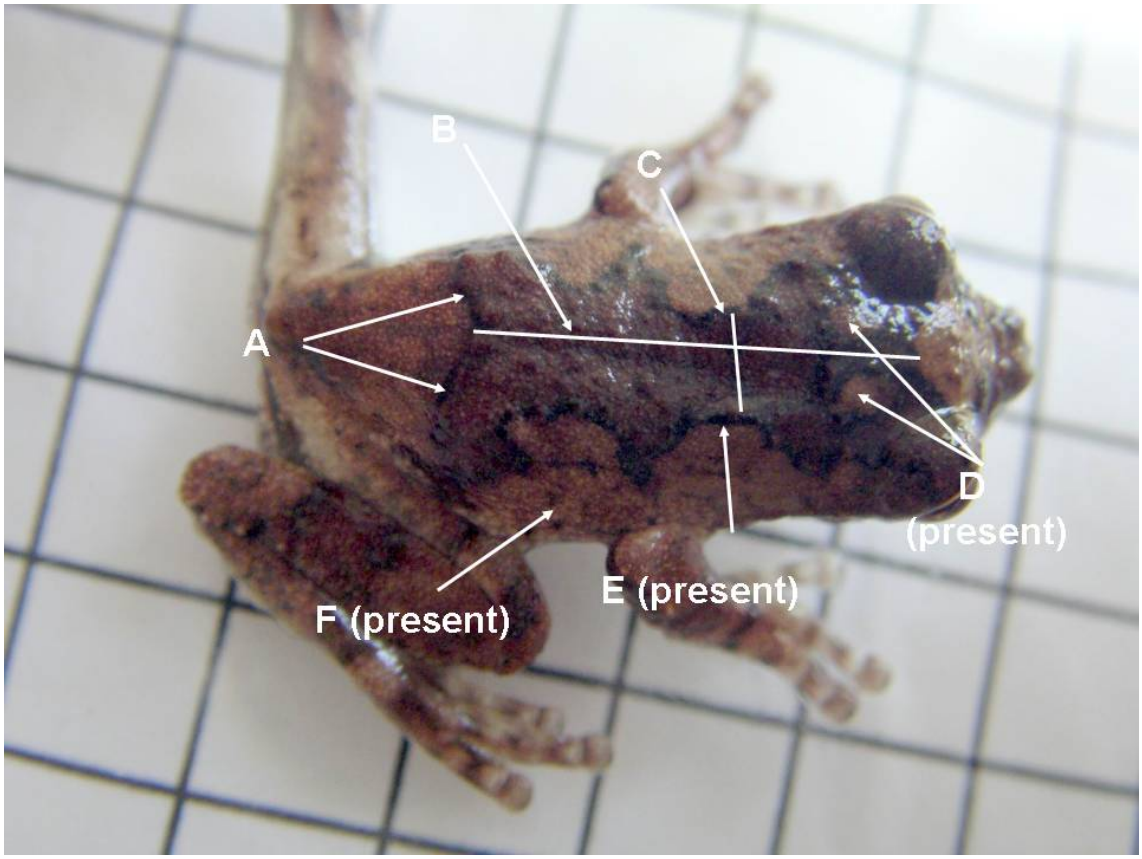


Figure 2.2 Measured aspects of the dorsal pattern in *Litoria genimaculata* used to create binary state variables analysed in Figure 2.3.

A-shape of the posterior line of the dorsal hourglass

B- length of the dorsal hourglass along mid-dorsal line

C- the narrowest part of the dorsal hourglass

D- dots between eyes

E- straight line within dorsal hourglass

F- dorsal dark lined circles

****

Adult frogs

A population of adult *L. genimaculata* in Murray Upper National Park (S18°11′,E145°52′) that are currently being studied and toe-tipped by Dr. Andrea Phillott, was used to determine whether PIM was effective in the field. I conducted three field trips; the first trip in December 2006 for two nights, the second in February 2007 for two nights and the third in October 2007 for four nights. Frogs were toe-tipped in a unique manner according to Phillott (pers.comm.). During the first survey night, digital images of the dorsal pattern of every *L. genimaculata* captured were taken with the same camera and stage as described for photographing juvenile frogs. Each frog was held by its left hind foot and placed on a small plastic bag (changed between frogs to avoid pathogen transfer) on the stage. The digital image number and the toe-tip number were recorded on site. The next day all images were printed (using a HP Photosmart 325 printer) and labelled on the reverse with the toe-tip number. Images were categorised according to the transect distance and frog dorsal pattern (presence or absence of dorsal hourglass shaped lines, circular-shaped marks between the eyes, and triangular-shaped marks between the nares) and sorted accordingly in a small album that was used on successive nights.

All *L. genimaculata* captured during the following nights were compared with existing photographs. Records were kept for correctly recognising either "new captures" or "previously caught with correct recognition of individual". To avoid possible biases in identification of marked individuals, I was not present during the inspection of frogs for toe-tipping. When handling the frogs I held them by the hind feet to avoid observing if toe-tipping had occurred. I was unaware of the toe-tipping sequence used so could not identify frogs according to their unique number. After comparison of the frog with photographs of those captured on previous nights, I categorised frogs as previously marked or unmarked. During the second field trip (February 2007), frogs were compared with photographs from the first field trip (December 2006) to determine whether dorsal patterns changed during the two month recapture interval.

****

During the last field trip in October 2007, the time taken to compare captures with existing images and to photograph frogs new to the study was recorded. These were compared with times required to toe-tip or recognise previously marked frogs of a number of species (*L. genimaculata*, *L. nannotis*, *L. rheocola* and *Nyctimystes dayi*) (Phillott, unpublished data) using two-sample t-tests. I tested the hypothesis that PIM performed better than random classification of frogs by using the overall proportions of previously-captured and not-previously-captured frogs caught on nights after the first night of the two sets of surveys to establish the proportions of frogs in each category that would have been placed in each class if decisions were made at random. I used a chi-squared goodness-of-fit test to compare the observed numbers of frogs in each category with the expected number,

Results

Juvenile frogs

The mean initial SVL of *L. genimaculata* metamorphs was 3.55cm (range=3.00–4.50cm). During the nine week study the 20 juvenile *L.genimaculata* grew an average of 0.84cm (range= 0.5-1.0cm). Most juvenile frogs possessed a dorsal pattern distinct from that of other individuals based on the character set I quantified; two pairs of individuals and one group of three did not (Figure 2.3). The juveniles that could not be distinguished quantitatively were easy to differentiate visually based on other aspects of their patterns, such as the locations of skin ridges and tubercles (Figure 2.4). The dorsal pattern remained constant through time within individuals (Figure 2.5), but overall colour varied considerably, changing from brown to green or the reverse in 75% of individuals over the course of the experiment (Figure 2.6, Table 2.1). No individual changed colour prior to week four, and at least one individual changed from brown to green and back to brown, and the reverse, within two weeks, indicating that at least after week three, colour can change in either direction within one week. Under the null hypothesis that colour change occurs in each frog at random times, the data for each week should reflect random alterations to the data for the preceding week. If colour changes are constrained to occur at similar times among frogs, either by internal developmental mechanisms or by subtle changes in the light environment in the

laboratory, changes between weeks should differ significantly from a random set of colour shifts. My comparison of observed versus random expected changes was significant (χ^2=12.66, df=6, p= 0.049), indicating that the timing of colour changes was non-random.

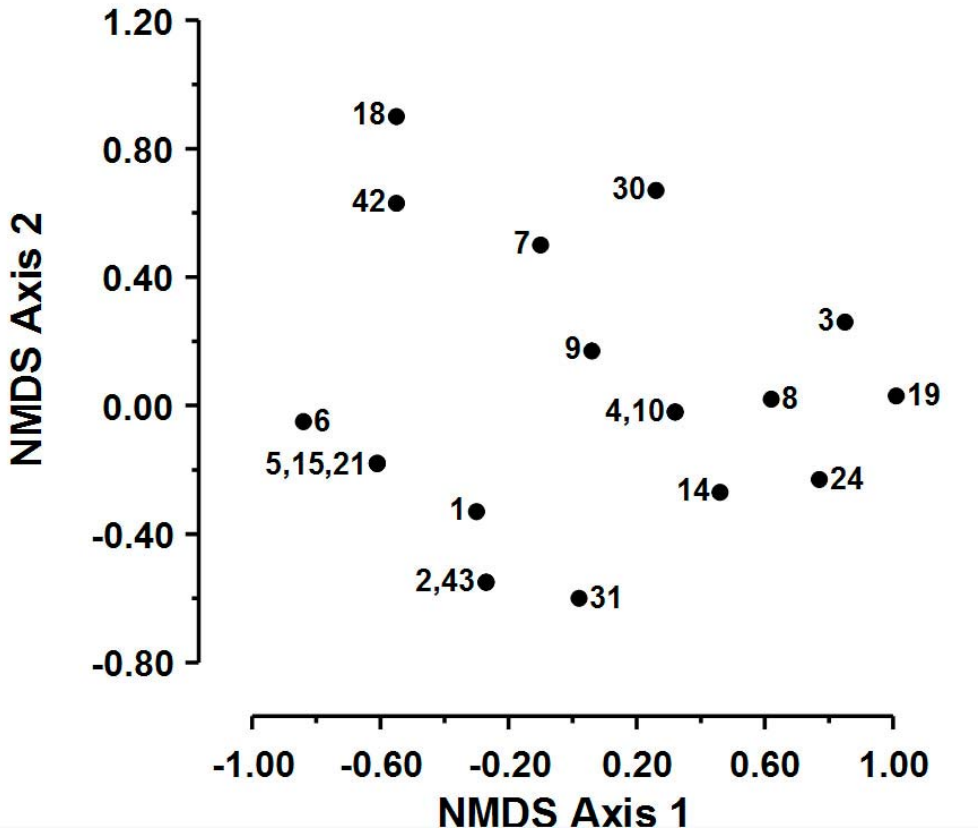
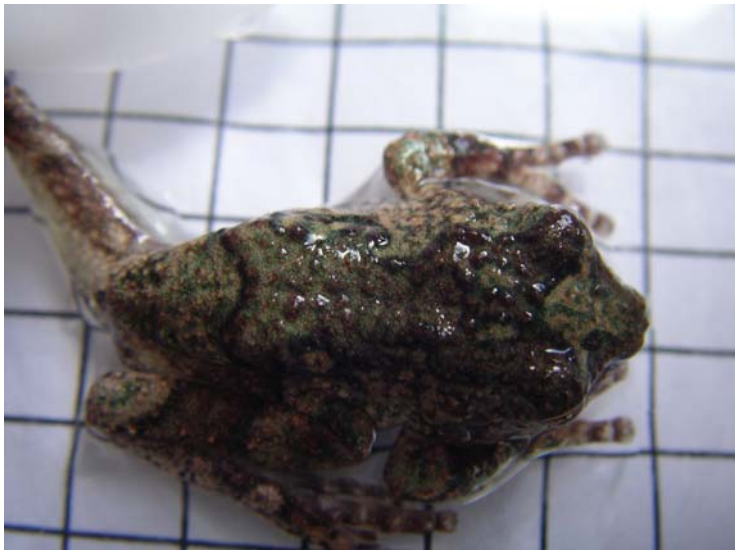


Figure 2.3 Results of non-metric multidimensional scaling ordination of Euclidean distances among individuals for dichotomised measurements of *Litoria genimaculata* juvenile dorsal pattern. Most individuals could be distinguished solely on these measurements, which did not change over the 9 week period of measurement.

a)



b)

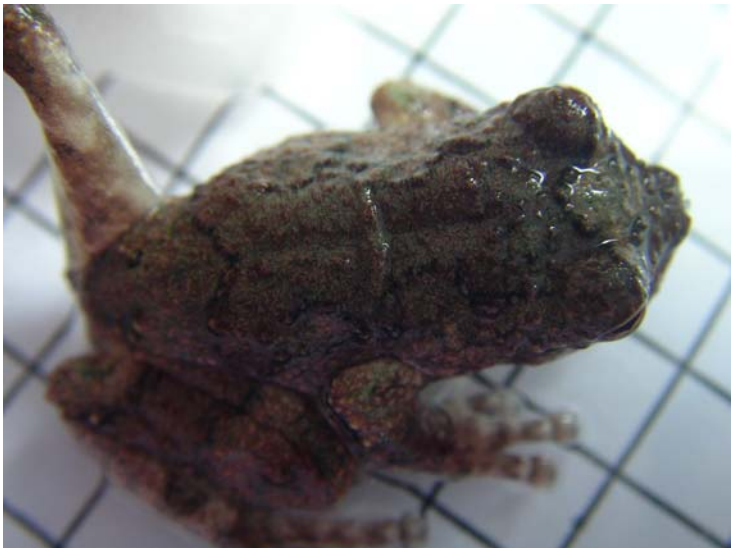


Figure 2.4 Dorsal pattern of two juvenile *Litoria genimaculata* - *Lg* 2 (a) and *Lg* 4 (b). In addition to the features we quantified (Figure 2.2), many distinctive ridges and tubercles were present.

a)

b)

Figure 2.5 Persistence of the dorsal pattern in a juvenile *Litoria genimaculata* (*Lg* 43) at week one (a) and week nine (b).

a)

b)

Figure 2.6 Constant dorsal pattern with temporal variation in colour of a juvenile *L. genimaculata* (*Lg* 3) a) week two (pattern colour brown), b) week nine (pattern colour green).

****

Table 2.1 Temporal variation in dorsal pattern background colour of 20 *Litoria genimaculata* juveniles during the nine-week study period. Brown background indicated by 1, green by 0, missing data or not calculated by --. Expected proportions were calculated for weeks 4-9 by applying mean rates of transition from brown to green (0.2247) and green to brown (0.4138) calculated for the intervals between weeks three to four through eight to nine to the data from the preceding week.

Frog	**Week**								
	1	2	3	4	5	6	7	8	9
Lg 1	1	1	1	1	1	1	0	1	0
Lg 2	1	1	1	0	0	0	0	1	1
Lg 3	1	1	1	1	1	1	1	1	0
Lg 4	1	1	1	1	1	1	0	1	0
Lg 5	1	1	1	1	1	1	0	1	1
Lg 6	1	1	1	1	1	1	1	1	1
Lg 7	1	1	1	1	1	1	1	1	1
Lg 8	1	1	1	1	1	1	1	1	1
Lg 9	1	1	1	1	1	1	1	1	1
Lg 10	1	1	1	1	1	1	1	1	0
Lg 14	1	1	1	0	0	0	0	1	0
Lg 15	1	1	1	1	1	1	1	1	1
Lg 18	1	1	1	1	0	0	0	1	0
Lg 19	1	1	1	1	0	0	0	1	--
Lg 21	1	1	1	1	1	1	1	1	1
Lg 24	1	1	1	0	0	0	0	1	--
Lg 30	1	1	1	0	1	0	0	1	1
Lg 31	1	1	1	0	1	1	0	1	0
Lg 42	1	1	1	1	1	0	0	0	0
Lg 43	1	1	1	1	1	1	1	1	1
Observed green	0	0	0	5	5	7	11	1	8
Observed brown	20	20	20	15	15	13	9	19	10
Expected green	--	--	--	4.49	6.12	6.71	6.92	7.00	6.32
Expected brown	--	--	--	15.51	13.88	13.29	13.08	13.00	11.68
Contribution to chi-square	--	--	--	0.07	0.29	0.02	3.68	7.90	0.69

****

Adult frogs

I photographed 63 adult *L. genimaculata* during the study. At capture, 98% were correctly recognised as new to the study (Table 2.2). PIM clearly performed much better than random assignment of individuals to categories (Table 2.2, $\chi^2 = 20.19$, df=3, $p < 0.001$). Only two new individuals were incorrectly regarded as previously captured due to the similarity in dorsal pattern and no distinction in hourglass pattern from a previously captured frog (Figure 2.7).

Twelve individuals were recaptured on 13 occasions. Correct recognition of these known individuals was low (62%; Table 2.2). In one frog this was due to a change in dorsal pattern as a black circular-shaped marking on the left flank was lost and a black line on the right flank was gained during the two month recapture interval (Figure 2.8). Incorrect recognition of the remaining individuals was due to human error; however, the majority of those individuals had no distinct hourglass pattern and instead possessed only random blotches. Overall, 53% of the frogs captured had no hourglass shape within their dorsal pattern present. No change in pattern colour was observed during the study. The toe-tip number was misread on one of the 13 recapture events.

The time taken to search through photographs and recognise an individual as new to the sample or previously photographed increased with the sample size (Figure 2.9). Time required to take a digital image was significantly greater than that to toe-tip a frog (t=3.549, df=31, p=0.001, Levene's tests for equality of variances satisfied). Similarly, searching images to recognise an individual was significantly slower (t=10.052, df=29.287, p=0.000, equal variances not assumed) than determining the unique number of a previously toe-tipped animal.

****

Table 2.2 Recognition of new and recaptured *Litoria genimaculata* in Murray Upper National Park, northern Queensland, Australia using PIM (photographic identification method). Night count restarted for trip 3 as the long interval between trips 2 and 3 meant there were no recaptures between trips. Note that much of the contribution to the chi-squared test of the null hypothesis that PIM classification was equivalent to random assignment of individuals as new or recaptured comes from the large excess of recaptured individuals correctly identified as such. The chi-squared test is significant (χ^2=20.19, df=3., p<0.001).

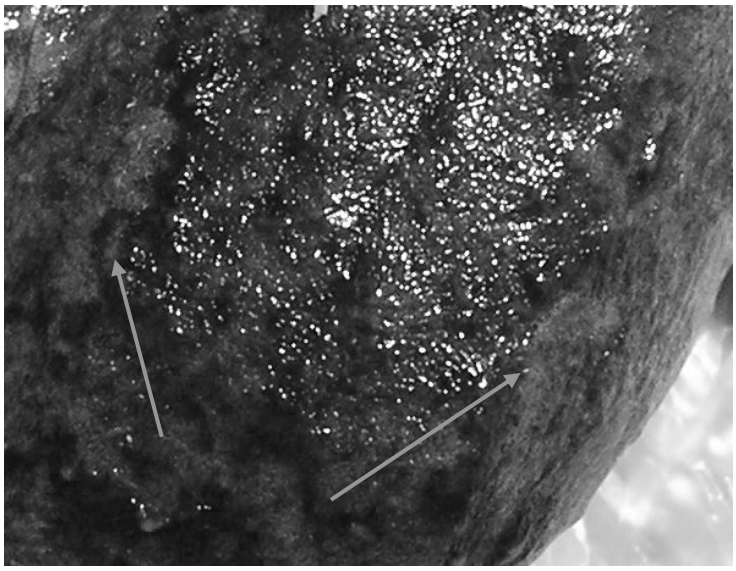
Trip	Night	**New capture, identified as**		**Recaptures, identified as**	
		New capture	Recapture	New	Recapture
1	1	10	--	--	--
	2	14	0	0	1
2	3	5	2	1	1
	4	4	0	1	2
3	1	7	--	--	--
	2	5	0	--	--
	3	6	0	1	2
	4	10	0	2	2
Total		61	2	5	8
Total excluding nights 1		44	2	5	8
Expected		35.86	10.14	10.14	2.86
Contribution to chi-square		1.85	6.53	2.60	9.21

a)

b)

Figure 2.7 The lack of a distinct dorsal hourglass and similar blotches in two adult *Litoria genimaculata* resulted in the incorrect identification of the newly captured animals as a previously captured animals (false positive error), a) *Lg* 62, b) *Lg* 64.

a)



b)

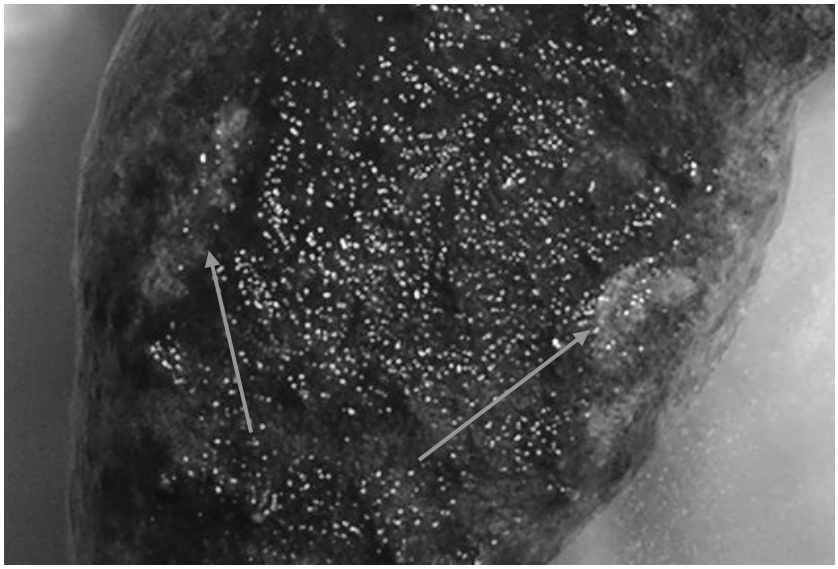


Figure 2.8 Dorsal pattern change in a *Litoria genimaculata* individual within two months, a) December 2006 where a clear black circle is seen on the left flank and no black marking on the right flank, b) February 2007 with no black circle on the left flank and a black marking on the right flank.

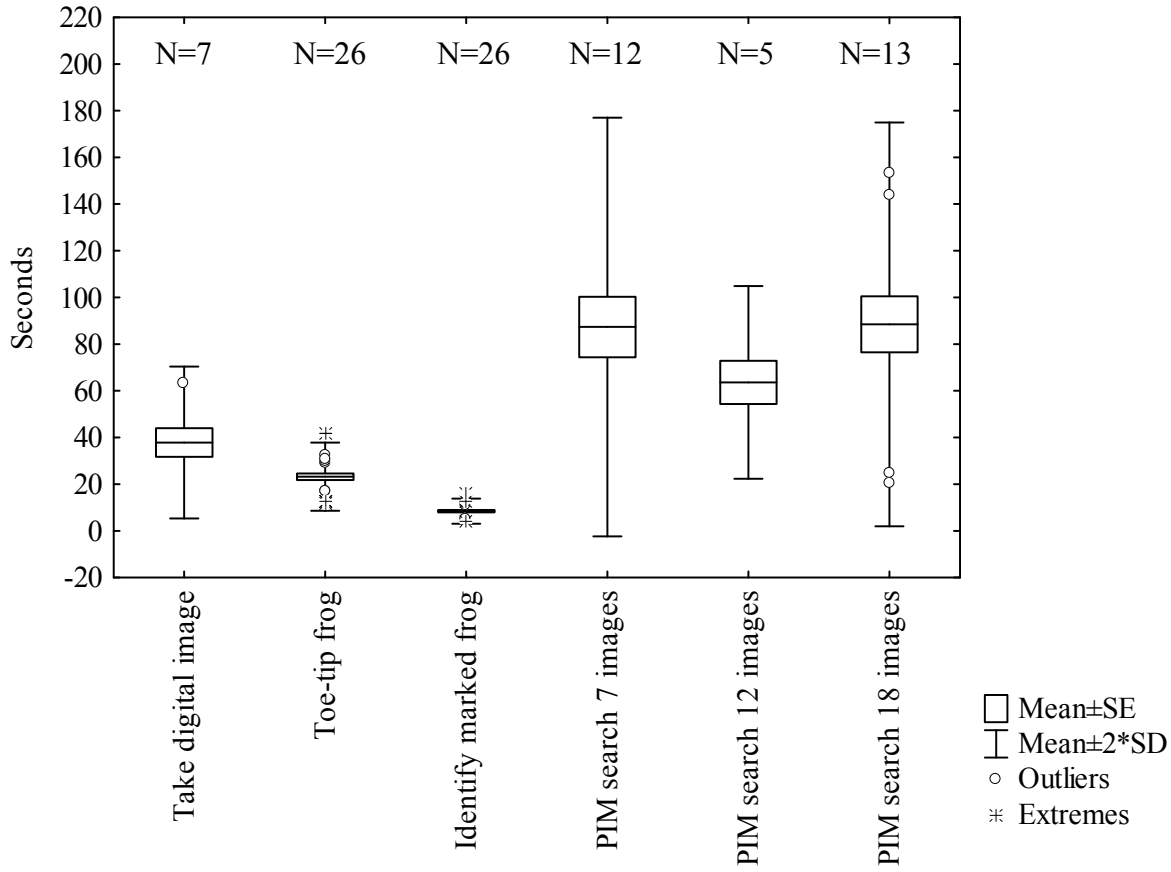


Figure 2.9. Time taken to apply toe-tipping and PIM (Photographic Identification Method) to adult frogs.

Discussion

There was sufficient dorsal pattern differentiation among 20 juvenile *L. genimaculata* to visually distinguish among them and hence this age-group appears to be suitable for PIM. My results do not rule out the possibility that dorsal patterns may change over longer periods of time or with further growth.

I took care not to focus on colour as this can vary greatly within the same individual. Hoffman and Blouin (2000) suggested that the darkening and lightening of existing skin tones is fairly common in anurans, but that actual colour changes are rare, usually in one direction, and age related. However, I observed dorsal colour change in both directions (green to brown and vice versa) in juvenile frogs over the nine-week observation period. This was also observed by Hoffman and Blouin (2000) in laboratory populations of *Hyla regilla*.

Skin colour in amphibians is due to the presence of dermal chromatophore units, containing layers of xanthophores, iridiophores and melanophores (pigment cells). Colour changes occur when pigments are rearranged within the chromatophores, due to light, temperature, hormonal, and other stimuli (reviewed by Frost-Mason et al., 1994). The significant temporal correlations I found across juveniles indicate that they were responding with some degree of synchrony to either internal or external cues. These could be part of a regular ontogenetic pattern initiated by hormonal changes occurring at fixed times after metamorphosis. However, changes in weeks three to four through six to seven were only moderately synchronous, while during the week seven to eight period there was a much higher degree of synchrony, with all brown frogs remaining brown and all but one green frog becoming brown. This suggests that the frogs were responding to an external cue, perhaps a subtle change in the light environment in the laboratory.

When applied to adult *L. genimaculata*, PIM proved most reliable (98% correct recognition) when comparing a new individual with a collection of images of previously captured frogs, as overall pattern shape was used during the comparison. The one frog

that was not correctly recognised as new to the study was similar in appearance to a previously captured animal and resulted in a false match or false positive error.

I did not observe any colour change in adults captured on both the first and second field trips, however, the sample size was limited (n=6) and occurred within the austral summer. Colour change in *Pseudacris regilla* (Pacific tree frogs) was attributed to seasonal change (Wente and Phillips, 2005) so a longer study period with more frequent observations would be required to detect colour polymorphism in adult *L. genimaculata*.

The reliability of PIM as a method to recognise previously captured adult *L. genimaculata* was lower (62% correct recognition) as smaller and more subtle markings were compared. Human error contributed to 4 of the 5 unrecognised frogs (false negative errors). One *L. genimaculata* in my study was not recognised as previously captured and photographed due to temporal variation in the dorsal pattern during the two month recapture interval. Other studies have found both substantial (Reaser, 1995) and only minor (Stephenson and Stephenson, 1957; Hagstrom, 1973; Healy, 1975; Denton and Beebee, 1993; Doody, 1995; Bradfield, 2004) change in adult markings over time. The causes of all of these pattern changes are unknown.

In conclusion, both studies suggest that PIM in *L. genimaculata* has to be used with care as a) dorsal markings did change and b) a large proportion of recaptured individuals were misidentified, mainly due to lack of a distinct hourglass pattern. The PIM method was successful in classifying individuals as previously uncaptured and hence, depending on the type and duration of the study, may be suitable. In order to use PIM during the peptide study (Chapter 3 and 4), extra precautions were included to avoid multiple sampling of frogs.

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Have anuran skin peptide defences against the emerging amphibian pathogen *Batrachochytrium dendrobatidis* responded to natural selection?

Abstract

The disease chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), has been implicated as the proximate cause of many amphibian declines around the globe. However, its effects vary widely among species; some can persist despite infection while others are driven to local extinction. One possible factor contributing to variable susceptibility is interspecific differences in innate immune defences, especially rate of production and effectiveness of antimicrobial peptides (AMPs) against *Bd.* I examined these in two sympatric species of Australian frogs, *Litoria genimaculata* and *L. rheocola.* I took samples from 216 frogs, from upland populations that suffered declines and disappearances caused by chytridiomycosis and have subsequently recovered and recolonised, and from lowland populations that did not decline, although chytridiomycosis is now endemic. I hypothesised that strong selection pressure exerted by *Bd* could have caused the AMPs of upland populations to diverge from those of lowland populations. I quantified peptide secretion using spectrophotometry and challenged *Bd* cultures with AMPs. Based on the concentration of AMPs needed to completely inhibit *Bd* growth (IC_{100}), seasonal variation in peptides secreted per surface area and AMP effectiveness against *Bd* was detected in both anuran species, suggesting that their innate immune defences may be affected by seasonal changes in physiology, or may be adapted to seasonal fluctuations in environmental microbiota. Even though, overall protection (mL of AMPs at IC_{100}/cm^2) of frogs against *Bd* did not differ significantly between high and low elevation populations of either species, more frogs had effective AMPs at high elevations, suggesting that the stronger selection imposed by *Bd* in high elevation populations has not led to the evolution of different and more effective AMPs, but may have led to a higher proportion of frogs possessing AMPs that are effective against *Bd*. If natural selection has acted to reduce the vulnerability of the high elevation populations to *Bd,* it has altered a different factor, such as microenvironment selection, frequency of frog to frog interactions, or avoidance of disease propagules.

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Introduction

Chytridiomycosis is a disease of amphibians, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*). It is defined as an emerging infectious disease (EID) as it has recently increased its geographical and host species ranges (Morse, 1995; Daszak et al., 2001; Dobson and Foufopoulos, 2001). Several studies have confirmed the fatal outcome of chytridiomycosis in various frog species including *Dendrobates tinctorius*, *Rana muscosa, Litoria caerulea*, *L. chloris* and *Mixophyes fasciolatus* (Berger et al., 1998; Nichols et al., 2001; Woodhams, 2003; Rachowicz et al., 2006). However, the effect of *Bd* infection varies widely among species; ranging from no apparent effect at the population level to local extinction (Richards et al., 1993; McDonald and Alford, 1999; Lips et al., 2005a).

Variation in innate immune defences among species could contribute to the observed variation in susceptibility. A key component of the innate immune system in amphibians is antimicrobial peptides (AMPs), short proteins that hinder initial microbial invasion of the epidermis (Hoffman et al., 1999). A single species can have 20 different types of AMPs, which vary in their effectiveness against viral, fungal and bacterial pathogens (Vanhoye et al., 2003), allowing numerous combinations of AMPs that provide unique innate defences for each species.

Several studies have shown that AMPs of frogs can kill or inhibit the growth of *Bd* zoospores (Rollins-Smith et al., 2002a; Rollins-Smith et al., 2003; Woodhams et al., 2007). There are several aspects of AMPs that can be compared among frog species (Woodhams et al., 2006; Woodhams et al., 2007). First, the quantity of peptides (which includes similar sized peptides and AMPs) a frog secretes per surface area, given a specific stimulus. Second, the effectiveness of AMPs at inhibiting the growth of *Bd* zoospores. This is measured as the minimal concentration of AMPs needed to absolutely inhibit the growth of *Bd* zoospores (IC_{100}). Third, the overall level of protection against *Bd*, which considers both the amount of peptides secreted per surface area and the IC_{100}. Woodhams et al. (2006) compared the amount of peptides secreted, effectiveness, and overall level of protection afforded by AMPs against *Bd* among five

Australian frog species, *L. lesueuri*, *L. genimaculata, L. nannotis*, *L. rheocola* and *Nyctimystes dayi*, that have suffered from chytridiomycosis-related declines to different extents. They found that *N. dayi* and *L. rheocola*, which had declined to a greater degree than the other species, secreted lower quantities of peptides per surface area, and needed greater concentrations to effectively inhibit *Bd*, than did *L. lesueuri* and *L. genimaculata.* The results of Woodhams et al. (2006) suggest that differences among frog species in innate immune defences may have affected the pattern of decline among species. However, their sample sizes were small for most species (usually below ten individuals), so they may have overlooked intraspecific variation. In addition, some individuals they sampled were infected with *Bd*, which could have altered their peptide secretions.

Northern Queensland is an ideal area to study chytridiomycosis as frog populations have been extensively monitored before, during and after epidemics, so their responses to these events are well documented. *Litoria genimaculata* and *L. rheocola* have both experienced population bottlenecks. *Litoria rheocola* suffered local extinctions at high elevation sites throughout its range during initial outbreaks of chytridiomycosis in the 1990s, while populations below 400 m were stable (McDonald and Alford, 1999). Populations of this species are slowly recovering at high elevations, most likely by recolonising from lowland populations (Woodhams and Alford, 2005). *Litoria genimaculata* populations at high elevations declined but did not reach local extinction and have subsequently recovered (McDonald and Alford, 1999; McDonald et al., 2005). The lack of declines in both species at low elevations is most likely due to less favourable environmental conditions for chytridiomycosis outbreaks (Berger et al., 2004; Woodhams and Alford, 2005; Kriger and Hero, 2007b).

I hypothesised that if AMPs played an important role in the survival of *L. genimaculata* and *L. rheocola* individuals infected with *Bd*, then strong selection pressure could have caused the AMPs of upland populations to diverge, increasing their innate protection against *Bd* when compared to lowland populations. I compared AMP production, effectiveness and overall protection against *Bd* between L. *genimaculata* and

L. rheocola at high and low elevation sites, to test this hypothesis and to provide a better general understanding of intraspecific variation in AMPs.

Materials and Methods

Study Species

Litoria genimaculata (Hylidae), the green-eyed tree frog, occurs between Townsville and Cooktown in Queensland, Australia (Barker et al., 1995). It is a stream dwelling rainforest species, with males frequently perching on vegetation along creeks and also higher in the canopy (Richards and Alford, 2005; Rowley and Alford, 2007b). *Litoria rheocola*, the common mistfrog, occurs between Murray Upper National Park and Cooktown (Frith and Frith, 1991) and shows a stronger association with rainforest streams than *L. genimaculata* (Richards et al., 1993).

Study sites

Skin peptides were collected from frogs at five sites at three different locations (Figure 3.1, Table 3.1). Each site consisted of a 400 meter transect along a creek. These sites were selected as frog monitoring data already exists. *Litoria genimaculata* at Birthday Creek (Paluma National Park) have been monitored at a variety of intervals since 1989 and regularly (two consecutive nights during winter and summer) since 2001, except in 2003 and 2004 (Richards and Alford, 2005; Woodhams and Alford, 2005). Standard frog surveys, two nights each in winter and summer, have been conducted since 2001 (except 2003 and 2004) at unnamed creeks at Bridge I and Bridge XI in Murray Upper National Park and Windin N Creek and Frenchman Creek in Wooroonooran National Park (Woodhams and Alford, 2005, Alford and Bell, unpubl.).

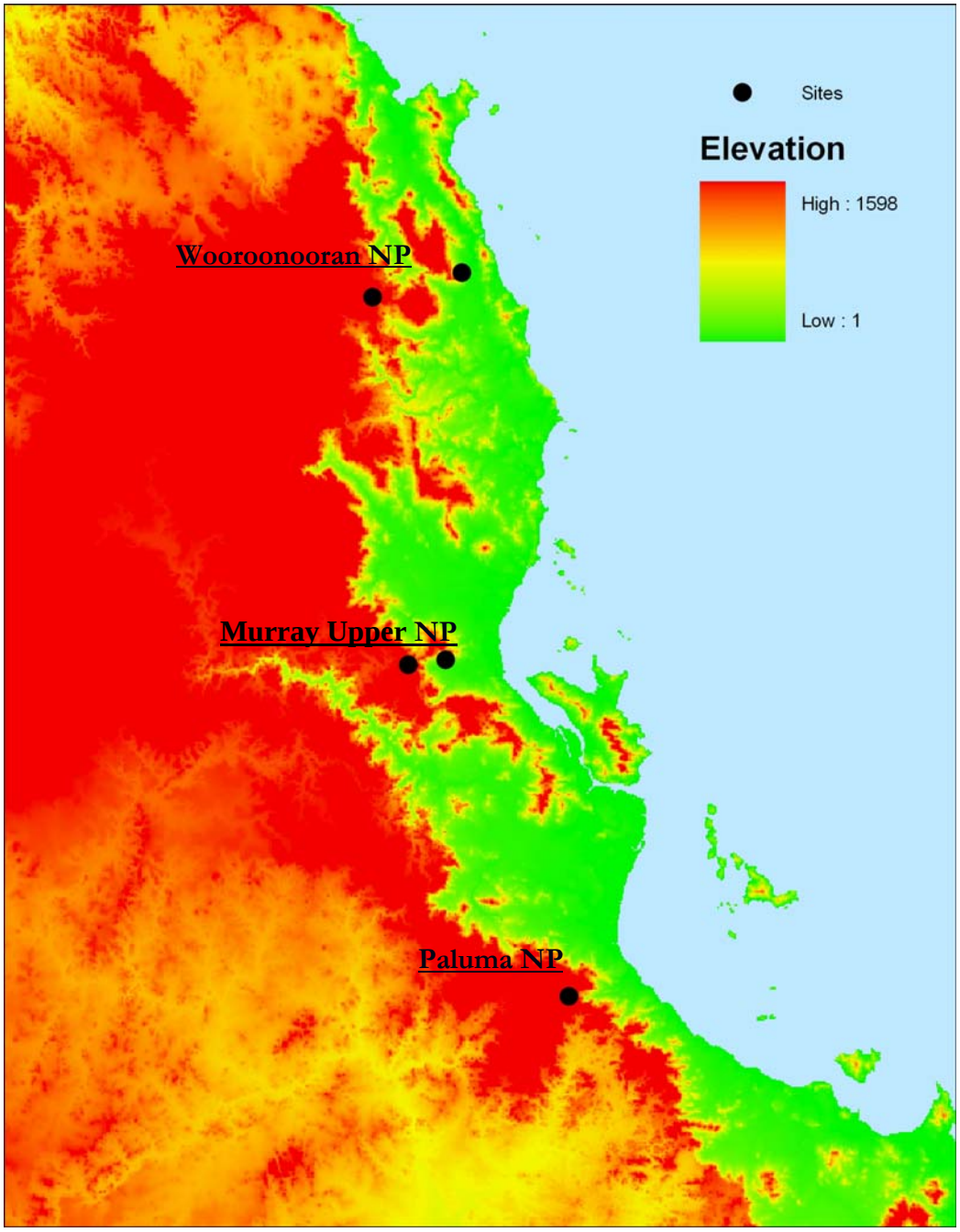

Figure 3.1 Three National Park areas (encompassing 5 study sites) in northern Queensland where *Litoria genimaculata* and *L. rheocola* were captured to sample skin peptides.

Table 3.1 Locations and descriptions of the five study sites where *Litoria genimaculata* and *L. rheocola* skin peptides were collected.

National Park	Site	Elevation	GPS coordinates	Site description	Frog species
Paluma Range	Birthday Creek	800 m	S18º58′54″ E146º10′02″	Simple notophyll vine forest creek	*L. genimaculata*
Murray Upper	Bridge I	100 m	S18º12′11″ E145º53′00″	Simple mesophyll vine forest creek	*L. genimaculata*
Murray Upper	Bridge XI	850 m	S18º12′55″ E145º47′48″	Simple mesophyll vine forest creek	*L. genimaculata*
Wooroonooran	Frenchman Creek	40 m	S17º18′32″ E145º55′16″	Complex mesophyll vine forest creek	*L. genimaculata* and *L. rheocola*
Wooroonooran	Windin N Creek	750 m	S17º21′57″ E145º42′54″	Complex mesophyll vine forest creek	*L. genimaculata* and *L. rheocola*

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Collection and processing of peptides

Field

Collection of male *L. genimaculata* and *L. rheocola* skin peptides occurred in 2006 and 2007. Only skin peptides of males were collected as sightings of females are rare and it would not have been possible to collect a sufficiently large sample to statistically compare the effects of sites, infection status and sex. Skin peptides from *L. genimaculata* were collected from all five sites in winter 2006 (non-breeding season) and summer 2007 (breeding season). Breeding season was determined according to the number of *L. genimaculata* males calling and presence of females, but is also in agreement with several studies (Woodhams and Alford, 2005; 2007). Skin peptides were collected from *L. rheocola* at only two sites (Windin N Creek and Frenchman Creek), as this species does not occur at the other sites. Skin peptides from *L. rheocola* were collected in summer 2007 and winter 2007. *Litoria rheocola* has a less well-defined breeding season than *L. genimaculata* (Phillott, pers. comm.). I heard male *L. rheocola* calling in both seasons.

Frogs were captured at night using small plastic bags inverted over the investigator's hand and transferred into larger 230mm × 305mm resealable bags (1 frog per bag). The resealable bags, containing the frogs, were numbered and the location of each frog on the transect was marked with flagging tape so that individuals could be released at the point of capture after peptide collection. For peptide collection, frogs were temporarily removed from the creek to a nearby vehicle because this process required chemicals that needed to be used away from the creek in case of accidental spillage. A maximum of ten frogs were sampled per night. Snout-vent length (SVL) of each frog was measured, the frog was weighed (using a high precision pocket balance PS-50) and swabbed using a sterile tubed dry swab (Medical Wire and Equipment, Corsham, Wiltshire UK) that was run across frog's hands, feet, thighs and ventral surface twice, for qPCR assay to quantify infection with *Bd* (Boyle et al., 2004). PCR analysis was carried out at the School of Veterinary and Biomedical Sciences, James Cook University.

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Peptide collection followed a protocol described by Rollins-Smith et al. (2006). Fifty mL of collecting buffer was placed in a labeled 250 mL specimen jar. Frogs were subcutaneously injected dorsally (0.01 mL/g frog weight) with norepinephrine bitartrate salt (Sigma N-5785) in phosphate buffered saline to 10 nM to enhance protein secretion, then placed in the pre-prepared specimen jar. To avoid cross-contamination of samples, a separate jar was used for each frog. After 15 minutes the frog was removed, rinsed with creek water and placed back in its original resealable bag.

Immediately after frogs were removed from the specimen jar, 1 mL of 50% hydrochloric acid was added to the 50 mL collecting buffer to acidify the solution. The solution was then passed through two connected Sep-Pak filters (Plus C-18 cartridges) at approximately one drop per second to collect cationic and hydrophobic peptides. Prior to the field trip, the filters were activated with 10 mL methanol and 10 mL buffer A and stored in 50 mL plastic tubes with 2 mL buffer A. After use, the Sep-Pak filters were placed back into the labeled 50 mL plastic tubes, stored on ice at approximately 6ºC and transported back to James Cook University for further processing (Rollins-Smith et al., 2006).

Following peptide collection, digital images of *L. genimaculata* were taken (for detailed description see Chapter 2) to allow use of the photographic identification method (PIM) to eliminate the possibility of taking samples from the same individuals on successive nights. A pilot study (Chapter 2) indicated that the photographic identification method can be a reliable means of avoiding re-sampling if a distinct hourglass shape in the dorsal pattern is present. To further decrease the chance of sampling individuals more than once, I avoided sampling individuals with any similarity in dorsal pattern to those captured on previous nights. After digital images were taken, frogs were released at their collection point on the transect. *Litoria rheocola* does not show a distinct ventral or dorsal pattern that allows recognition of individuals. To avoid re-sampling individuals, frogs of this species were either collected in one night, or at different locations along the creek. For both species it is possible that there may have been some recaptures between seasons, however, mark-recapture studies by other investigators

(Richards and Alford, 2005) indicate that long-term recapture rates for these species are very low, making it very unlikely that enough individuals were multiple-sampled to seriously compromise the independence of the data across seasons. If any individuals were double-sampled, their peptide production should not have been affected by previous sampling. The volume of norepinephrine injected does not completely deplete the frog of its peptides and recovery time is well within the minimum of five months that elapsed between sampling trips (Rollins-Smith et al., 2005).

Laboratory

The samples were eluted from the Sep-Pak filters with 21 mL buffer B (Rollins-Smith et al., 2006) using two peristaltic pumps (Gilson Minipuls). Of the total 21 mL sample, 20 mL was placed into a centrifuge tube and stored at - 4ºC. The remaining 1 mL was added to a sterile Eppendorf microtube to determine total skin peptides per sample using a Micro Bicinchoninic Acid (BCA) protein assay (Pierce, Rockford, III., USA) following manufacturer's instructions (Biotechnology, 1997). Bradykinin synthetic peptides were used as standards instead of bovine serum albumin (BSA), due to better detection of smaller antimicrobial peptides (Rollins-Smith et al., 2002b). Each protein assay plate was read on a spectrophotometer at an absorbance of 540 nm, as recommended by the manufacturer, and concentration of the peptides was calculated.

The 20 mL peptide samples were concentrated using a rotary evaporator with a water bath, set at approximately 50ºC, to remove the acetonitrile added during elution. Afterwards, samples were stored at - 80ºC for at least 24 hours prior to freeze-drying (Freeze-dryer Model Operon). Peptides are partially hydrophobic and hence I freeze-dried samples to a volume that would allow me to reconstitute the samples to 5 mg/mL. This method differed from that of Rollins-Smith et al. (2006), where samples were completely dried prior to reconstitution.

The peptide samples were reconstituted with sterile high performance liquid chromatography (HPLC) water to 5 mg/mL. Samples were filter sterilised using a 0.22 μm Millex filter.

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Bd growth inhibition assays were performed to a) determine if AMPs were present in the peptide samples and to b) test and quantify their effectiveness against *Bd* cultures. Isolates of *Bd* (Tully-*L. rheocola* 06-LB-1) were cultured following the protocol of Longcore et al. (1999) but at a nutrient concentration of 50% as it increased *Bd* culture growth. Zoospores were harvested by flooding agar plates with 2 mL of TGhL for ten minutes (Boyle et al., 2003) followed by vacuum filtration through autoclaved 20 μm spectra/mesh nylon filters (Spectrum 722-05067-000) to remove any zoosporangia that might influence culture growth. The concentration of zoospores was determined by counting live and moving zoospores in three subsamples using a haemocytometer. TGhL was added until a final concentration of *Bd* zoospores of 10×10^6 mL^{-1}. Peptide samples were diluted to 100, 250, 500 and 1000 μg/mL. Ninety-six-well tissue culture treated microtitre plates (Corning Incorporation) were used for growth inhibition assays. Each plate contained three peptide samples at each of the four dilutions (five replicates each) with *Bd*, ten replicates of *Bd* culture at 5×10^5 mL^{-1} zoospores (positive control), five replicates of non-viable *Bd* culture that was heat treated for 30 minutes at 60 ºC (negative control), and 19 replicates of the TGhL culture medium (background absorption correction; Table 3.2).

Each plate was read daily on the spectrophotometer at an absorbance of 492 nm until one day after maximum growth of the *Bd* culture (day x). Each well of the plate was observed daily under 10 × and 20 × magnification and observations were recorded, including those of bacterial contamination and inhibition of *Bd*. Any well that was noted as contaminated was excluded from analysis as it resulted in higher spectrophotometer readings.

Percentage inhibition was calculated by first subtracting the average absorbance for the negative control from each well at day 0 and day × using the formula:

$$1 - [(\text{day } x - \text{day } 0)/(\text{day } x - \text{day } 0 + \text{positive control})] \times 100$$

An average was taken of all uncontaminated wells per peptide sample per concentration.

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Table 3.2 96-well plate layout for *Batrachochytrium dendrobatidis* (*Bd*) growth inhibition assay. Three peptide samples could be run concurrently.

+ = positive control (50µl *Bd* + 50µl HPCL water)

- = negative control (50µl *Bd* heat killed + 50µl HPCL water)

* = background absorption correction (100µl TGhL)

	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	+	+	+	empty	empty	-	-	-	-	-
B	+	+	+	+	+	*	*	*	*	*	*	*
C	Peptide sample 1 (µg/ml)				Peptide sample 2 (µg/ml)				Peptide sample 3 (µg/ml)			
	1000	500	250	100	1000	500	250	100	1000	500	250	100
D	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
E	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
F	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
G	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
H	*	*	*	*	*	*	*	*	*	*	*	*

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Statistical analysis

Total skin peptides secreted (determined by micro BCA protein assay):

To investigate whether body mass influenced total peptide secretion, linear regression between total peptides secreted (μg) and body mass (g) per frog was performed separately for each species. Total peptide secretion per surface area (cm^2) was calculated by estimating surface area using the equation of McClanahan and Baldwin (1969).

$$\text{Surface area } (cm^2) = 9.9 \times (\text{weight in grams})^{0.56}$$

The data was $\log_{10}$ transformed before any statistical analysis. I performed a Mann-Whitney U Test (MWU-test) to test for differences in total peptide secretion per surface area between *L. genimaculata* and *L. rheocola*. Because there were no infected *L. genimaculata* individuals in summer at low elevation, a three way ANOVA, including all three factors (infection status, elevation and season) could not be performed. I therefore performed two two-way ANOVAs (infection status and elevation for the winter samples; infection status and season for samples at high elevation) and a t-test between infected and uninfected *L. genimaculata* at low elevation in winter to test for any effect of infection and determine whether this variable could be safely excluded from further analyses. None of the analyses were significant and infection status was not included in the final two-way ANOVA (season and elevation). Only a few infected *L. rheocola* were found during the study and consequently infection status could not be incorporated into the factorial analysis for this species.

Inhibition:

Some samples for each species showed no evidence of inhibition of *Bd* at the concentrations tested. The results for these samples are not commensurate with the results of samples that did show inhibitory activity, but the proportions of samples that fell into each category are a potentially important measure of levels of protection against *Bd.* I therefore carried out a series of categorical analyses. I used Fisher's exact tests for each species to determine whether infection status affected the proportions of

samples that fell into each category. If this was not significant, I proceeded to combine the data from infected and uninfected individuals, and used separate Fisher's exact tests to determine whether the proportion of samples showing anti-*Bd* activity differed among sites, elevations, and seasons.

Effectiveness of AMPs

The standard statistic considered in medical and toxicological literature is the LC_{50}, the concentration of an agent that is lethal to 50% of the target population (Sanchez-Bayo and Goka, 2007). However, this seems irrelevant in the study of AMPs; a frog surrounded by a concentration of AMPs that reached the LC_{50} for *Bd* zoospores could presumably still become infected and possibly die as a result of chytridiomycosis. I therefore calculated and analysed the IC_{100}, the concentration necessary to completely inhibit the growth of *Bd*. This is the same as the MIC (minimal inhibitory concentration) described in Rollins-Smith et al. (2003), but potentially less confusing. There were several samples at which no concentration of AMPs tested produced 100% inhibition of *Bd*. In these cases, I used extrapolation to predict peptide concentrations where 100% inhibition would occur ($x = (100/b)/m$, where x is the unknown concentration of peptides, b = y intercept and m= slope of a regression of percent inhibition on concentration.

Because I measured the inhibitory activity of AMPs at few concentrations only and had to use extrapolation to estimate the IC_{100} for some samples, the data were not truly on an interval scale. For a conservative analysis, I categorised the estimates of IC_{100} into four groups, forming a log series of concentrations: 1 = 50-174 μg/mL, 2= 175-374 μg/mL, 3= 375-749 μg/mL and 4= 750+ μg/mL. I used Fisher's exact tests for each species' data to determine whether there were differences between the IC_{100}s of peptides produced by infected and uninfected frogs, between upland and lowland populations and between summer and winter (following the same procedure described in the previous section).

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Overall protection of frogs

Once the IC_{100} was calculated, the overall protection of the frog against *Bd* was estimated as the total number of mL of solution at the IC_{100} that would be produced per unit surface area:

Overall protection (mL at IC_{100} per cm^2) = (total mass of peptides/ SA × $1/IC_{100}$).

The data was $\log_{10}$ transformed before statistical analysis. I repeated the analysis as described in the "total skin peptides secreted" section to determine whether infection status significantly contributed to the variation.

Results

qPCR assay to determine prevalence of *Bd* and intensity of infection

A total of 160 *L. genimaculata* and 56 *L. rheocola* were captured to determine prevalence and intensity of infection with *Bd* and to collect skin peptides. Fifty-six *L. genimaculata* and 13 *L. rheocola* tested positive for *Bd*. Except for frogs at Paluma, prevalence of *Bd* was significantly lower in summer compared to winter (Fisher's exact test, $p<0.01$ and $p<0.01$, respectively; Figure 3.2). Intensities of *Bd* infection in low elevation *L. genimaculata* populations were generally, although not significant, higher during the winter season (Mann-Whitney U-test, $Z=0.43$, $p=0.66$; Figure 3.3). Higher prevalence of *Bd* in high elevation *L. rheocola* populations was found during winter at Windin N creek (Mann-Whitney U-test, $Z=-0.64$, $p=0.4$; Figure 3.4). Overall, *L. genimaculata* had a significantly higher intensity of infection with *Bd* than did *L. rheocola* (Mann-Whitney U-test, $Z=4.17$, $p<0.001$).

Total skin peptides secreted

The total amount of peptides secreted by *L genimaculata* and *L. rheocola* were not significantly correlated with body mass ($r^2=0.03$ and 0.001, respectively; Figure 3.5). Site (ANOVA, $F_{4, 150} = 4.58$, $p<0.01$ and $F_{1, 52} = 4.60$, $P = 0.04$) and season (ANOVA, $F_{1, 150} = 5.53$, $p=0.02$ and $F_{1, 52} = 5.68$, $P = 0.02$, respectively) had significant effects on total peptide secretion per surface area in both *L. genimaculata* and *L. rheocola* (Figures 3.6 and 3.7, respectively). In addition to the significant main effects, there were

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significant interactions between the effects of site and season on *L. genimaculata* and *L. rheocola* total peptide secretion per surface area (ANOVA, $F_{4, 150} = 6.89$, $p<0.01$ and $F_{1, 52} = 4.6$, $p=0.04$, respectively); this was not sufficient to remove the main effect of site or season. Overall, *L. genimaculata* secreted a significantly lower quantity of peptides per surface area than *L. rheocola* (Mann-Whitney U-test, $p<0.01$; Figure 3.8).

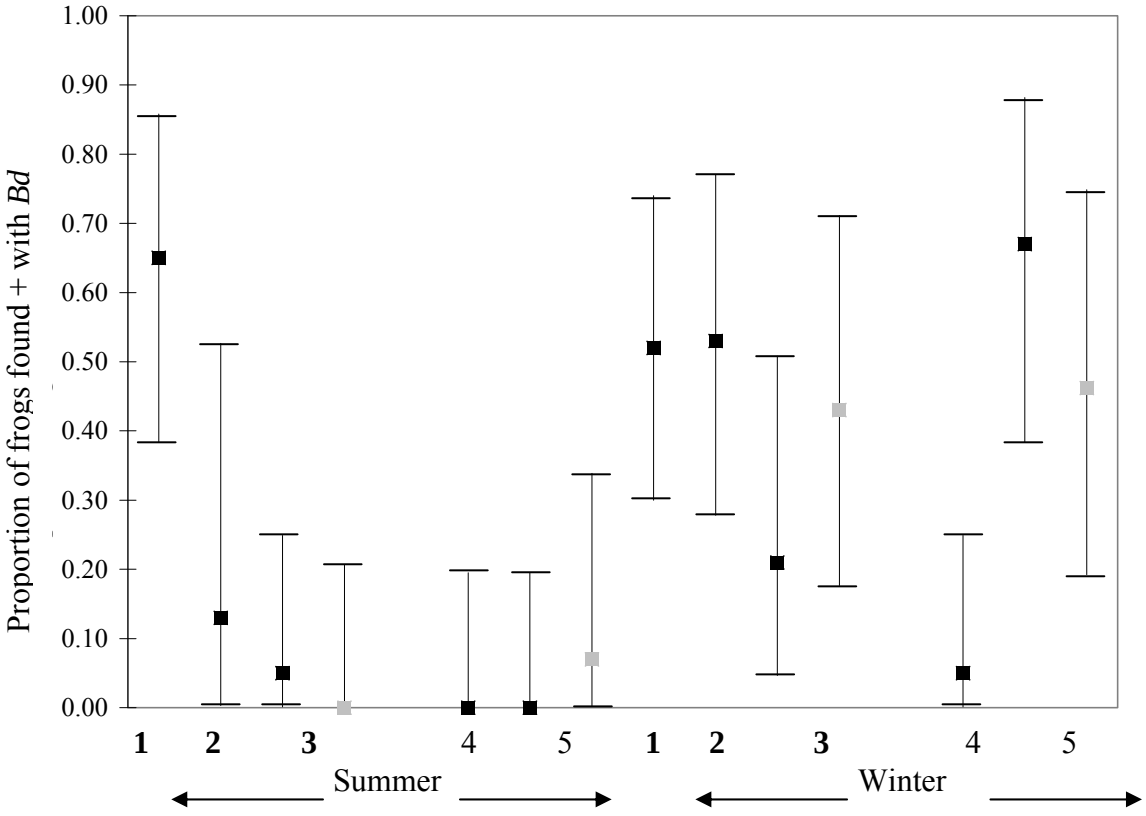

Figure 3.2 Prevalence of *Batrachochytrium dendrobatidis* (means and 95% confidence limits) in *Litoria genimaculata* (■) and *L. rheocola* (▒) individuals from which skin peptides were collected during two seasons at three high elevation sites (**1**= Birthday Creek, **2**= Murray Upper Bridge XI, **3**= Windin N Creek) and two low elevation sites (4= Murray Upper Bridge I, 5= Frenchman Creek).

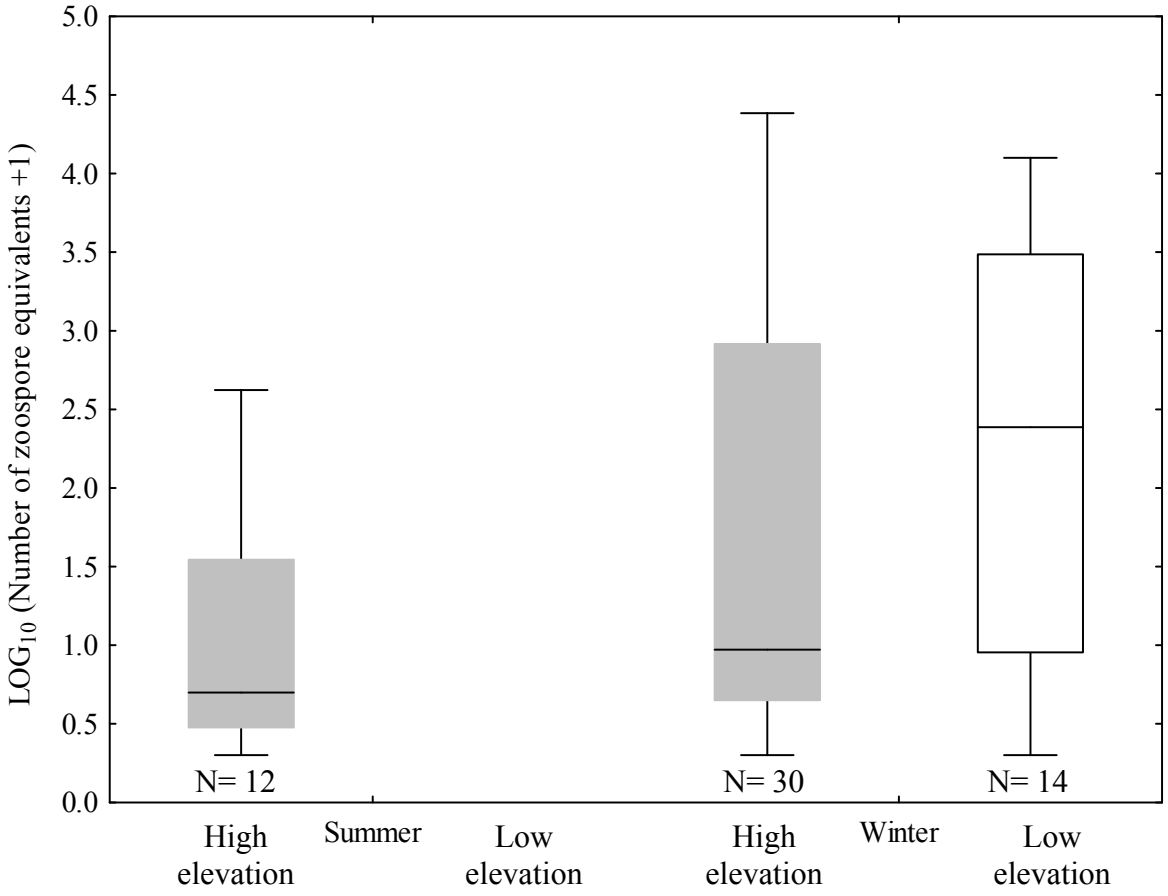

Figure 3.3 *Batrachochytrium dendrobatidis* infection intensity in *Litoria genimaculata* during summer and winter at high (▒) and low (□) elevations. In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

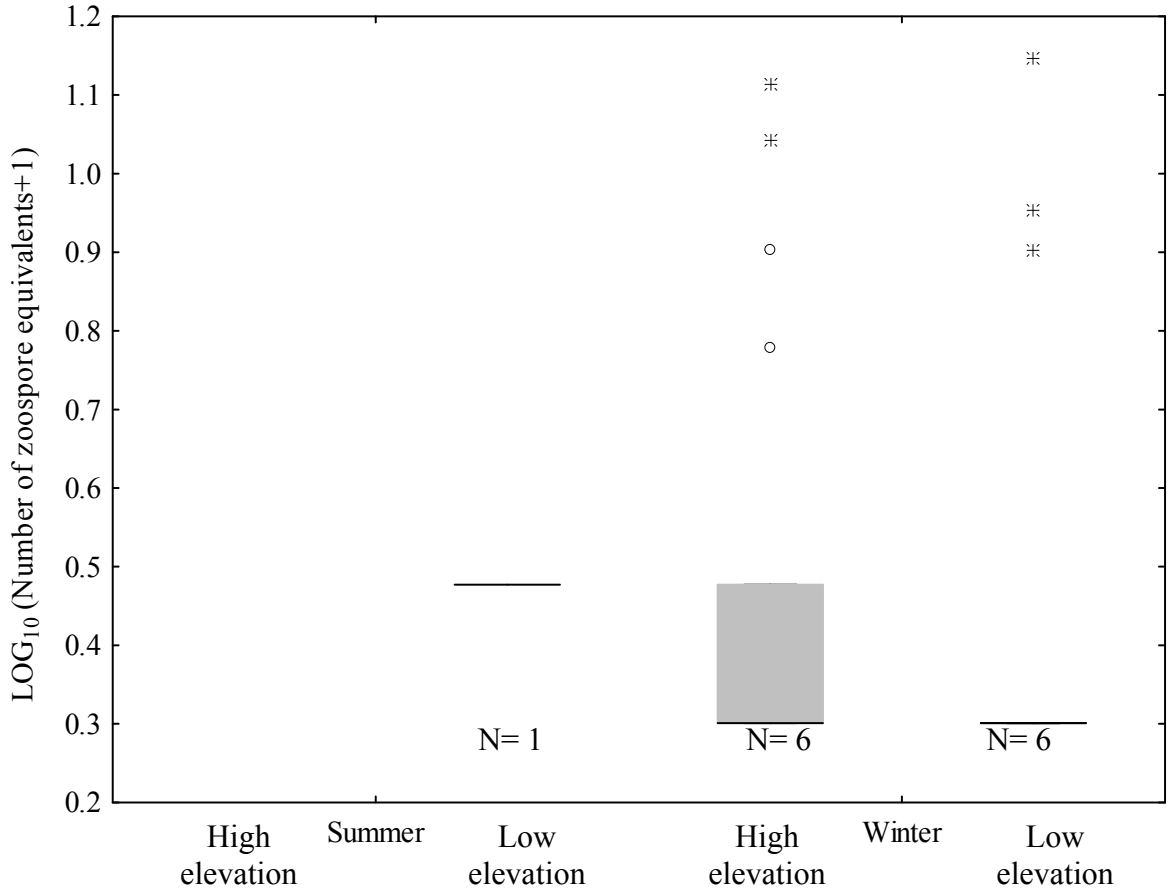

Figure 3.4 *Batrachochytrium dendrobatidis* infection intensity in *Litoria rheocola* during summer and winter at high (▭) and low (□) elevations. In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

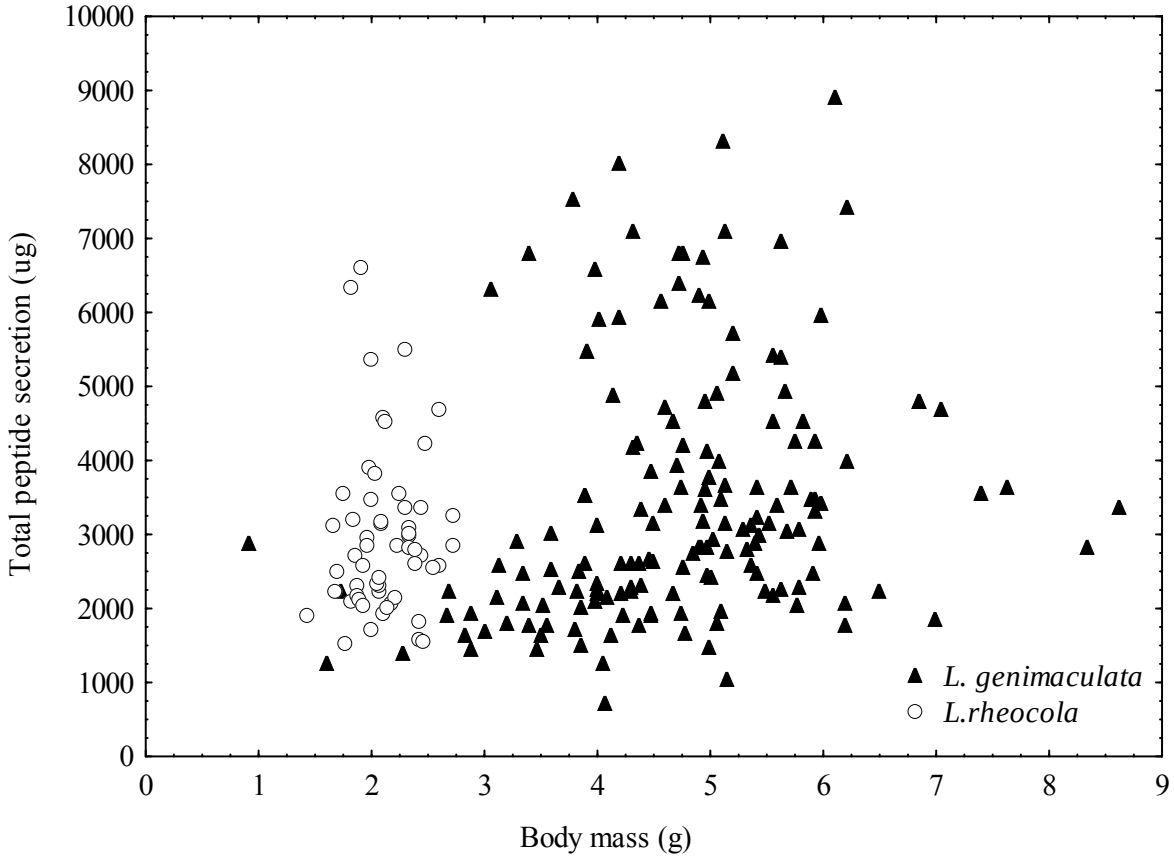

Figure 3.5 Relationship between total peptide secretion (μg) and body mass (g) in *Litoria genimaculata* ($r = 0.2$, $p = 0.004$; $r^2 = 0.05$) and *L. rheocola* ($r = 0.03$, $p = 0.8$; $r^2 = 0.001$).

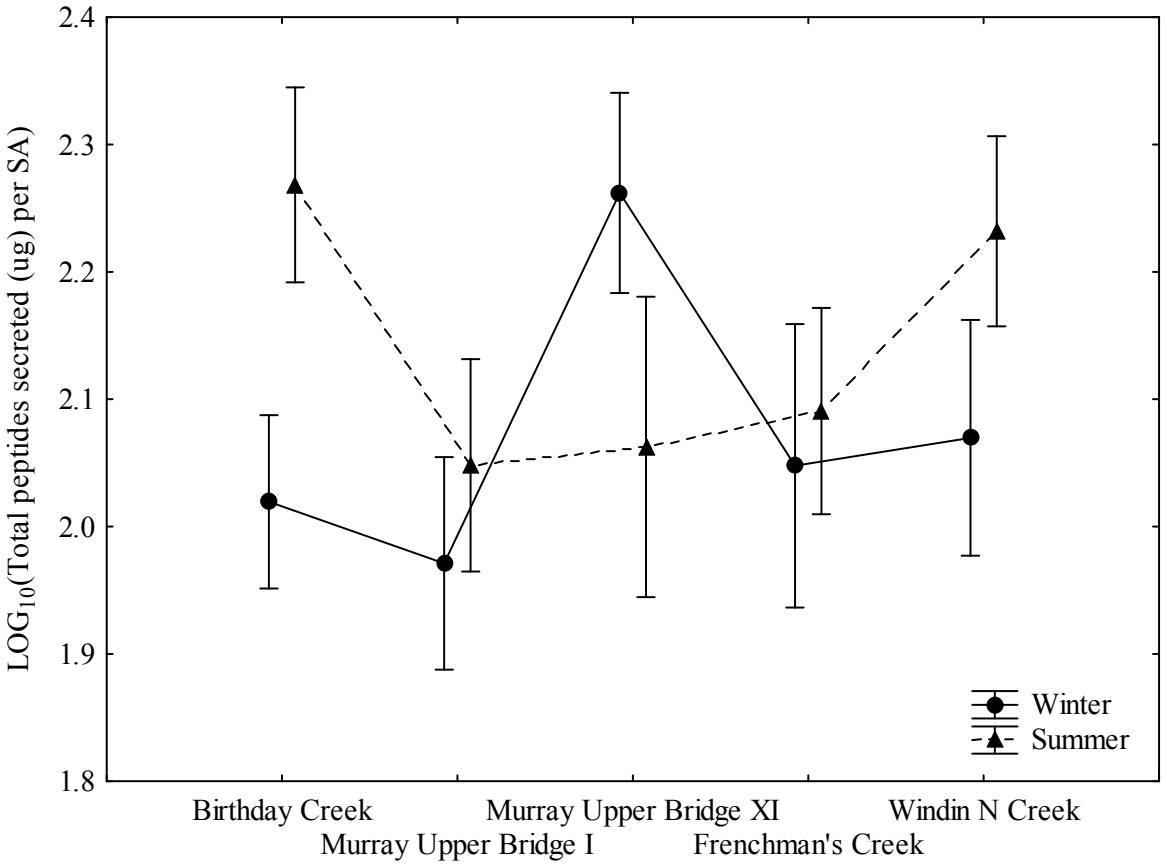

Figure 3.6 Total peptides secreted (μg) per surface area (cm^2) by *Litoria genimaculata* (means and 95% confidence limits) at three lowland sites (Murray Upper Bridge I and Frenchman Creek) and two upland sites (Birthday Creek, Murray Upper Bridge XI and Windin N Creek). The lines between data points are to aid in visualisation of the results only.

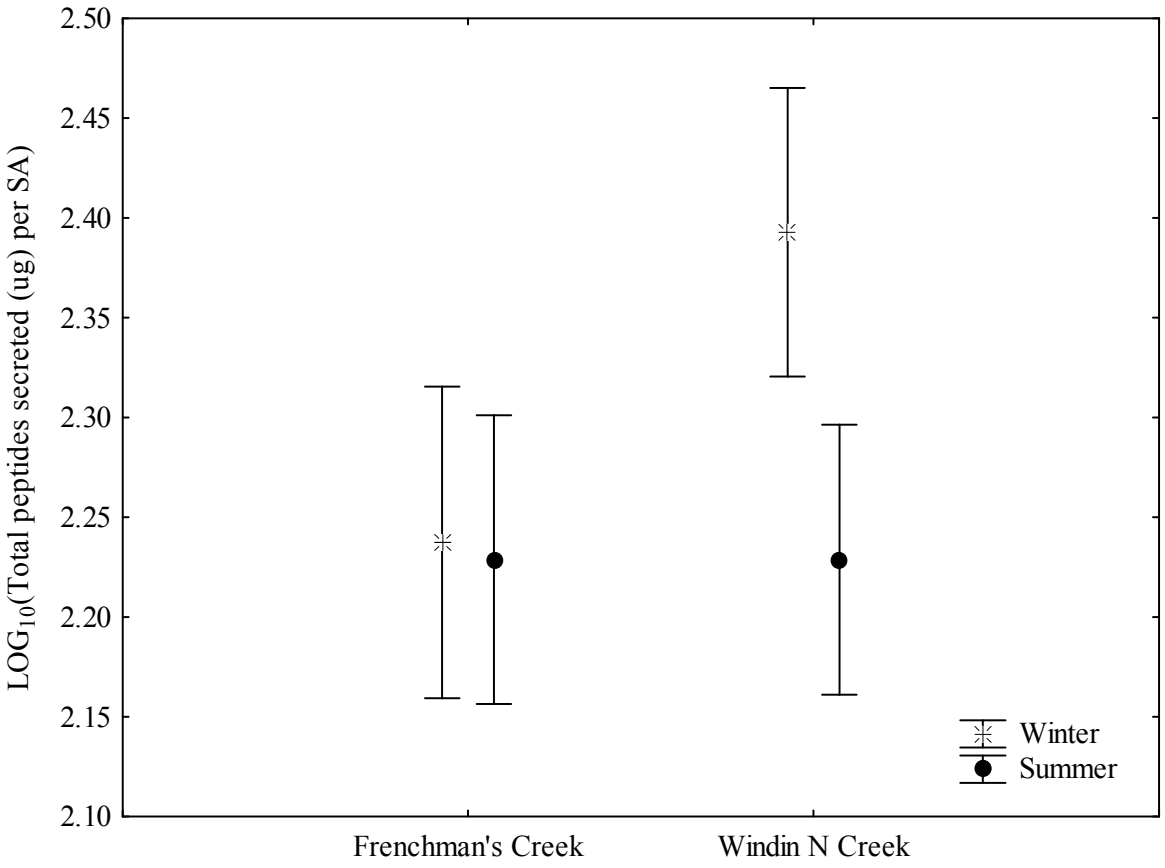

Figure 3.7 Total peptides secreted (μg) per surface area (cm^2) by *Litoria rheocola* (means and 95% confidence limits) during two different seasons at one lowland site (Frenchman Creek) and one upland site (Windin N Creek).

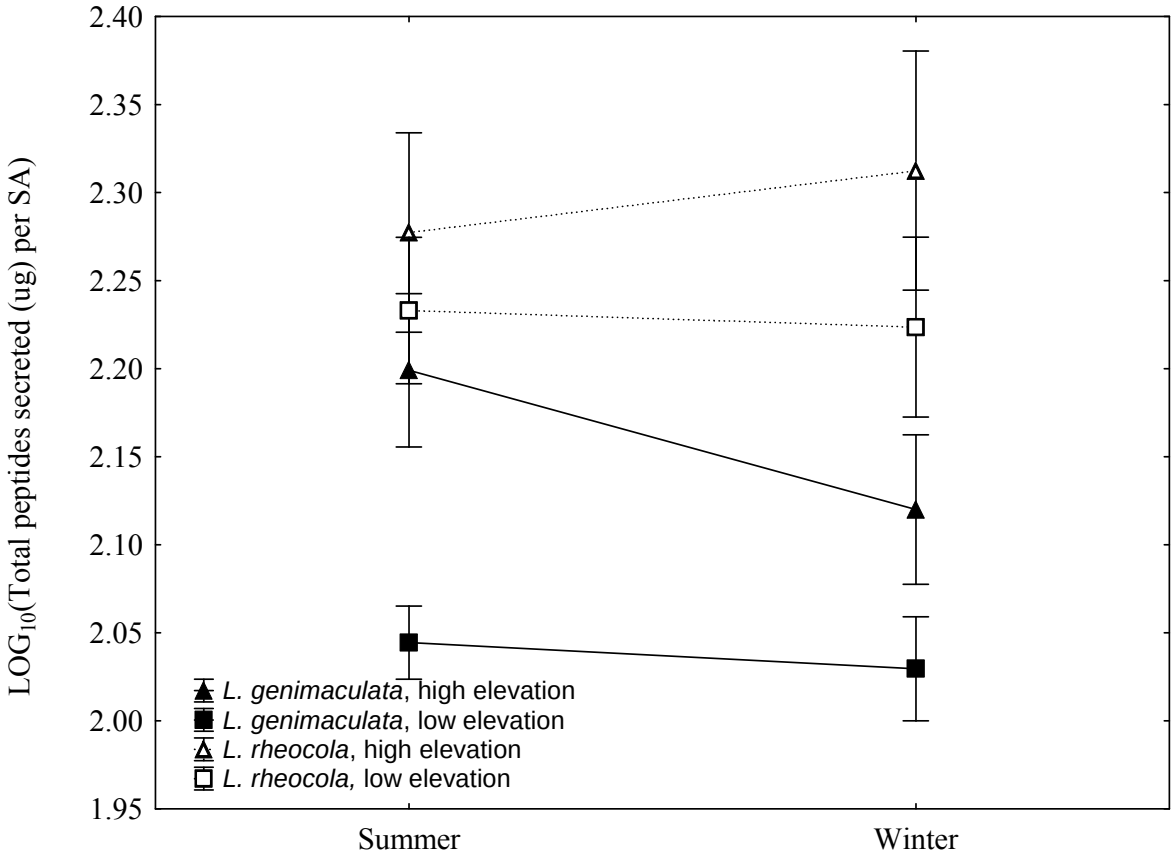

Figure 3.8. Total amount of peptides secreted (μg) per surface area (cm^2) by *Litoria genimaculata* and *L. rheocola* (means and 95% confidence limits) in winter and summer at low and high elevations. The lines between data points are to aid in visualisation of the results only.

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Proportion of samples showing inhibition of *Bd*

One hundred and twenty four of the 206 skin peptide samples showed at least some inhibition of *Bd*. The proportions of peptide samples that did and did not inhibit *Bd* differed significantly between *L. genimaculata* and *L. rheocola* (Fisher's exact test, $p<0.01$), where more peptide samples of *L. rheocola* inhibited *Bd*. The proportions of peptide samples that did and did not inhibit *Bd* did not differ significantly between infected and uninfected *L. genimaculata* (Fisher's exact test, $p=0.06$; Figure 3.9). However, the proportion of *L. genimaculata* samples inhibiting *Bd* was affected significantly by season (Fisher's exact test, $p=0.01$; Figure 3.9) and elevation (Fisher's exact test, $p<0.01$; Figure 3.9). During winter, a higher proportion of *L. genimaculata* peptide samples showed at least some inhibition of *Bd*. Infected and uninfected *L. rheocola* did not differ significantly in proportion of peptide samples that inhibited *Bd* (Fisher's exact test, $p=0.7$). In *L. rheocola*, there were also no significant differences between winter and summer (Fisher's exact test, $p=0.5$) and between high and low elevation sites (Fisher's exact test, $p=0.3$).

Effectiveness of AMPs (IC_{100})

There was a significant overall difference in IC_{100} between *L. genimaculata* and *L. rheocola* (Fisher's exact tests, $p<0.01$) where more peptide samples of *L. genimaculata* required higher concentrations to achieve total inhibition of *Bd*. This difference persisted when the data for each season were examined separately (Figure 3.10). In summer and winter more peptide samples of *L. genimaculata* required higher concentrations to achieve total inhibition of *Bd* than *L. rheocola* (Fisher's exact test, $p=0.04$; $p=0.04$, respectively). Within species, there was no significant difference in AMP effectiveness between infected and uninfected *L. genimaculata* and *L. rheocola* (Fisher's exact tests, $p=0.6$ and $p=0.4$, respectively). There was also no effect of site on AMP effectiveness for either species (Fisher's exact tests, $p=0.2$ and $p=0.6$, respectively). There was a significant seasonal effect on IC_{100} in *L. genimaculata* (Fisher's exact test, $p=0.03$); more peptide samples in summer required higher concentrations to achieve total inhibition of *Bd* (Figure 3.10). There was no seasonal effect within *L. rheocola* samples (Fisher's exact test, $p=0.2$).

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Overall protection of frogs

Litoria rheocola had significantly higher levels of overall protection against *Bd* (mL of AMPs at IC_{100}/cm^2) than *L. genimaculata* (Mann-Whitney U-test, $p<0.01$; Figure 3.11). *Litoria genimaculata* overall protection did not significantly differ among seasons or elevations (ANOVA, $F_{1, 76} = 2.98$, $p=0.09$, $F_{1, 76} = 45.0$, $p=0.50$, respectively) and was not affected by the infection status of frogs (Figure 3.12). There were no significant effects of season or elevation on overall AMP protection of *L. rheocola* (ANOVA, $F_{1, 40} = 3.04$, $p=0.09$, $F_{1, 40} = 0.01$, $p=0.94$, respectively), although the apparent interaction (Figure 3.13) between these factors (season and elevation) approached significance (ANOVA, $F_{1, 40} = 3.88$, $p=0.06$).

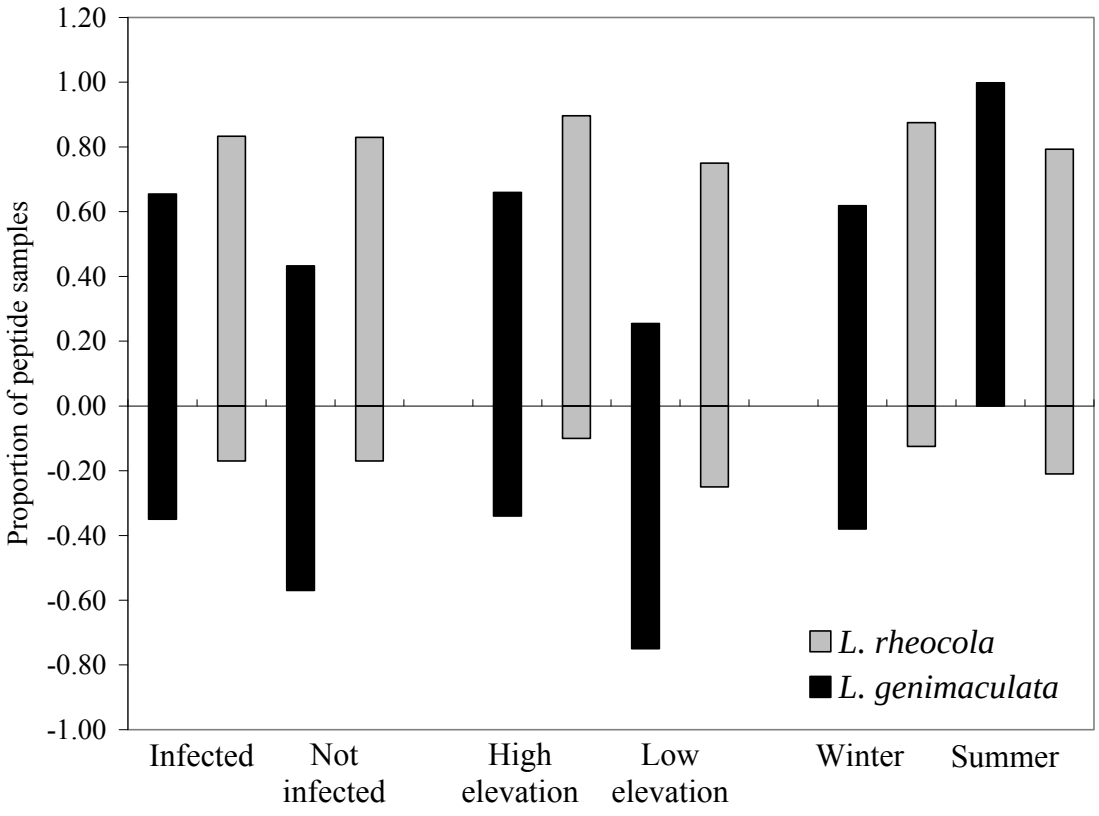

Figure 3.9. Proportion of peptide samples from *Litoria genimaculata* (■) and *L. rheocola* (▒) that did (positive value) and did not (negative value) inhibit *Batrachochytrium dendrobatidis* during challenge assays.

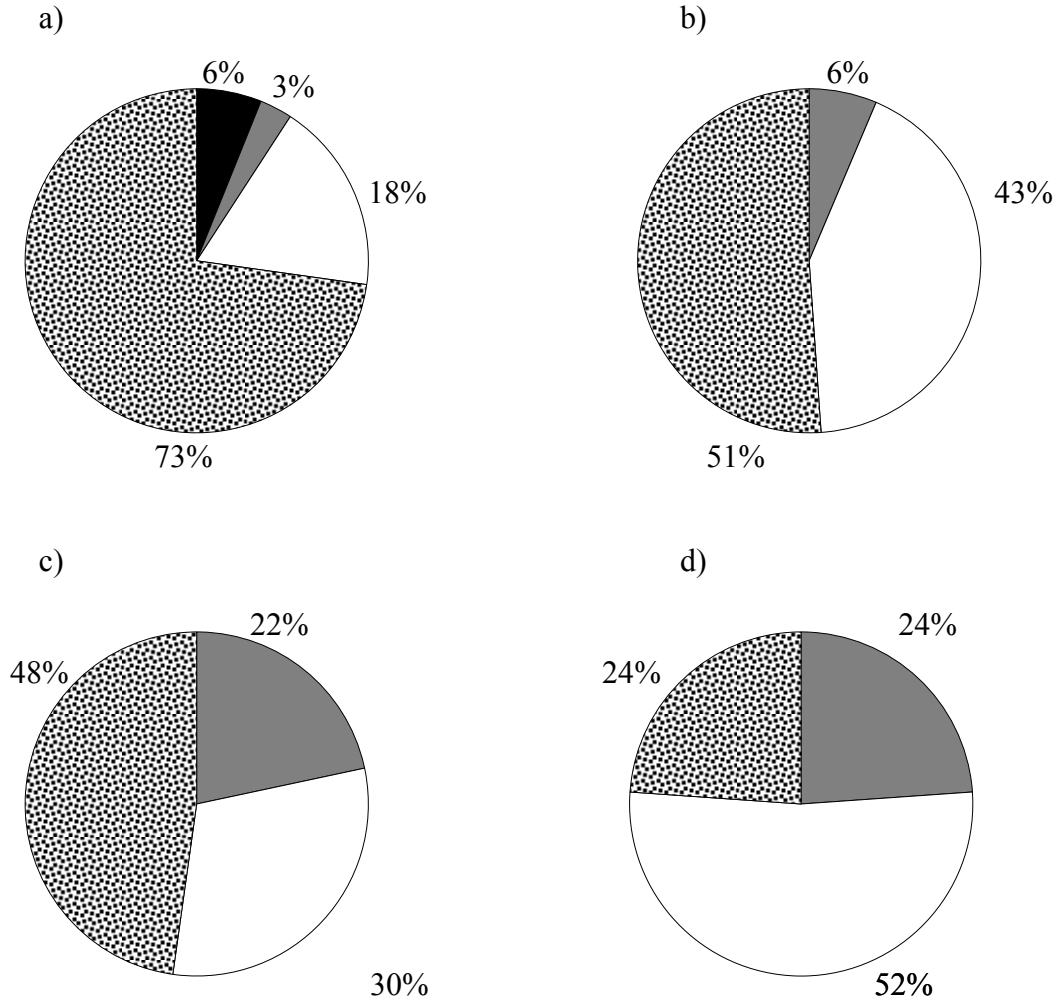

Figure 3.10. Proportions of peptide samples from *Litoria genimaculata* in summer (a) and winter (b) and from *L. rheocola* in summer (c) and winter (d) at the four different inhibitory concentrations, 1 (■) = 0-174 µg/ml, 2 (▒) = 175-374, 3 (□) = 375-749 µg/ml and 4 (▨) = 750+ µg/ml, that completely inhibited *Bd* (IC_{100}).

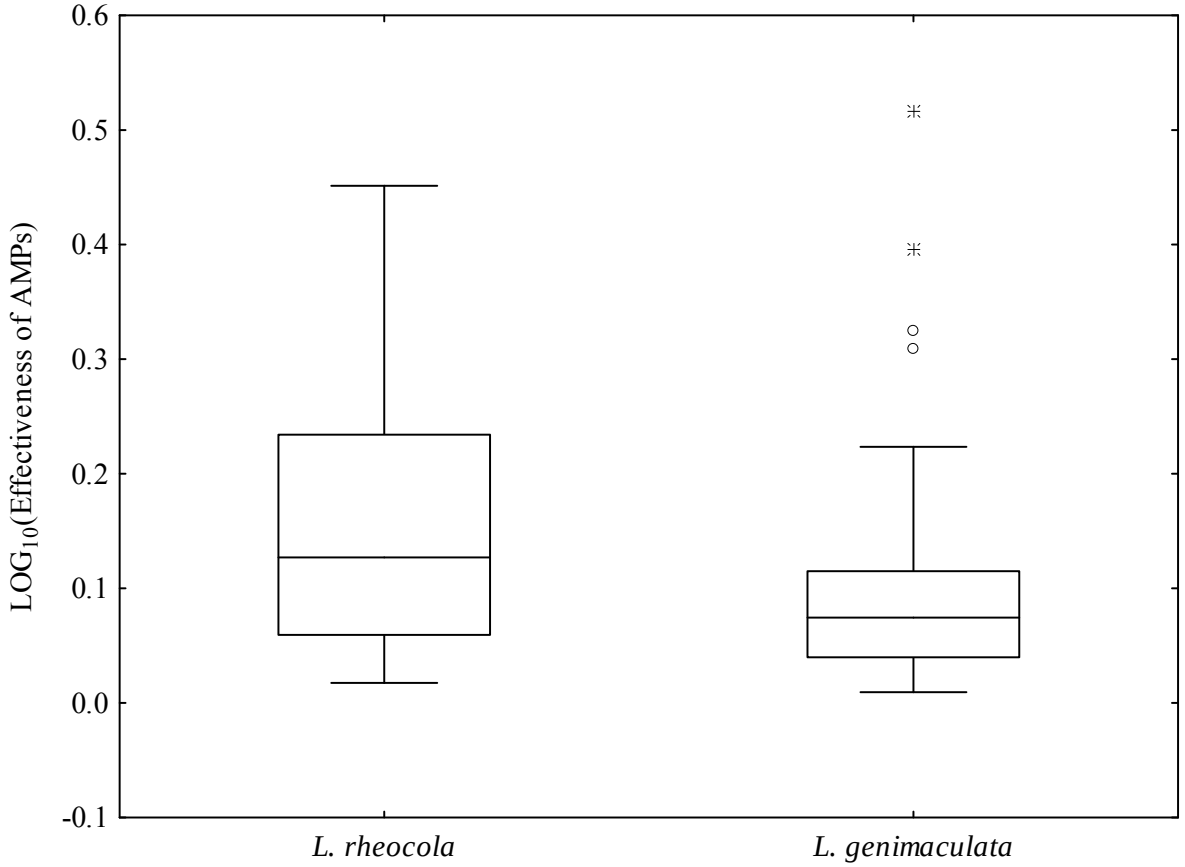

Figure 3.11. Overall protection afforded by AMPs (mass of peptides/ SA × 1mL/IC_{100}) in *Litoria genimaculata* and *L. rheocola*. In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

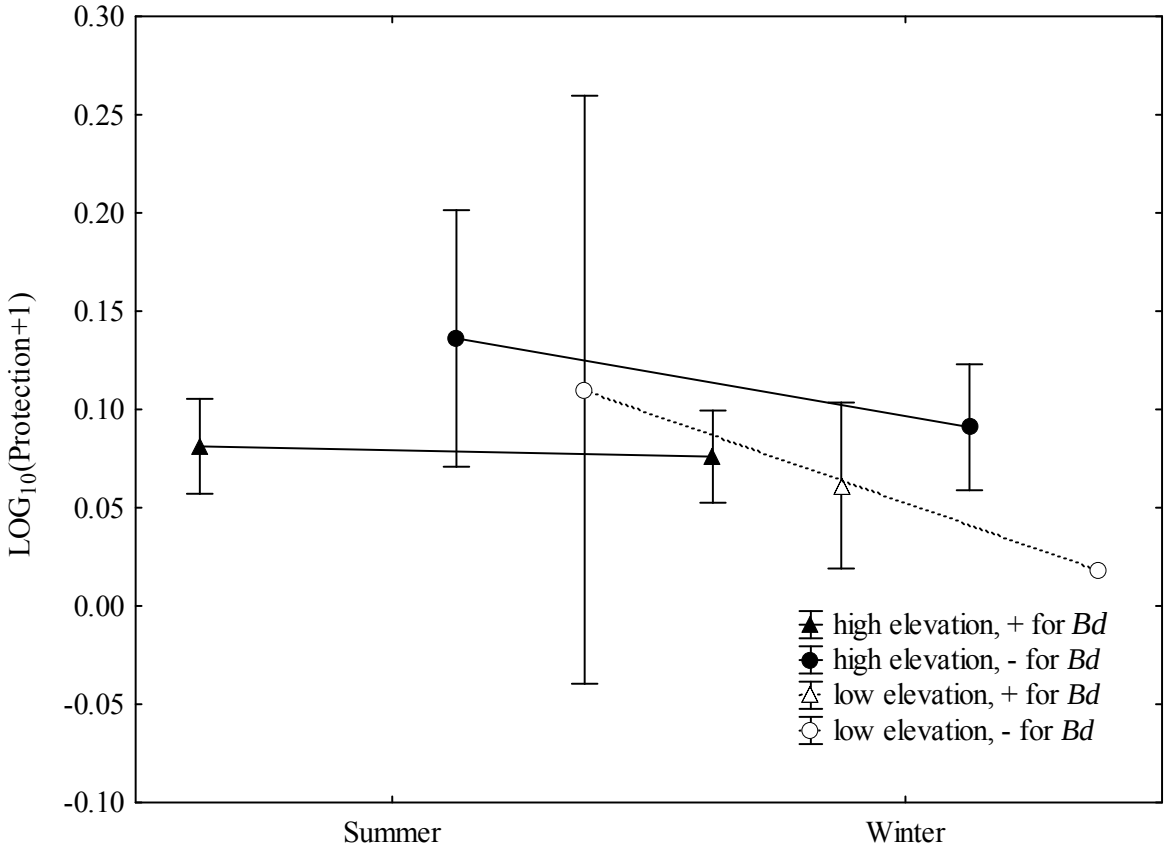

Figure 3.12. Overall protection afforded by AMPs (total peptides secreted × ($1/IC_{100}$) / SA) of infected (positive for *Batrachochytrium dendrobatidis*) and uninfected (negative for *Batrachochytrium dendrobatidis*) *Litoria genimaculata* (means and 95% confidence limits) collected during two different seasons at high and low elevation sites. Infection status was determined by qPCR assay. The lines between data points are to aid in visualisation of the results only.

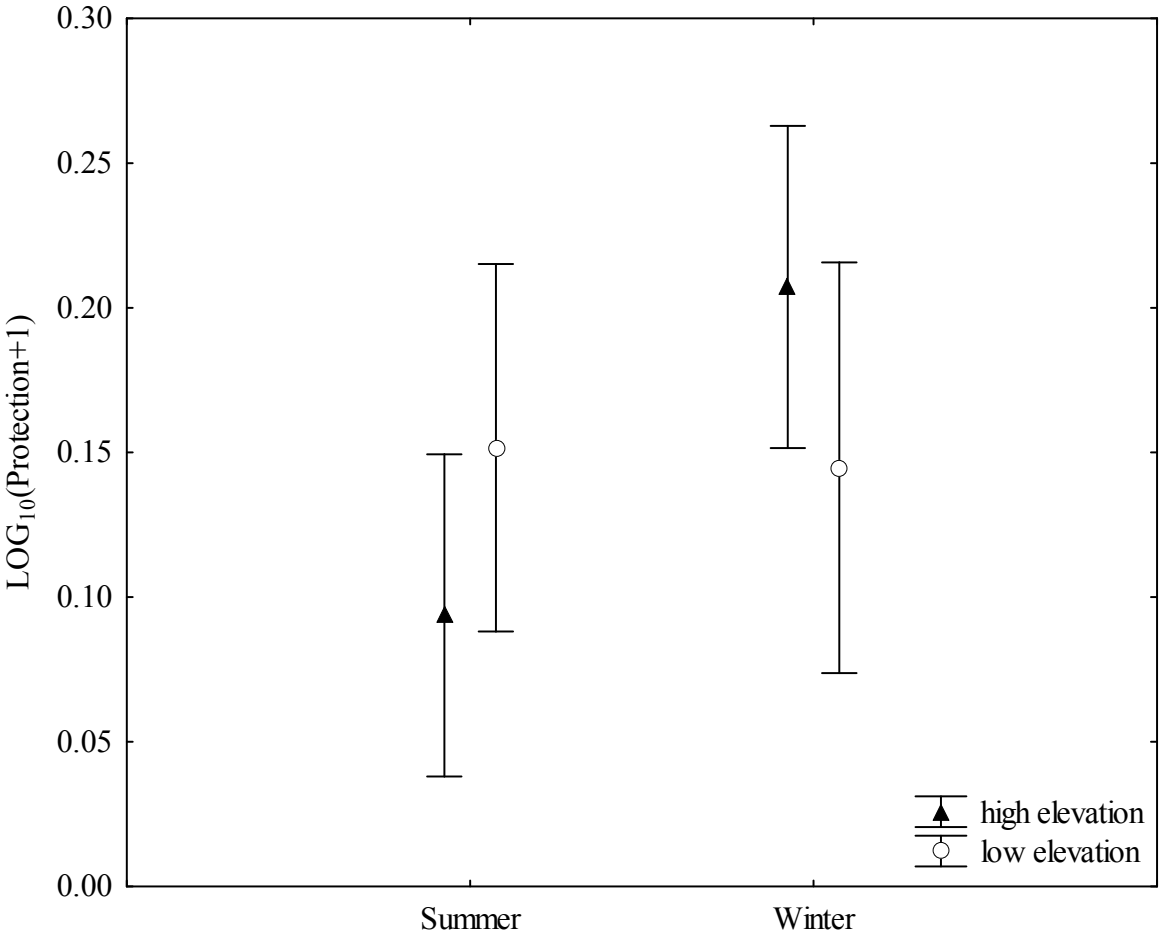

Figure 3.13. Overall protection afforded by AMPs (total peptides secreted × ($1/IC_{100}$) / SA) in *Litoria rheocola* (means and 95% confidence limits) from samples collected during summer and winter at high and low elevation sites.

Discussion

Effects of season and elevation on the prevalence of *Bd,* which is usually greater in winter and at higher elevations, have been observed in multiple anuran species in Queensland and New South Wales, Australia (Berger et al., 2004; Retallick et al., 2004; McDonald et al., 2005; Woodhams and Alford, 2005; Kriger and Hero, 2007b). These patterns are most likely driven by temperature, since lower temperatures are more suitable for *Bd* survival and reproduction (Longcore et al., 1999; Berger et al., 2004). Lower prevalence of *Bd* infection was detected in summer at most sites, except at Birthday Creek, Paluma National Park, where a high prevalence occurred in *L. genimaculata* in summer. The reasons why frogs at Paluma demonstrated such high prevalence of *Bd* infection in summer remained unclear. Woodhams (2003), who used the same transects, calculated the prevalence to be 10 percent or less, which is much lower than recorded during the 2005-2007 period of my study. It is possible that previous studies may have underestimated prevalence (McDonald et al., 2005; Woodhams and Alford, 2005). This is because these studies used histology on removed toe clips to detect *Bd* infection rather than the more efficient qPCR assays (Annis et al., 2004; Hyatt et al., 2007), as the latter method had not been developed for detecting and quantifying *Bd* during the period in which they collected data. Low intensity of infection, as was observed in *L. rheocola*, may result in even lower prevalence estimates when histology is used as a diagnostic technique (Hyatt et al., 2007). Alternatively, environmental conditions during my study period may have been more favourable for *Bd* compared to the time period of McDonald et al. (2005) and Woodhams and Alford (2005) study.

Litoria genimaculata and *L. rheocola* peptide secretion per surface area differed among sites and seasons. Several hypotheses could explain the high seasonal variation. It may be an anuran physiological response to decreased temperatures in winter, resulting in smaller amount of peptide per surface area being produced or released during that season. Seasonal variation could also represent an adaptive response to changes in the abundance or composition of the microbial assemblage that the frogs are exposed to. Seasonal variation has been documented in microbial assemblages in the rainforests of

northern Queensland; for example Paulus et al. (2006) found that microfungal assemblages on decaying leaves of four rainforest tree species at Wooroonooran National Park, northern Queensland, differed significantly between the winter and summer seasons. Studies in other habitats have demonstrated seasonal variation in methanotrophs (a form of bacteria) and epiphytic yeasts (Glushakova and Chernov, 2007; Singh and Kashyap, 2007). The combination of seasonal fluctuations in both quantity secreted and effectiveness suggests that the composition of the AMPs secreted changed seasonally, which would be expected if the seasonal changes were adaptive to fluctuations in the microbiota.

Directional selection, resulting in novel antimicrobial peptide gene sequences, has been observed in *Rana pipiens* (Tennessen and Blouin, 2007). This study concluded that selection may result in affected species producing more effective AMPs against novel microbial organisms. The situation in *L. genimaculata* is more complex. When individuals possessed AMPs that were effective against *Bd,* AMPs from upland populations were not significantly more effective than AMPs from lowland populations, suggesting that selection has not altered the nature of the AMPs possessed by upland frogs. However, significantly greater proportions of individuals in upland populations possessed AMPs that were effective against *Bd*; and at both upland and lowland sites, the proportion of individuals with anti-*Bd* AMPs was significantly higher in winter. This suggests that selection may have acted to increase the production of existing anti-*Bd* AMPs during winter and in upland populations of *L. genimaculata*. Further studies are needed, investigating the composition of different suites of AMPs in *L. genimaculata* and *L. rheocola*, at high and low elevation sites, in order to determine whether their AMPs have been influenced by natural selection after exposure to *Bd*.

While *L. rheocola* demonstrated a slower population recovery than *L. genimaculata* after the appearance of chytridiomycosis, the species' innate immune defences appear likely to be more successful at inhibiting *Bd* infection than that of *L. genimaculata*. *Litoria rheocola* secreted more peptides per surface area, more individuals secreted peptides that inhibited *Bd,* and overall protection was significantly higher than in

L. genimaculata. This is in contrast to the findings presented by Woodhams (2003) who observed the opposite trend. However, he did not subcutaneously inject norepinephrine but used a norepinephrine immersion technique, which is significantly less effective (Woodhams, 2003). Additionally, his limited sample size did not allow him to account for other influencing factors, such as season or elevation, which may have affected his results.

This is the first large study on AMPs of two sympatric frog species and results suggest that natural selection caused by the effects of chytridiomycosis on different populations may have acted on the amounts of peptides secreted by *L. genimaculata*. The fact that neither the effectiveness of peptides nor the proportions of individuals secreting effective peptides differed between upland and lowland populations of *L. rheocola* may support the idea that this species recolonised from lowland populations (Woodhams and Alford, 2005). The selection pressure experienced by this species while possible recolonising high elevation habitat, is unclear. The results suggest that perhaps they experienced a stronger natural selection pressure than *L. genimaculata*, resulting in more successful innate immune defences inhibiting *Bd* infection. On the other hand, it is possible that other factors, such as microenvironment selection, frequency of frog to frog interactions, or disease avoidance behaviour, may also influence the susceptibility of these frog species to *Bd*.

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Antimicrobial peptide defence and profile in *Litoria genimaculata* (Anura: Hylidae): comparison amongst five different geographical locations.

Abstract

Amphibians have been and are still declining globally. One of the contributing factors is chytridiomycosis, an amphibian skin disease caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*). Host behaviour, including habitat selection, interaction with other individuals and microenvironment selection, possibly influence exposure time to *Bd* and the outcome of *Bd* infection, and may have contributed to the variable susceptibility to infection among amphibians observed in the wild. Antimicrobial peptides (AMPs), produced by the granular glands in amphibians, are a component of the amphibian innate immune defence against microbial organisms, including *Bd*. I took skin peptide samples from a total of 86 *Litoria genimaculata*, an Australian tree frog, from three upland populations that have suffered declines caused by chytridiomycosis and have subsequently recovered, and from two lowland populations that did not decline, although *Bd* is now endemic. I investigated the quantity of peptides that frogs secreted per surface area, how effective the peptides were against *Bd*, based on the concentration of AMPs needed to completely inhibit *Bd* growth (IC_{100}) and the overall protection provided by AMPs to frogs (mL of AMPs at IC_{100}/cm^2). Furthermore, I compared the AMP profiles (summary of all of the skin peptides secreted quantified by mass spectrometry) amongst the *L. genimaculata* populations. I hypothesised that if AMPs play an important role in the survival of *L. genimaculata* individuals infected with *Bd*, then strong selection pressure could have caused the AMP profiles of upland populations to diverge, resulting in possible increased innate protection against *Bd* compared to lowland populations. Total skin peptide secretion per surface area was significantly negatively correlated with prevalence of *Bd*. A similar trend was observed with overall protection. However, infected individuals did not always secrete smaller amounts of peptides per surface area or have less overall protection than uninfected individuals from the same site. This suggests that, while some aspects of innate immune defence are correlated with prevalence of *Bd*, once an individual is infected it may not necessarily change its peptide secretion or overall protection afforded by AMPs

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produced. Multiple Response Permutation Procedure analyses showed that skin peptide profiles differed across elevations within latitudes and along a latitudinal gradient, suggesting that there are background differences in AMPs among populations and that the different histories of natural selection experienced by upland and lowland populations of *L. genimaculata* may have led to differences in the nature of the AMPs secreted by individuals.

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Introduction

It is recognized that more than 30 percent of amphibian species are declining (IUCN, 2006; Smith et al., 2006), and that these declines occurred and are occurring at a greater rate than in any other animal group (Blaustein and Wake, 1990; Wake, 1991; Blaustein, 1994; Meyer et al., 1998; Daszak et al., 2005). Numerous studies have confirmed that chytridiomycosis, an amphibian disease caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), has contributed to the rapid amphibian declines (Mutschmann et al., 2000; Bosch et al., 2001; McDonald et al., 2005; Lips et al., 2006). At the same time, some amphibians persist despite infection (Richards et al., 1993; McDonald and Alford, 1999; Kriger and Herman, 2006).

Several behavioural factors, such as habitat selection, interaction with other individuals and microenvironment selection, have been suggested to possibly influence risk of exposure to *Bd* and the outcome of *Bd* infection (Woodhams et al., 2003; Rowley and Alford, 2007b; Rowley and Alford, 2007a). For example, Rowley and Alford (2007b) observed a more restricted habitat use and increased frog to frog interaction in *Litoria nannotis*, which has experienced severe declines due to chytridiomycosis, compared with two sympatric species with less or little decline, *L. genimaculata* and *L. lesueuri*. This suggests a correlation between vulnerability to *Bd* and habitat selection and frequency of frog to frog interactions. Even though no population decline due to *Bd* has been recorded in *L. lesueuri*, the species is highly susceptible to *Bd* infection with a prevalence of 26 percent (Kriger and Hero, 2007b; Kriger et al., 2007b), suggesting that in this species *Bd* infection may not be fatal at all times (Kriger and Herman, 2006).

One factor that may hinder initial microbial invasion, or influence *Bd* infection, is the action of antimicrobial peptides (AMPs), found in the granular glands of amphibians (Boman, 1995; Hoffman et al., 1999; Zasloff, 2002). AMPs of several anuran species kill *Bd* zoospores *in vitro* (Rollins-Smith et al., 2002a; Rollins-Smith et al., 2003; Rollins-Smith and Conlon, 2005; Woodhams et al., 2005; Woodhams et al., 2007). It is possible, although no study has confirmed this yet, that although AMPs may not completely prevent initial invasion of the epidermis by *Bd* zoospores, they may reduce

the rate of self re-infection as the zoosporangia release motile zoospores capable of infecting the host animal. Hence AMPs may prevent the initial infection reaching a critical density that becomes fatal through physiological disruption (Voyles et al., 2007).

To date, several hundred unique amphibian AMPs have been isolated and characterised, including their molecular mass, sequence and inhibition of some microbes (see online database http://www.bbcm.univ.trieste.it). Each type of AMP can differ in inhibiting epidermal invasion by a variety of bacteria, protozoa and fungi (Nicolas and Mor, 1995; Simmaco et al., 1998; Batista et al., 1999; Bowie et al., 1999; Kim et al., 2001; Zasloff, 2002; Chan et al., 2006). Consequently, knowing a species' peptide profile (identified by mass spectrometry analysis), followed by identifying each peptide's inhibitory success against *Bd*, can assist in understanding a species' defence against *Bd*. Woodhams et al. (2006) conducted a matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry analysis on several Panamanian anuran species and observed greater inter - than intra-specific variation.

Selection pressure has been found to change genes encoding for AMPs (Tennessen, 2005; Tennessen and Blouin, 2007), resulting in a diversification (Duda et al., 2002) which may increase effectiveness against a target pathogen. Investigating the AMP profiles and inhibitory success against *Bd* of one species, at different locations, with different decline histories and therefore different selection pressures, may give us a greater understanding of the importance of AMPs as a defence against *Bd* infection.

Litoria genimaculata, the green-eyed tree frog, is a rainforest stream dweller that occurs on the northern part of the east coast Australia (Cogger, 2000). Several studies have investigated the antibiotic activity and structure of Maculatin, a particular type of peptide produced by this species (Rozek et al., 1998; Chia et al., 2000; Brinkworth and Bowie, 2003; Niidome et al., 2004; Woodhams et al., 2005). Woodhams et al. (2005) challenged *Bd* with pure synthetic Maculatin 1.1 and, depending on the *Bd* isolate, achieved a total inhibition of zoospore growth at 25 to 50 μm. To date, no study has

investigated *L. genimaculata* AMP profiles (using MALDI) from populations from different geographical areas with different decline histories. This species suffered declines at high elevation sites during initial outbreaks of chytridiomycosis in the 1990s, while at low elevations (below 400 m), due to less favourable environmental conditions for *Bd* survival (Berger et al., 2004; Woodhams and Alford, 2005; Kriger and Hero, 2007b), populations appeared to be stable (McDonald and Alford, 1999; McDonald et al., 2005). The well documented decline pattern of *L. genimaculata*, where high elevation populations experienced a bottleneck in numbers, makes it an ideal species to investigate AMP profiles and AMP effectiveness against *Bd*. I hypothesised that if AMPs played an important role in the survival of *L. genimaculata* individuals infected with *Bd*, then strong selection pressure could have caused the AMP profile of upland populations to diverge, resulting in a possible increase in innate protection against *Bd* compared with lowland populations.

Materials and Methods

Sites and frog species

For a detailed description of the study species and study sites see Chapter 3.

Collection and processing of skin peptides

Skin peptides of male *L. genimaculata* were collected during September 2005 at three high elevation sites (Birthday Creek at Paluma National Park; Bridge XI at Murray Upper National Park and Windin N Creek at Wooroonooran National Park) and two low elevation sites (Bridge I at Murray Upper National Park and Frenchman Creek at Wooroonooran National Park). All frogs were swabbed using a sterile tubed dry swab (Medical Wire and Equipment, Corsham, Wiltshire UK) that was run across frog's hands, feet, thighs and ventral surface twice, for qPCR assay to quantify infection with *Bd* (Boyle et al., 2004). Quantitative PCR analysis was carried out at the School of Veterinary and Biomedical Sciences, James Cook University.

The collection of the skin peptides was conducted as described in Chapter 3. The processing of the sep-pak filters was carried out in collaboration with Dr. L.A. Rollins-

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Smith and conducted by Dr. Doug Woodhams at Vanderbilt University, Nashville, USA, following the same protocol as I have previously described. Due to a processing error, the inhibition potential of eleven samples could not be determined.

MALDI mass spectrometry readings of peptides

The matrix-assisted laser desorption/ionization (MALDI) mass spectrometry of the skin peptides secreted *by L. genimaculata* was conducted at Vanderbilt University, Nashville, USA and followed the procedure of Woodhams et al. (2006).

Statistical analysis

Prevalence of *Bd* and intensity of infection

A Fisher's exact test was used to determine if there was a significant difference in prevalence of *Bd* between high and low elevation populations. A Mann-Whitney U-test was performed to determine if there was a significant difference in infection intensity between high and low elevation populations.

Total skin peptides secreted

Total peptide secretion per surface area (SA) (cm^2) was calculated as per chapter three, using the equation from McClanahan and Baldwin (1969) to represent surface area.

$$\text{Surface area } (cm^2) = 9.9 \times (\text{weight in grams})^{0.56}$$

The data were $\log_{10}$ transformed before any statistical analysis. I performed a factorial ANOVA to test for the effect of infection and site on total peptides secreted per surface area. A *posthoc* (unequal N HSD) was performed to evaluate further effects. Additionally, I used a Pearson's R correlation analysis to explore the linear relationship between intensity of infection ($\log_{10}$ number of zoospore equivalents+1) and median peptide secretion per surface area for each site. A Spearman Rank correlation analysis was used to explore the relationship between prevalence and median peptide secretion per surface area (per site). The Spearman Rank correlation analysis was preferred as only five data points (sites) that exhibited a strong pattern were compared, but did not fulfil the assumption of Pearson's R correlation analysis.

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Effectiveness of AMPs

I calculated the effectiveness of AMPs as the inhibitory concentration where no *Bd* growth occurred (IC_{100}), as described in Chapter 3. There were several samples in which no concentration produced 100% inhibition of *Bd*. In these cases, I used extrapolation to predict the peptide concentrations where 100% inhibition would occur ($x = (100/b)/m$, where x is the unknown concentration of peptides, b = y intercept and m= slope of a regression of percent inhibition on concentration. I categorised the IC_{100} into four groups, forming a log series of concentrations: 1 = 50-174 μg/mL, 2= 175-374 μg/mL, 3= 375-749 μg/mL and 4= 750+ μg/mL. I used Fisher's exact tests to determine if there was a difference between the IC_{100}s of peptides produced by infected and uninfected frogs, amongst sites and between highland and lowland populations.

Overall protection of frogs

Once the IC_{100} was calculated, the overall protection of the frog against *Bd* was estimated as the total number of mL of solution at the IC_{100} that would be produced per unit surface area:

$$\text{Overall protection (mL at } IC_{100} \text{ per cm}^2) = (\text{total mass of peptides/ SA} \times 1/IC_{100}).$$

The data were $\log_{10}$ transformed before statistical analysis. I conducted a Pearson's R correlation analysis to explore the relationship between intensity of infection ($\log_{10}$ number of zoospore equivalents+1) and median overall protection of frogs at each site. I also conducted a Spearman Rank correlation analysis to explore the relationship between prevalence and median overall protection per site.

MALDI mass spectrometry analysis of peptides

The output file of the MALDI mass spectrometry readings consisted of an average of 50,000 molecular mass values (mass to charge ratio; m/z) per peptide sample as it determined molecular mass to the 6th decimal place. Studies on anuran peptides predominantly use molecular mass rounded to the nearest whole number (Rozek et al., 1998; Chia et al., 2000; Brinkworth and Bowie, 2003; Brinkworth et al., 2003; Niidome

et al., 2004; Woodhams et al., 2005). I therefore rounded the molecular mass to the nearest whole number and used the maximum intensity value (monoisotopic mass) for each subsequent group. The intensity value is greatly influenced by the volume of the sample used. Even though a consistent volume (0.5 μL) per sample was used, small pipetting differences may influence the intensity reading. Since it is the pattern of relative intensity at different molecular weights that is important, I adjusted the intensity value to fall within a range from 0 to 100. Graphically exploring the data before and after the adjustments showed that rounding the data to the nearest whole number and standardising intensity did not change the overall pattern of the mass spectrometry reading (Figure 4.1). Due to the nature of the data, with few peaks (representing intensity of specific AMPs) and a large amount of background noise, I created a presence/absence data set by molecular mass (peak present = 1, peak absent = 0). Each sample was graphically observed in order to determine where to set the breakpoint of relative intensity that separated background noise from genuine peaks. A two way ANOVA with site and infection status as categorical factors was conducted on the total number of peaks per sample using Statistica (version 8). Additionally, a dissimilarity distance matrix, using Euclidean distance measurements, was conducted and used for the multidimensional scaling (MDS) technique. Blossom statistical software (version 2007 12.21) was used to perform a multi-response permutation procedure (MRPP) on the coordinates of the data in the first two dimensions of the MDS, using site, elevation and infection status as the grouping variables.

Currently, five skin peptides of *L. genimaculata* have been described, including their molecular weight (MW); Maculatin 1.1 (MW= 2143 and 2145), Maculatin 1.1.1 (MW = 1973 and 1975), Maculatin 1.2 (MW 2358 and 2360), Maculatin 2.1 (MW = 1878) and Maculatin 3.1 (MW = 2392 and 2395) (Rozek et al., 1998; Brinkworth and Bowie, 2003). I used Fisher's exact tests to determine whether infection status, elevation or site affected the presence of these five known skin peptides.

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Results

qPCR assay to determine prevalence of *Bd* and intensity of infection

There was no significant difference in prevalence of *Bd* between high and low elevation site (Fisher's exact test, p=0.26). The highest prevalence of *Bd* was observed at a lowland site (Frenchman Creek; Figure 4.2). Intensities of infection with *Bd* were greater at the high elevation sites (Mann-Whitney U-test, Z=1.9, p=0.05; Figure 4.3).

Total skin peptides secreted per surface area

Total peptides secreted per surface area did not differ significantly between infected and uninfected individuals (ANOVA, $F_{1, 76} = 0.07$, p=0.80), but varied significantly amongst sites (ANOVA, $F_{4, 76} = 6.98$, p<0.001; Figure 4.4). The *L. genimaculata* population at Frenchman Creek secreted significantly smaller amounts of peptides per surface area than individuals at any other sites (Table 4.1). The relationship between *Bd* infection intensity (Log_{10} number of zoospore equivalents+1) and total peptide secretion (μg) per surface area (SA) was not significant at four sites (Birthday Creek, r = 0.06, p = 0.87; r^2 = 0.003; Bridge IX, r = -0.46, p = 0.21; r^2 = 0.21; Frenchman Creek, r = 0.0719, p = 0.8435; r^2 = 0.0052 and Windin N Creek, r = -0.8455, p = 0.3586; r^2 = 0.7149; Figure 4.5) but was unable to be calculated for the fifth site (Bridge I) as only one individual tested positive for *Bd*. There was a significant negative correlation across sites between prevalence of *Bd* and the total peptides secreted per surface area (Spearman Rank correlation, r_s=-1, p=0.02).

Effectiveness of peptides (IC_{100})

Of the 86 samples, only 61 could be tested for effectiveness; of these, four samples did not show any inhibition. The effectiveness of peptides was significantly different between infected and uninfected *L. genimaculata* (Fisher's exact test, p<0.01) with uninfected individuals having greater numbers of samples with more effective peptides (lower inhibitory concentrations needed to inhibit *Bd* growth; Figure 4.6). Infected individuals did not show a significant difference in the effectiveness of peptides (IC_{100}) amongst sites (Fisher's exact test, p=0.28) or elevation (Fisher's exact test, p=0.79). Uninfected individuals did not show a significant difference in the effectiveness of

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peptides (IC_{100}) amongst sites (Fisher's exact test; p=0.16) or elevation (Fisher's exact test; p=0.88).

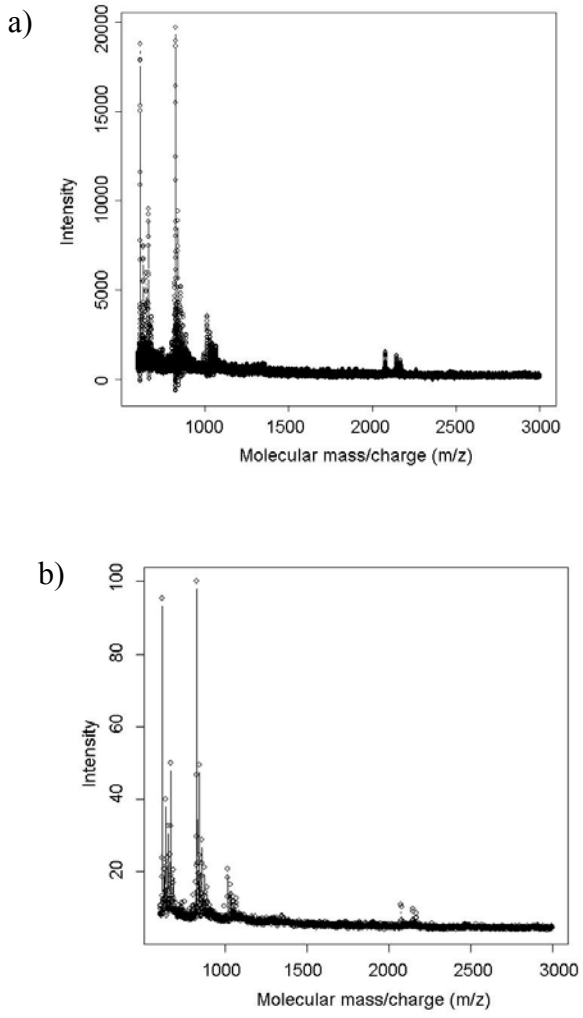

Figure 4.1. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry reading of skin peptides of a *Litoria genimaculata* individual. a) raw data and b) after molecular mass were grouped to the nearest whole number, reducing it from 50,000 to 3,000 values (note that the range stays the same) and intensity was standardised from 0 to 100.

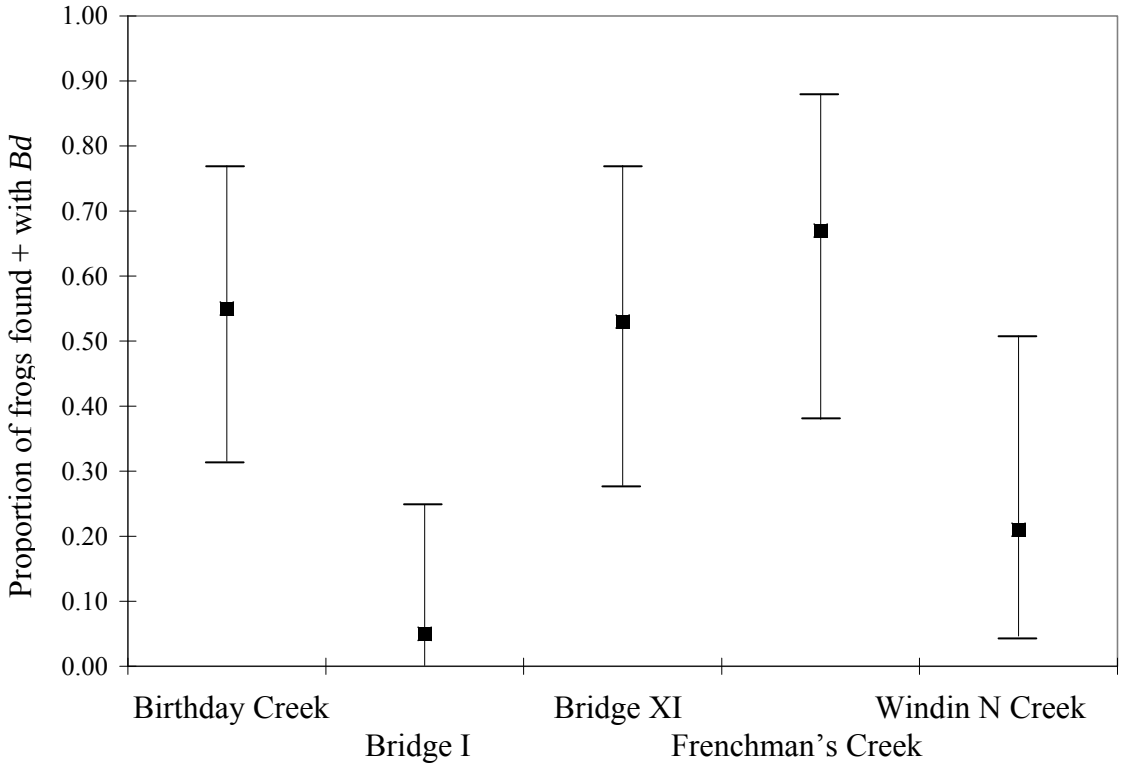

Figure 4.2. Prevalence of *Batrachochytrium dendrobatidis* in *Litoria genimaculata* individuals (means and 95% confidence limits) from which skin peptides were collected at three high elevation sites (Birthday Creek, Bride XI and Windin N Creek) and two low elevation sites (Bridge I and Frenchman Creek).

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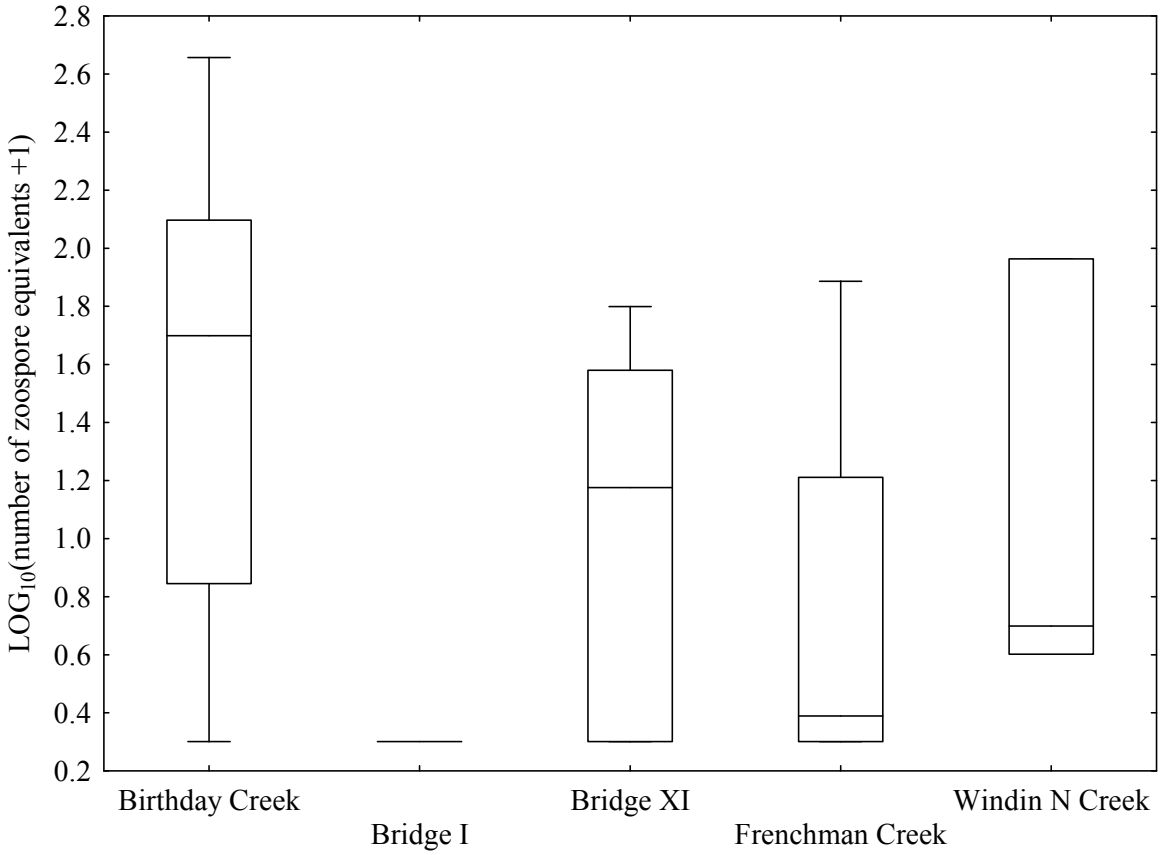

Figure 4.3 *Batrachochytrium dendrobatidis* infection intensity in *Litoria genimaculata* at three high elevation sites (Birthday Creek, Bride XI and Windin N Creek) and two low elevation sites (Bridge I and Frenchman Creek). In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

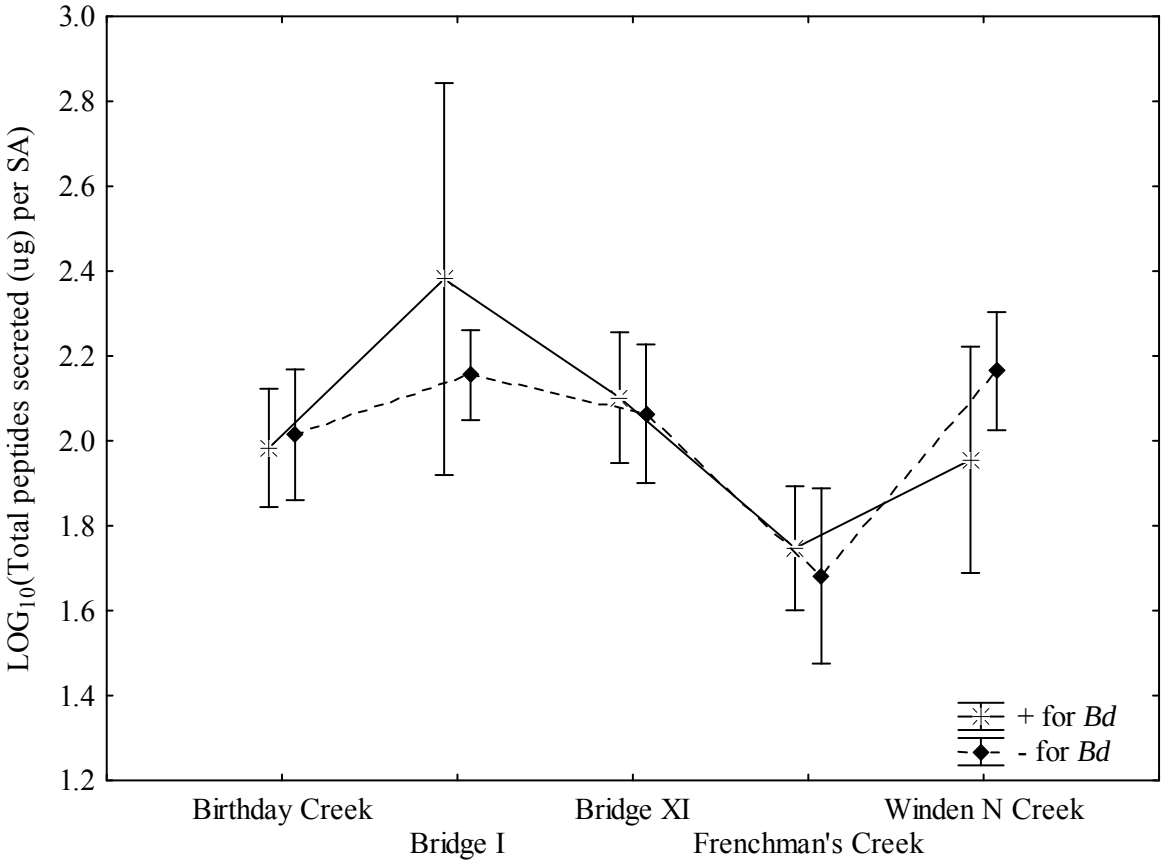

Figure 4.4. Total amount of peptides secreted (μg) per surface area (cm^2) by infected and uninfected *Litoria genimaculata* (means and 95% confidence limits) at three high elevation sites (Birthday Creek, Bridge XI and Windin N Creek) and two lowland sites (Bridge I and Frenchman Creek). The lines between data points are to aid in visualisation of the results only.

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Table 4.1. *Posthoc* analysis (unequal N HSD) of total amount of peptides secreted (μg) per surface area (cm^2) by *Litoria genimaculata* at three high elevation sites (Birthday Creek, Bridge XI and Windin N Creek) and two lowland sites (Bridge I and Frenchman Creek). Significant values (p value) are in bold.

***Posthoc* Unequal N HSD, df = 76**					
Sites	Birthday Creek	Bridge I	Bridge XI	Frenchman Creek	Windin N Creek
Birthday Creek		0.16	0.81	**0.02**	0.63
Bridge I	0.12		0.84	**<0.01**	0.98
Bridge XI	0.81	0.84		**<0.01**	0.99
Frenchman Creek	**0.02**	**<0.01**	**<0.01**		**<0.01**
Windin N Creek	0.63	0.98	0.99	**<0.01**	

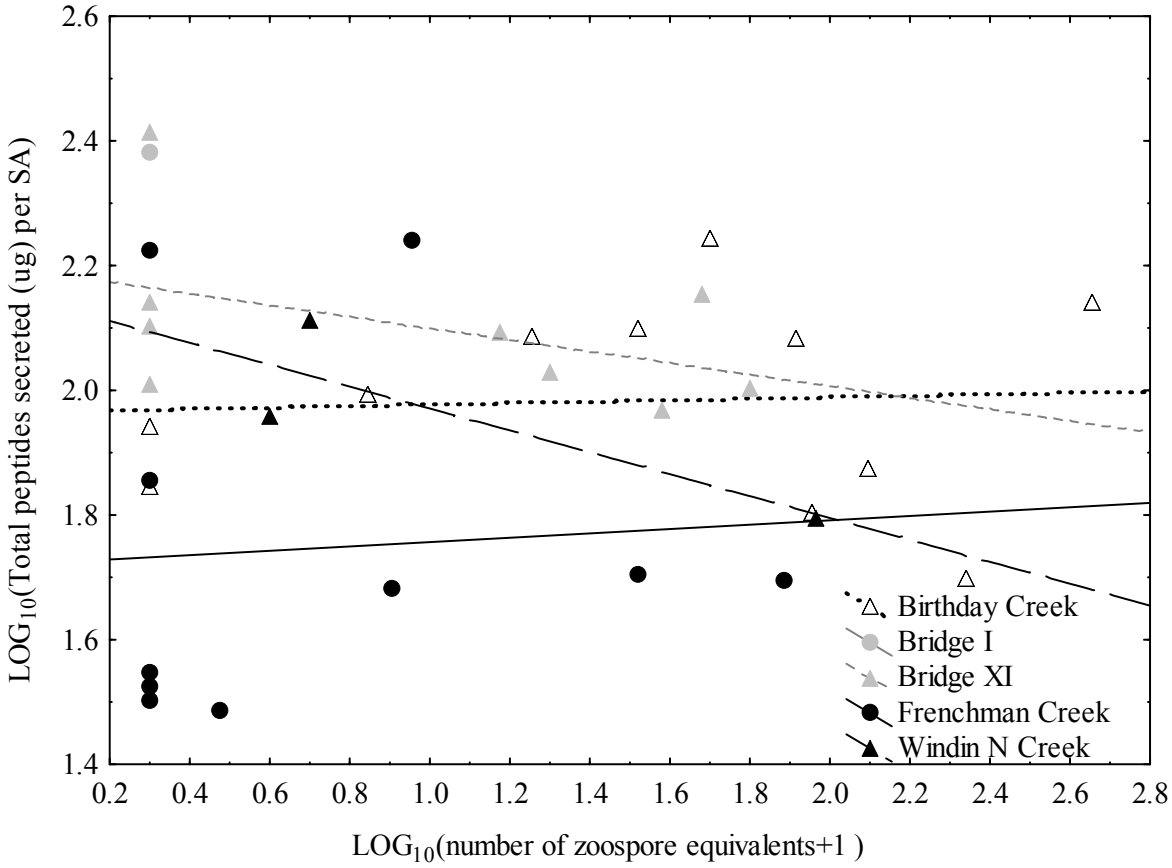

Figure 4.5. Relationship between *Batrachochytrium dendrobatidis* (*Bd*) infection intensity (Log_{10} number of zoospore equivalents+1) in *Litoria genimaculata* and total peptide secretion (μg) per surface area (SA) at Birthday Creek ($r = 0.06$, $p = 0.87$; $r^2 = 0.003$), Bridge IX ($r = -0.46$, $p = 0.21$; $r^2 = 0.21$) Frenchman Creek ($r = 0.0719$, $p = 0.8435$; $r^2 = 0.0052$) and Windin N Creek ($r = -0.8455$, $p = 0.3586$; $r^2 = 0.7149$). Note that only one individual tested positive for *Bd* at Bridge I and no correlation was therefore calculated.

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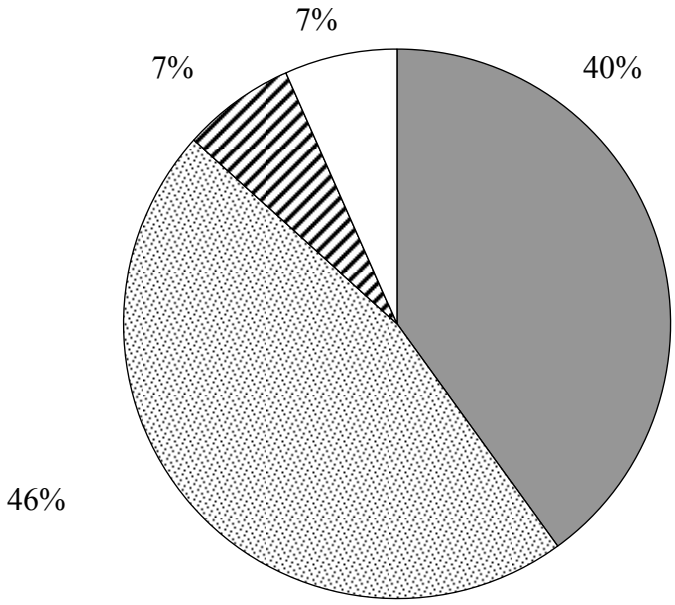
a)



b)

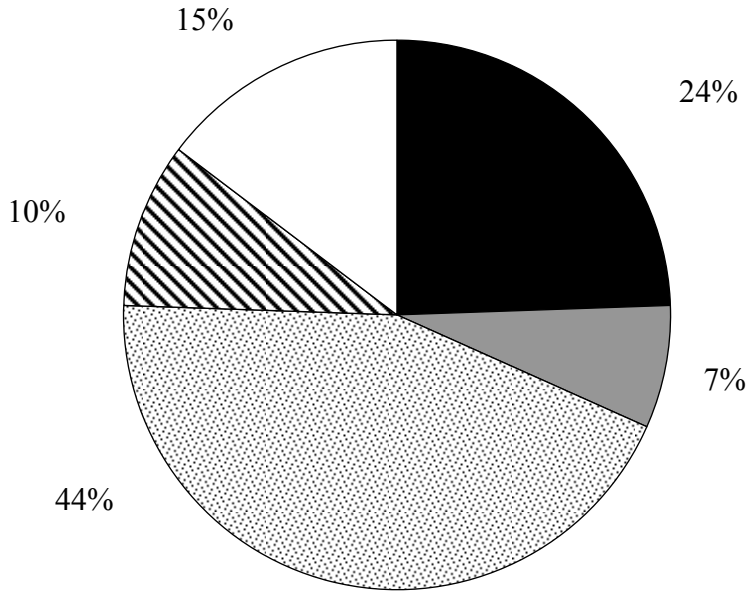


Figure 4.6. Proportion of peptide samples in infected (a) and uninfected (b) *Litoria genimaculata* in the five different inhibitory concentration groups, 1 (■) = < 100 μg/ml, 2 (▩) = 100-174 μg/ml, 3 (▩) = 175-374,μg/ml and 4 (▩) = 375-749 μg/ml and 5(□) = 750+ μg/ml, where no *Batrachochytrium dendrobatidis* growth occurred (IC_{100}).

Overall protection of frog

Infection status had no significant effect on overall protection afforded by AMPs of the frog (ANOVA, $F_{1, 61}$ =0.27, p=0.61), however, overall protection afforded by AMPs varied significantly amongst sites (ANOVA, $F_{4, 61}$=4.45, p<0.01; Figure 4.7). Individuals at Frenchman Creek had the lowest overall protection and were significantly different compared with frogs at Bridge I (*posthoc* unequal HSD, df= 61, p<0.01) and Birthday Creek (*posthoc* unequal HSD, df= 61, p=0.02; Table 4.2). The overall protection of *L. genimaculata* was also significantly different between Bridge I and Bridge XI (*posthoc* unequal HSD, df= 61, p=0.01).

The relationship between *Bd* infection intensity (Log_{10} equivalent number of zoospores+1) and overall protection (mL of AMPs at IC_{100} per cm^2) was not significant at four sites (Birthday Creek, r = 0.2193, p = 0.5171; r^2 = 0.0481; Bridge IX, r = -0.5140, p = 0.2379; r^2 = 0.2642; Frenchman Creek, r = 0.1667, p = 0.6931; r^2 = 0.0278 and Windin N Creek, r = 0.0597, p = 0.9620; r^2 = 0.0036; Figure 4.8) and could not be calculated for the fifth site (Bridge I) as only one individual tested positive for *Bd*. There was no significant correlation between prevalence of infection and overall protection (Spearman Rank correlation, r_s=-0.7, p=0.23).

Mass spectrometry analysis of peptides

The Vanderbilt Laboratory could not perform mass spectrometry analysis on all 86 peptide samples. A total of 69 samples were analysed (Table 4.3). There was a significant difference in the total number of peaks (indicating presence of specific peptides) amongst the five sites (ANOVA, $F_{4, 59}$ = 4.9, p<0.01; Figure 4.9). Infection status of *L. genimaculata* did not have a significant effect on the total number of peaks quantified during mass spectrometry (ANOVA, $F_{1, 59}$ = 0.9, p=0.35). MDS ordination (Euclidean distance measure) indicated a geographical effect (Figure 4.10). Statistically this was supported; there were significant differences in the total number of peaks amongst sites (MRPP, δ = 0.97, p< 0.01) and between lowland and upland populations (MRPP, δ = 1.18, p= 0.04). Infection status of the frogs did not significantly influence peptide expression in *L. genimaculata* (MRPP, δ = 1.21, p= 0.55). Maculatin 1.1 was

the most common of the described peptides, found in 57 of the 69 individuals (Table 4.4), followed by Maculatin 1.1.1 (n=18) and Maculatin 2.1 (n=2). Maculatin 1.2 and 3.1 were not detected from any of the 69 *L. genimaculata*. There was a significant difference in the occurrence of the three described Maculatin peptides amongst sites (Fisher's exact test, $p=0.02$) and elevations (Fisher's exact test, $p<0.001$) but not between infected and uninfected individuals (Fisher's exact test, $p=0.7$). The highest occurrence of a non-described peptide was at a molecular mass of 2075 in 46 *L. genimaculata* individuals.

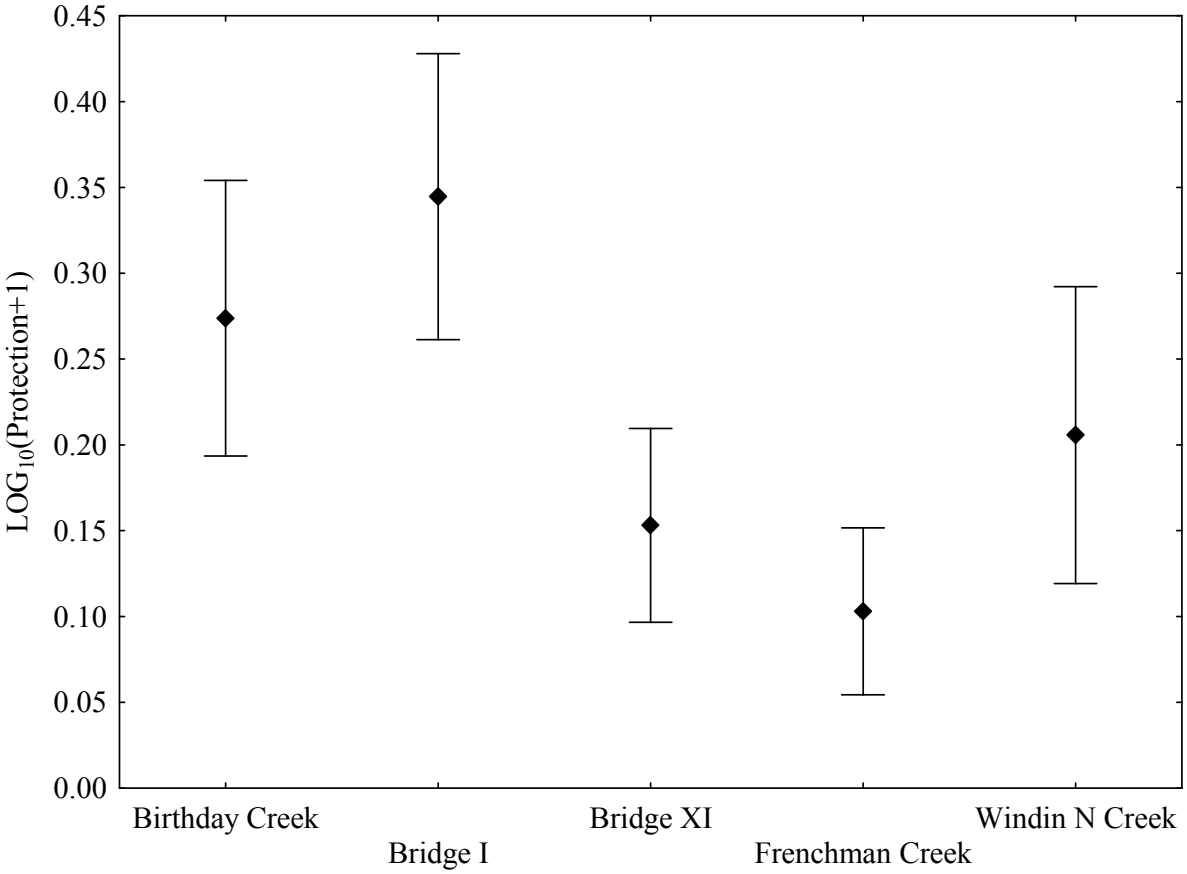


Figure 4.7. Overall protection afforded by AMPs (total peptides secreted × ($1/IC_{100}$) / SA) in *Litoria genimaculata* (means and 95% confidence limits) at three high elevation sites (Birthday Creek, Bridge XI and Windin N Creek) and two lowland sites (Bridge I and Frenchman Creek).

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Table 4.2. *Posthoc* analysis (unequal N HSD) of overall protection afforded by AMPs (total peptides secreted × ($1/IC_{100}$) / SA) in *Litoria genimaculata* at three high elevation sites (Birthday Creek, Bridge XI and Windin N Creek) and two lowland sites (Bridge I and Frenchman Creek). Significant results (p value) are in bold.

***Posthoc* Unequal N HSD, df = 61**					
Sites	Birthday Creek	Bridge I	Bridge XI	Frenchman Creek	Windin N Creek
Birthday Creek		0.69	0.16	**0.02**	0.66
Bridge I	0.69		**0.01**	**<0.01**	0.09
Bridge XI	0.16	**0.01**		0.89	0.85
Frenchman Creek	**0.02**	**<0.01**	0.89		0.33
Windin N Creek	0.66	0.09	0.85	0.33	

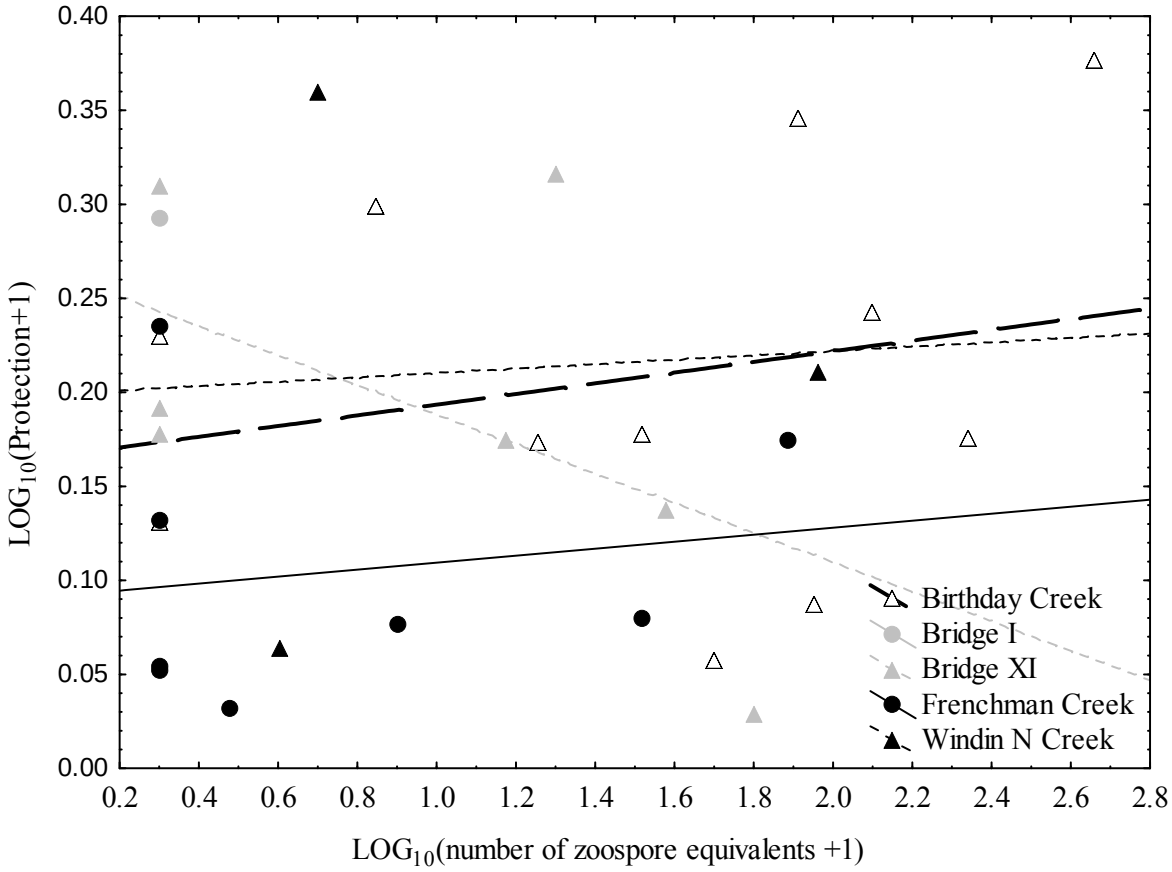

Figure 4.8. Relationship between *Bd* infection intensity (Log_{10} number of zoospore equivalents+1) in *Litoria genimaculata* and overall protection afforded by AMPs (mL at IC_{100} per cm^2) at Birthday Creek ($r = 0.2193$, $p = 0.5171$; $r^2 = 0.0481$), Bridge IX ($r = -0.5140$, $p = 0.2379$; $r^2 = 0.2642$) Frenchman Creek ($r = 0.1667$, $p = 0.6931$; $r^2 = 0.0278$) and Windin N Creek ($r = 0.0597$, $p = 0.9620$; $r^2 = 0.0036$). Note that only one individual tested positive for *Bd* at Bridge I and no correlation was therefore calculated.

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Table 4.3. Number of *Litoria genimaculata* skin peptide samples on which Dr. Doug Woodhams at Vanderbilt University, Nashville, USA, conducted MALDI mass spectrometry.

Location	**Elevation**	**+ for *Bd***	**- for *Bd***
Birthday Creek	High	11	9
Bridge I	Low	1	17
Bridge XI	High	4	6
Frenchman Creek	Low	3	4
Windin N Creek	High	3	11
Total		**22**	**47**

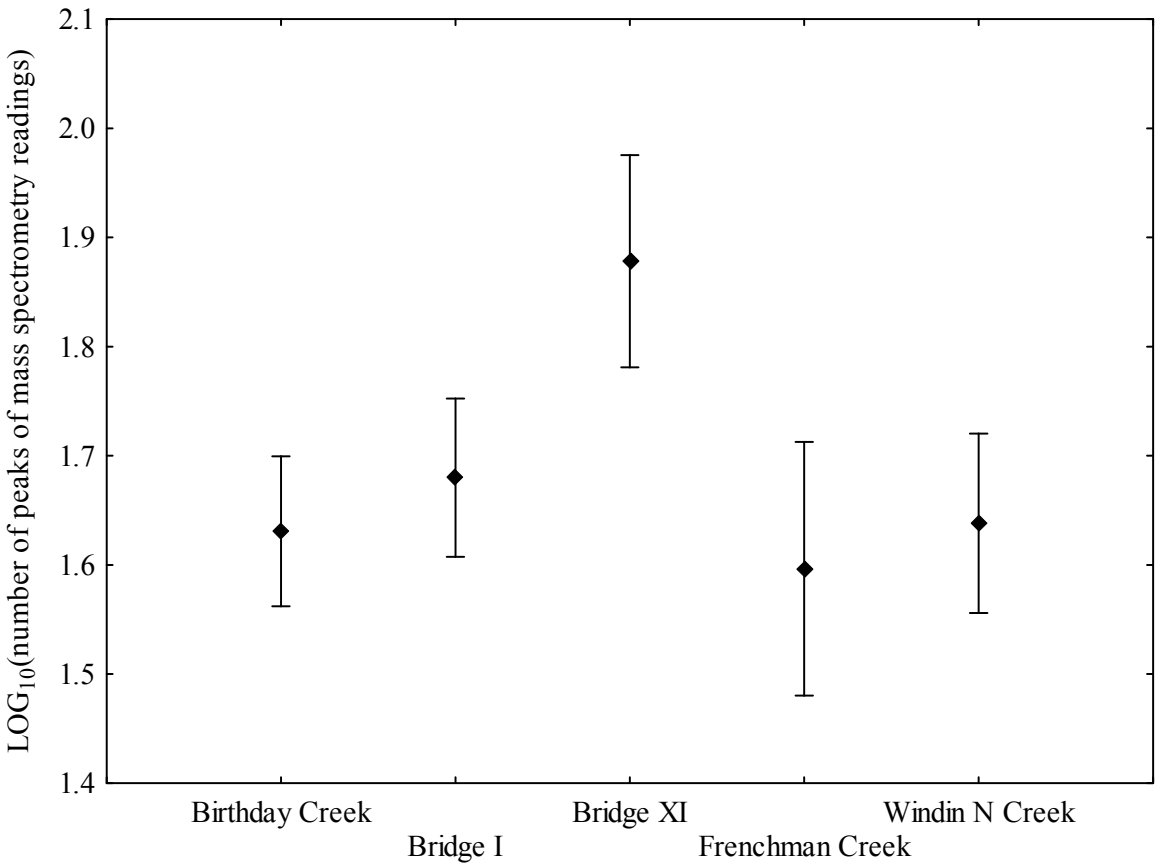

Figure 4.9. Total numbers of peaks (means and 95% confidence limits) counted by MALDI mass spectrometry reading of the skin peptide samples collected from adult male *Litoria genimaculata* at three high elevation sites (Birthday Creek, Bridge XI and Windin N Creek) and two lowland sites (Bridge I and Frenchman Creek).

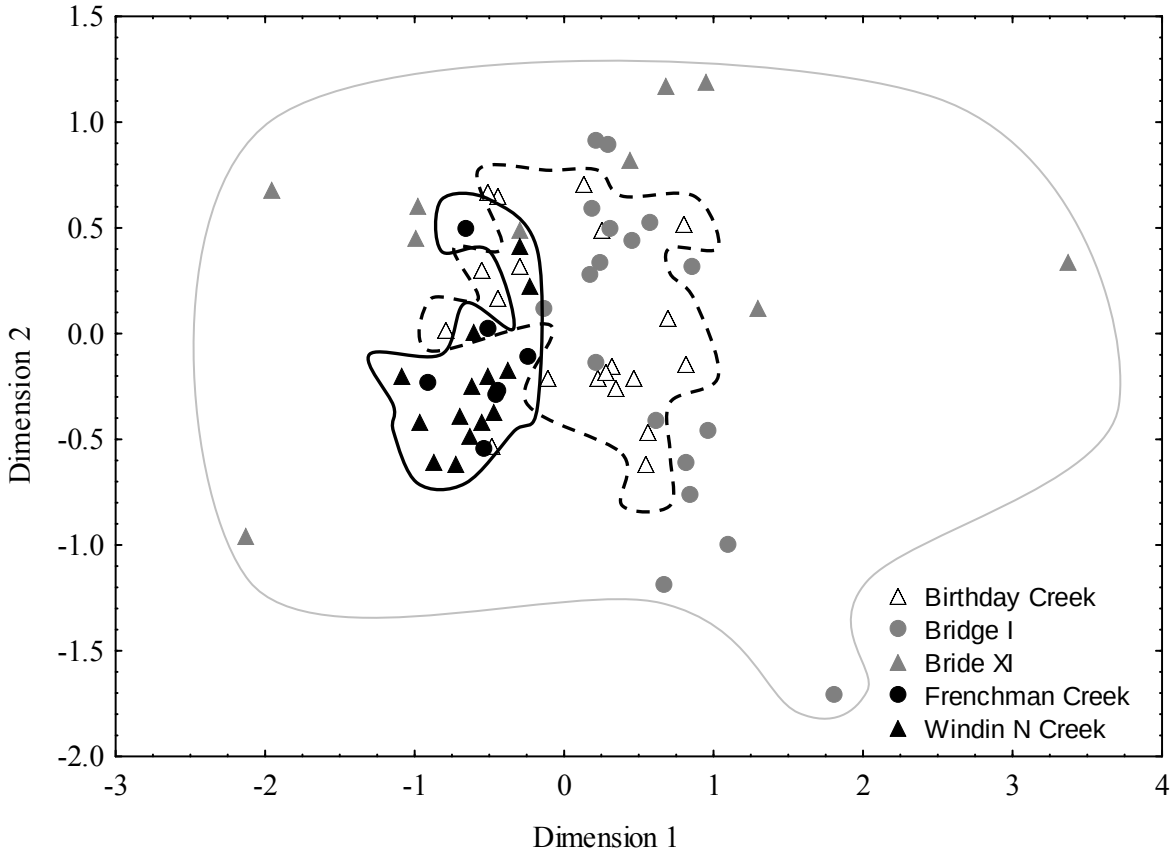

Figure 4.10. MDS ordination (Euclidean distance measure) on MALDI mass spectrometry reading of skin peptide samples from *Litoria genimaculata* at five sites: Birthday Creek, Paluma National Park (N=20), Bridge I and Bridge XI at Murray Upper National Park (N=18, N=10, respectively) and Frenchman Creek and Windin N Creek at Wooroonooran National Park (N=7, N=14, respectively). Triangle symbols represent high elevation sites, circles low elevation sites.

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Table 4.4. The sum of Maculatin peptides found in the skin secretion of 69 male *Litoria genimaculata* at three high elevation sites (Birthday Creek, Bridge XI and Windin N Creek) and two lowland sites (Bridge I and Frenchman Creek). Fisher's exact tests were conducted using all three Maculatin peptides for categories of infection, site or elevation.

Category		**Maculatin 2.1 (MW= 1878)**	**Maculatin 1.1.1 (MW=1973+ 75)**	**Maculatin 1.1 (MW=2143+ 45)**	**Fisher's exact test**
Infection Status	Negative for *Bd*	2	11	38	
	Positive for *Bd*	0	7	19	
					P=0.7
Site	Birthday Creek	0	5	18	
	Bridge I	1	0	11	
	Bridge XI	0	7	9	
	Frenchman Creek	1	0	7	
	Windin N Creek	0	6	12	
					P=0.02
Elevation	high elevation (>400m)	0	18	39	
	low elevation (<400m)	2	0	18	
					P<0.001
Number of individuals that had skin peptides at a particular molecular weight		**2**	**18**	**57**	

Discussion

The prevalence of *Bd* infection in *L. genimaculata* differed amongst sites, with less of an elevational pattern than previously observed in Australian anurans (Berger et al., 2004; Woodhams and Alford, 2005; Kriger et al., 2007b). September is the beginning of the *L. genimaculata* breeding season (Richards and Alford, 2005) in which higher numbers of mature males and females aggregate along the rainforest creek, resulting in an increase in frog to frog interactions (Richards and Alford, 2005). This may result in an increase in prevalence of *Bd* at lowland sites, such as detected at Frenchman Creek in Wooroonooran National Park. However, the population at another lowland site, Bridge I at Murray Upper National Park, had a low prevalence of *Bd* at the same time. Perhaps the beginning of the breeding season, which largely depends on climatic conditions (Richards and Alford, 2005), and subsequent frog interactions varied amongst sites resulting in this difference, or climatic conditions were more favourable for the prolonged presence of *Bd* at Frenchman Creek when compared with Bridge I. This is assuming that *Bd* can be transferred directly from frog to frog during skin contact, which has not yet been demonstrated.

The prevalence of *Bd* in *L. genimaculata* at each site was correlated with the peptide secretion per surface area. For example, the frog population at Bridge I (Murray Upper National Park), which had lowest prevalence of *Bd*, secreted the largest amount of peptides per surface area; whereas the population at Frenchman Creek (Wooroonooran National Park) with the highest prevalence of *Bd*, secreted the smallest quantity of peptides per surface area. A similar pattern was observed with overall protection (mL of AMPs at IC_{100} per cm^2), suggesting that the amount of peptide secreted per surface area and overall protection may affect vulnerability to *Bd* at the population level. It is possible, although there is no evidence yet, that *Bd* infection, which causes a thickening of the *stratum corneum* (Berger et al., 1998), may block ducts and hence reduce peptide secretion. On the other hand, within populations there were no significant correlations between intensity of infection and total peptide secretion per surface area or overall protection, which you would expect in the above mentioned scenario. Additionally, infected individuals did not always secrete smaller amounts of peptides per surface area

than uninfected individuals from the same site. This suggests that while the AMP component of the anuran innate immune defence is correlated with prevalence of *Bd*, once an individual is infected, its peptide levels may not affect or reflect the intensity of the infection.

I hypothesised that if AMPs played an important role in the survival of *L. genimaculata* infected with *Bd*, then strong selection pressure could have caused the AMP profile of upland populations to diverge, resulting in a possible increase in innate protection against *Bd* compared to lowland populations. My data provided some support for this hypothesis, since the MRPP analysis of the coordinates of each individual on the first two MDS axes showed significant differences between individuals from upland and lowland sites. However, this analysis revealed a stronger and more easily visualised (Figure 4.10) latitudinal gradient. Frogs at Wooroonooran National Park region (Frenchman Creek and Windin N Creek) and Paluma National Park (Birthday Creek) showed two distinct groups in terms of their AMP profile, suggesting lower variability within a site but distinct differences amongst regions. It is unclear how these differences reflect the frog's immune system defence against *Bd*, as each AMP would have to be isolated and challenged against *Bd*. The challenge assays of the skin secretion, which includes all AMPs that were used for the MDS analysis, did not reveal a similar regional pattern. However, the combination of a significant correlation between peptide secretion per surface area and infection intensity, and the elevational differences in peptide profiles demonstrated by the MDS and MRPP analyses, suggests that AMPs do play a role in *L. genimaculata* innate immune defence against *Bd* but that there are other factors, perhaps microenvironment selection or disease avoidance behaviour, that contribute to the comparatively lower vulnerability to *Bd* of this species when compared to sympatric species.

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A laboratory behaviour study on the effect of microenvironment selection by anurans on chytridiomycosis

Abstract

Experimental studies in the laboratory have established that elevated body temperatures, similar to those experienced by basking frogs, can clear individuals of chytridiomycosis, an amphibian disease caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*). Field observations have also shown that infection status and prevalence may be correlated with microenvironment selection by frogs. We designed a laboratory environment that allowed frogs to select from a range of thermal and hydric microenvironments during disease studies and investigated whether *Bd* infections progress similarly in frogs (*Litoria caerulea, L. genimaculata and L. wilcoxii)* housed in variable environments that provided a choice of environmental conditions and in frogs housed in constant environments that provided only constant, standard laboratory environmental conditions. We also investigated whether the infection status of frogs affected their choice of microenvironments (cold, warm or hot temperatures and low or high humidity) and substrates (ground, pond or wall), and whether they exhibited similar movement patterns (moved less or more than a body length between observations or did not move). In each species, the proportion of infected frogs housed in variable environments that decreased their infection load was greater than that for frogs housed in constant environments, although due to small sample sizes this trend was not significant. Thermal microenvironment selection differed significantly amongst the three species studied and can be linked to some extent to the decline patterns observed in the wild. *Litoria caerulea* (vulnerable to infection with *Bd* but no population declines due to chytridiomycosis have been detected) selected warm and hot environments significantly more often than *L. genimaculata* (vulnerable to infection with *Bd* with highland population declines followed by recovery). At night, infected *L. wilcoxii* moved significantly less than uninfected individuals and *L. genimaculata* infected by *Bd* selected different substrates than uninfected individuals. Six adult *L. genimaculata* lost their infections within 18 days while housed in the laboratory in constant, standard conditions, suggesting that other mechanisms, such as antimicrobial peptides, also influence the progress of chytridiomycosis.

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Introduction

Anurans experience repeated changes in body temperature and water balance as they modulate their internal environment through behavioural changes and microhabitat selection to meet their physiological needs (Duellmann and Trueb, 1994; Hillyard, 1999; Beck and Jennings, 2003). Since anurans' internal environments fluctuate daily, investigating their host-disease interactions in a constant laboratory environment is likely to lead to an inaccurate conclusion (Woodhams, 2003). However, in most previous work, this important aspect of amphibian biology has been largely ignored as the surrounding environment has been controlled and stabilised (Huchzermeyer et al., 1994; Cheatwood et al., 2003; Davidson et al., 2003). This may lead to imprecise descriptions of the impact of a disease at an individual and population level.

One emerging infectious disease that has been intensively investigated in the last decade is chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) (Berger et al., 1998; Woodhams and Alford, 2005; Voyles et al., 2007). Studies have confirmed the fatal outcome of chytridiomycosis in several Australian frog species, including *Litoria caerulea*, *L. chloris* and *Mixophyes fasciolatus,* under stable environmental conditions (23°C and 100% relative humidity) (Berger et al., 1998; Woodhams et al., 2007) that are ideal for *Bd* growth and reproduction (Longcore et al., 1999). However, none of these species show population declines due to chytridiomycosis in the wild (McDonald and Alford, 1999).

The discrepancy between laboratory and field studies raised the question of what factors influence the host-pathogen interaction and possibly alter the outcome of an infection with *Bd* (Woodhams et al., 2003; Retallick et al., 2004). Temperature (Woodhams et al., 2003; Berger et al., 2004) and humidity (Woodhams, 2003) can both influence the progress of chytridiomycosis in anurans. Woodhams (2003) and Woodhams et al. (2003) observed that experimentally infected *L. chloris* exposed to higher temperatures (37^{o}C) were cured of infection and that *Bd* is more pathogenic in high humidity.

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In the field, greater mortality of Australian frogs infected with *Bd* occurs during colder months (Bradley et al., 2002) and more stable populations of several susceptible species, such as *L. nannotis*, *L genimaculata* and *Nyctimystes dayi*, occur at lower altitudes where temperatures are higher compared to highland sites (McDonald and Alford, 1999). Relative humidity may be lower during the dry winter season compared to summer, but is still likely to be above the threshold (Woodhams, 2003) that reduces the progress of chytridiomycosis (Kenyon, pers.obs.). The results of the laboratory studies and patterns observed in the wild suggest that climatic conditions can influence the progress of chytridiomycosis.

Since microenvironment selection by infected frogs will alter the environment *Bd* is exposed to, and it is clear that the rates of growth and reproduction of *Bd* are influenced by temperature and might be affected by humidity; it is likely that frogs' microenvironment selection can affect the growth of *Bd* on individuals, or eliminate established infections. Rowley (2007) radio tracked and recorded body temperatures of wild *L. lesueuri, L. genimaculata* and *L. nannotis*, species which have experienced chytridiomycosis associated declines to different extents. Her study suggests that vulnerability of the three species to *Bd* infection may be correlated with their microenvironment selection.

Understanding the interactions of the pathogen *Bd* with wild frog populations requires a controlled and close study of the progress of chytridiomycosis in frogs exposed to a more naturalistic range of environmental conditions. This study was designed to evaluate whether allowing frogs access to a wide range of microenvironments alters the outcome of infections with *Bd*, and whether infection with *Bd* alters frogs' behaviour, including microenvironment selections and movement patterns. I examined three species that have experienced chytridiomycosis-associated declines to very different extents. All three Australian species, *L. caerulea, L. wilcoxii* and *L. genimaculata* are from the Family Hylidae. The green tree frog, *L. caerulea*, is a pond breeder and has been found positive for *Bd* in the wild but no populations have shown noticeable declines (Berger et al., 1998; Kriger and Hero, 2007a). The stony creek frog,

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L. wilcoxii, and the green-eyed tree frog, *L. genimaculata*, are rainforest stream breeders and prevalence of *Bd* is highly variable with season, location and elevation (Woodhams and Alford, 2005; Kriger and Hero, 2007b). *Litoria genimaculata* declined in the highland regions but has subsequently recovered (Richards and Alford, 2005), whereas no noticeable decline of *L. wilcoxii* has been detected throughout its range (McDonald et al., 2005). This study may assist in explaining why some anuran species have not experienced population declines even though they are susceptible to infection with *Bd*.

Materials and Methods

Experimental design

Trials of the experiment were carried out in two different types of terraria; Type I (constant environment) was designed to represent conditions normally experienced in laboratory studies where frogs have no hydric or thermal environmental choices. It consisted of a 250 × 350 × 150 mm plastic container with lid and airspace to allow ventilation. The containers were misted every second day to maintain a constant high humidity environment and had an open plastic container (115 mm diameter × 60 mm high) filled with rainwater as a pond. Type II (variable environment) allowed the frogs to select from a range of hydric and thermal conditions (Figure 5.1). We used three 1500 × 700 × 500 mm glass terraria that were divided halfway across the longest dimension with PVC walls to create six identical 750 × 700 × 500 mm terraria for the larger frogs, *L. caerulea* (average snout-vent length (SVL) 61mm, mass 21g). Smaller frogs, *L. wilcoxii,* (average SVL 38mm, mass 5.6g) and *L. genimaculata* (average SVL 39mm, mass 3.2g) were housed in 12 600 × 200 × 300 mm terraria. All terraria were divided lengthwise with a 4.5 mm acrylic wall to create separate high and low humidity areas. Two holes (50 diameter mm or 25 diameter mm for the smaller terraria) were drilled between these two sections to allow the frogs to freely move between them. The high humidity environment had an acrylic lid to trap moist air and maintain high relative humidity, whereas the less humid environment had a mesh lid, which allowed air exchange with the air-conditioned room, resulting in a dryer environment. A heat lamp (80 watt) was placed at one end of the terraria, centered over the wall between the high and low humidity areas, to create a thermal gradient. The larger terraria of type II

had two halves of a PVC pipe filled with rainwater as ponds (one in the lower humidity and one in the high humidity compartment). The smaller terraria of type II had an open plastic container (115 mm diameter × 60 mm high) in the centre of each less humid and high humidity environment compartment. The design of the varied hydric and thermal environment allowed microenvironment selection in relation to temperature, humidity and combinations of both to be investigated. I-button data loggers recorded the temperature and relative humidity hourly in both constant and variable environment terraria. Three digital cameras, viewing all of the type II terraria, were programmed to automatically take pictures at half hour intervals to monitor the locations of frogs in the terraria.

A total of six trials were conducted (Table 5.1). Four trials used the larger Type II terraria for *L. caerulea* and each trial consisted of six individuals in the constant and six individuals in the variable environments, resulting in 48 frogs in total. Using the smaller Type II terraria, one trial with *L. genimaculata*, (12 frogs in the constant environment and 12 in the variable environment) and one trial with *L. wilcoxii* (6 frogs in the constant environment as no more frogs were available, and 12 in the variable environment) were conducted. Each trial lasted for a minimum of eight days, except that for *L. genimaculata* which, due to an air-conditioning failure, was terminated at day 5.

All experimental trials were conducted at the freshwater compound, James Cook University, in one air-conditioned room. Room temperature fluctuated between 18-21°C at night and 21-24°C during the day. The light was automatically switched on at 7.30 am and off at 7.30 pm.

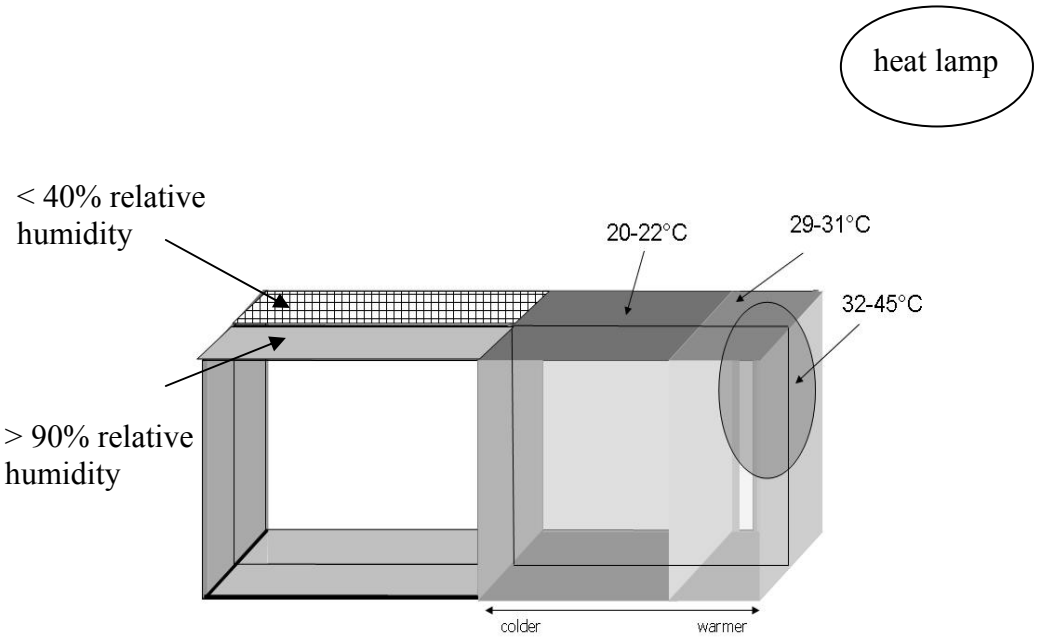

Figure 5.1 The design of the variable microenvironment terraria in which frogs could choose between two hydric (low and high relative humidity) and three thermal (cold, warm and hot) microenvironments.

Table 5.1 Terrarium size, frog species and sample size used during the six trials to monitor microenvironment selection and the progress of chytridiomycosis. In Type I terraria frogs were housed at a constant environment, Type II terraria frogs had a choice of hydric and thermal microenvironments.

Trial	Frog species	Size of type II terraria (variable environment)	Sample size Type II terraria	Sample size Type I terraria
1	*L. caerulea*	Large	6	6
2	*L. caerulea*	Large	6	6
3	*L. caerulea*	Large	6	6
4	*L. caerulea*	Large	6	6
5	*L. wilcoxii*	Small	12	6
6	*L. genimaculata*	Small	12	6

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Study animals

Litoria caerulea was the only species available as captive bred individuals at the time of the study. Subadults were obtained from captive populations maintained by Brendan Tiernan (Morphett Vale, South Australia) and Gerry Marantelli (Amphibian Research Centre, ARC, North Coburg, Victoria). In order to minimise impact on wild frog populations, we collected most *L. wilcoxii* and *L. genimaculata* from the wild in their aquatic life history stages and raised them to subadults. *Litoria wilcoxii* were raised from tadpoles collected at Crystal Creek, Paluma Range National Park, Queensland, Australia (S18°58′54″ E146°12′01″). *Litoria genimaculata* were raised from eggs or collected as adult male frogs from Birthday Creek, Paluma Range National Park (S18°58′54″ E146°10′02″). All frogs were swabbed (sterile tubed dry swab from Medical Wire and Equipment, Corsham, Wiltshire UK) before and after the experiment for diagnostic quantitative PCR analysis (Boyle et al., 2004) to determine their infection status for *Bd*. In order to eliminate the possibility of unintentional infection with *Bd*, all terraria and water containers were cleaned and waste water diluted with F10 Veterinary Disinfectant (Webb et al., 2007). Gloves were worn at all times and changed between each frog and terrarium handled. Frogs received crickets (*Acheta domesticus)* twice a week; once a week 0.5 mL of a liquid supplement mixture (2 mL Calcivet/100 mL rainwater) was applied dorsally to each frog. Calcivet (Vetafarm) contains 33 g/L calcium borogluconate, 2 g/L magnesium sulphate and 25,000 i.u./L vitamine D_3 and appears to be more effective than coating crickets with a phosphorus free calcium and vitamin D3 supplement powder. Frogs were provided with rainwater (collected and stored in a water tank) that was changed at least once a week.

Culture of *Bd* and experimental infection of frogs

Isolates of *Bd* (*L. les donna* 06-LB-1) were cultured in the School of Veterinary and Biomedical Science, James Cook University, following the protocol of Longcore et al. (1999) but using only half of the nutrients recommended as this appeared to increase culture growth. Two different methods were used to infect the frogs with *Bd*. *Litoria caerulea* were exposed to *Bd* zoospores in a bath following the procedure of Berger et al. (2005b). Zoospores were harvested by flooding agar plates containing *Bd* cultures

with 2 mL of dilute salt solution (DS) for 10 minutes (Boyle et al., 2003). The concentration of zoospores was determined by pre-sampling and counting motile zoospores with a haemocytometer in triplicate. A total concentration of 27,000 zoospores per mL was used for each *L. caerulea*. Control frogs were bathed in a solution obtained from agar plates with no *Bd* culture, flooded with the same volume of DS.

Unfortunately, when we applied the same procedure to *L. genimaculata* all individuals, including the control animals, died within the first four hours of the 24 hour *Bd* exposure period. Due to time constraints, we then collected 24 adult male *L. genimaculata* from the wild at the same location where we collected the eggs, so this study could be continued immediately. Frog populations at Birthday Creek, Paluma National Park, have been monitored for several years (Richards and Alford, 2005; Woodhams and Alford, 2005) and removing 24 adult frogs had a minimal impact on the population. Infection status was determined by qPCR assay of sterile tubed dry swabs (Medical Wire and Equipment, Corsham, Wiltshire UK) which were run across frogs' hands, feet, thighs and ventral surface twice. However, to avoid further mortality, we exposed the uninfected *L. genimaculata* and all *L. wilcoxii* to *Bd* using a sterile cotton swab that had been rolled three times across agar plates with *Bd* cultures and then wiped on the ventral surface of the frog. The control group were swiped with a sterile cotton swab that was rolled on sterile agar plates. No frog died using this exposure procedure; however the disadvantage was the unknown number of *Bd* zoospores to which the frogs were exposed. It has been observed that frogs develop clinical signs of chytridiomycosis at varying intensities of infection with *Bd* (Voyles et al., 2007) and for the purpose of this study it was sufficient to compare behaviour of frogs positive for *Bd* (but at a variable intensity of infection) with uninfected frogs.

Housing after experimental infection

All frogs were individually housed in constant environments (Type I terraria) for 10 days after their experimental exposure to *Bd*. I-button recordings were used to verify stable and constant hydric and thermal environments. Each frog was swabbed as

described above for qPCR assay to determine infection status, before being released into the variable environment terrarium and after the trial. Quantitative PCR assays for the first two trials were carried out at the Australian Animal Health Laboratory (AAHL), Geelong, Victoria. For the remaining trials, qPCR assays were carried out at the School of Veterinary and Biomedical Sciences at James Cook University, Townsville, Australia.

Statistical analysis

The temperature and humidity conditions in each type of terrarium prior to the study commencing were initially measured placing multiple i-button data loggers in each terrarium. In both the humid and less humid halves of the variable environment terraria, zones were determined that corresponded to three ranges of temperatures: cold, warm, and hot (during daylight hours when the heat lamps were on; temperature ranges for each zone are presented in results). For each trial of the experiment, the position of each frog within each terrarium was determined in each half-hourly photograph. For analysis, we divided the data into two main groups, 1) day data where a thermal and hydric gradient was present and 2) night data where no thermal gradient existed but the hydric gradient was present.

The effect of environment on infection was evaluated by determining, for each frog that was exposed to *Bd*, whether intensity of infection as measured by qPCR assay, increased or decreased during each trial. Frogs that did not become infected were not included in the analysis. We used an exact Zelen's test for homogeneity of odds ratios to evaluate if we could combine the data set for all species. We then used Fisher's exact test to evaluate whether the intensity of *Bd* infections changed differently in frogs housed in constant environment (Type I terraria) than it did in frogs that had a choice of thermal and hydric environments (Type II terraria).

From each digital image, data obtained for frogs in the variable-environment terraria were: which humidity environment they occupied (high or low), which temperature zone within that humidity environment (cold, warm or hot, during the day only), the

substrate they occupied (ground, wall or pond) and the distance they had moved since the previous photograph (nil movement, movement less than a body length or movement greater than a body length). These data allowed us to investigate frog microenvironment selection along several possible environmental axes alone and in combination. Several combinations, mostly relevant to host-pathogen dynamics, were included; we did not examine every possible combination to reduce the chances of Type I error. The axes and combinations we investigated were humidity alone, temperature alone, substrate alone, movement alone, temperature and humidity combined, humidity and substrate combined, and humidity and movement combined. The proportion of images in which each frog was found in each combination was calculated from the digital images.

We used Blossom statistical software (version 2007 12.21) for analysis and conducted a multi-response permutation procedure (MRPP) on the proportional data for each species separately using infection status as the grouping variable. We further investigated, using the same technique, differences in frog behaviour (using all different categories, described previously) among species and between night and day.

Results

The microenvironment within terraria

Eight days of hourly i-button readings of small and large Type II (variable environment) terraria designs showed that they presented similar ranges of microenvironments for frogs to select from. Relative humidity in the high humidity environment ranged from 70 to 90%, with an average of 84% for the small variable terraria and 92% for the large variable terraria. The relative humidity in the lower humidity environment was usually below 70%, with an average of 46% in the small, and 62% in the large, variable terraria (Figure 5.2). The relative humidity in the constant environment terraria ranged from 80-97% with an average of 91%.

The hot environment temperature fluctuated during the day from 28.0°C – 40.0°C in the small and 29.5°C -39.5°C in the large variable terraria (Figure 5.3). The warm

environment temperature fluctuated from 23.5°C -29.7°C in the small and 25.0°C - 30.5°C in the large variable environment terraria. The cold environment temperature fluctuated from 19.5°C -26.5°C in the small, 19.0°C -24.5°C in the large variable environment terraria and 19.0°C to 23.5°C in the constant environment terraria.

During trial 6 with *L. genimaculata*, which was terminated early because of a failure of the air-conditioner in the laboratory, temperature in the terraria reached 36.5°C.

qPCR assay to determine infection status before and after each trial

Experimental infections with *Bd* of all three species were not always successful (Table 5.2). It is unclear whether frogs did not become infected when exposed to *Bd* zoospores, or rapidly cleared infections within 10 days, prior to swabbing for the qPCR assay. Of the 24 adult *L. genimaculata* we collected in the wild, 13 were positive for *Bd* at the time of collection, but six tested negative 18 days later. During that period, frogs were housed in small containers where humidity was high and constant. Temperature in the laboratory was not recorded during that period, however there were no equipment failures and temperatures in the same room at other times ranged from 21.0^{o}C to 26.0^{o}C, with an average of 23.0^{o}C.

Changes in intensity of infection

The exact Zelen's test for homogeneity of odds ratios was not significant (ZE = 0.384, p= 1.0), indicating that the pattern of changes in intensity of infection did not differ significantly among the three species. We therefore combined the data for all three species (Table 5.3) and conducted a single Fisher's exact test of the hypothesis that the proportion of frogs whose intensity of infection increased and decreased during the trials was independent of whether individuals were housed in Type I or Type II terraria. Across all three species, there was a trend, although not significant (Fisher's exact test, p=0.17), that more individuals that had a choice of thermal and hydric environments reduced their infection load than frogs that were housed in constant environments (Table 5.3). In total, 11 frogs lost their initial *Bd* infections, of which seven had a choice of hydric and thermal microenvironments.

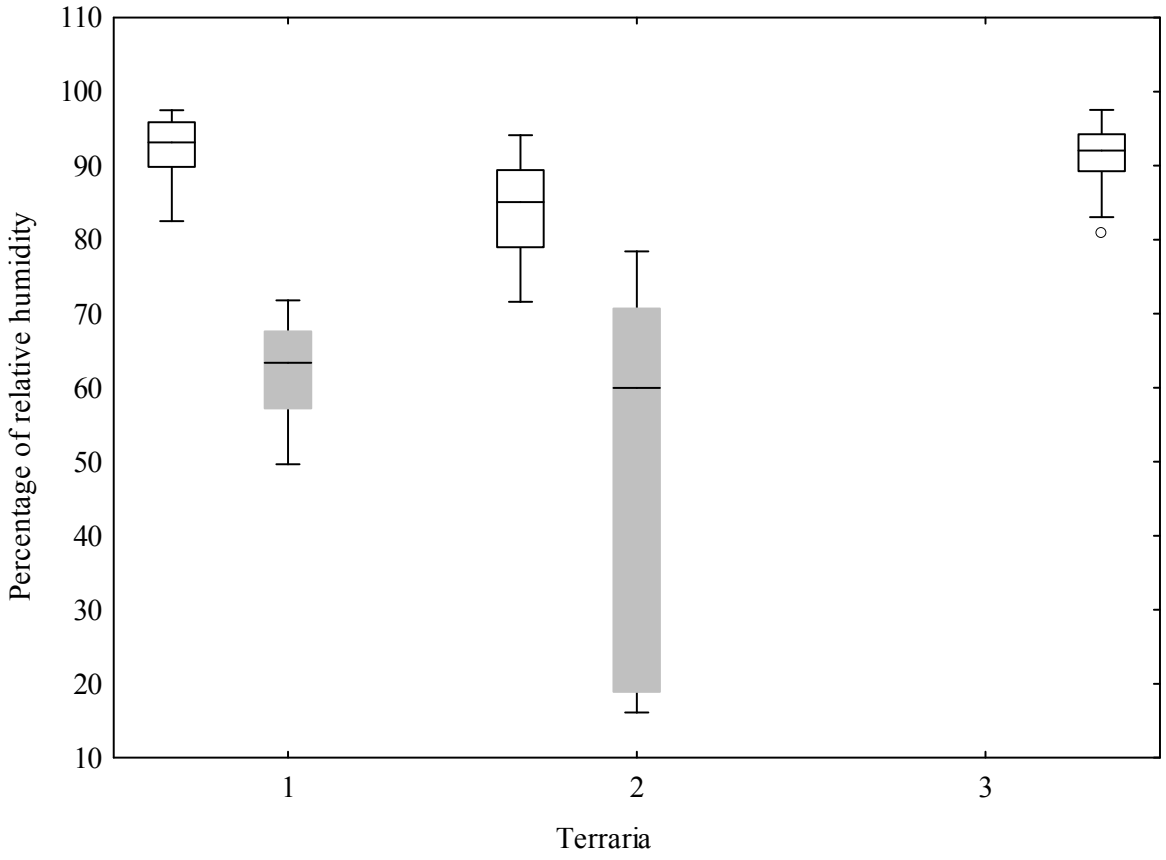

Figure 5.2. Relative humidity in the terraria used to monitor microenvironment selection by frogs. 1= larger variable microenvironment terraria (Type II), 2= smaller variable microenvironment terraria (Type II), 3= constant microenvironment terraria (Type I). The variable microenvironment terraria had both high relative humidity □ and low relative humidity ■. The constant microenvironment terraria had only high relative humidity □. In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries).

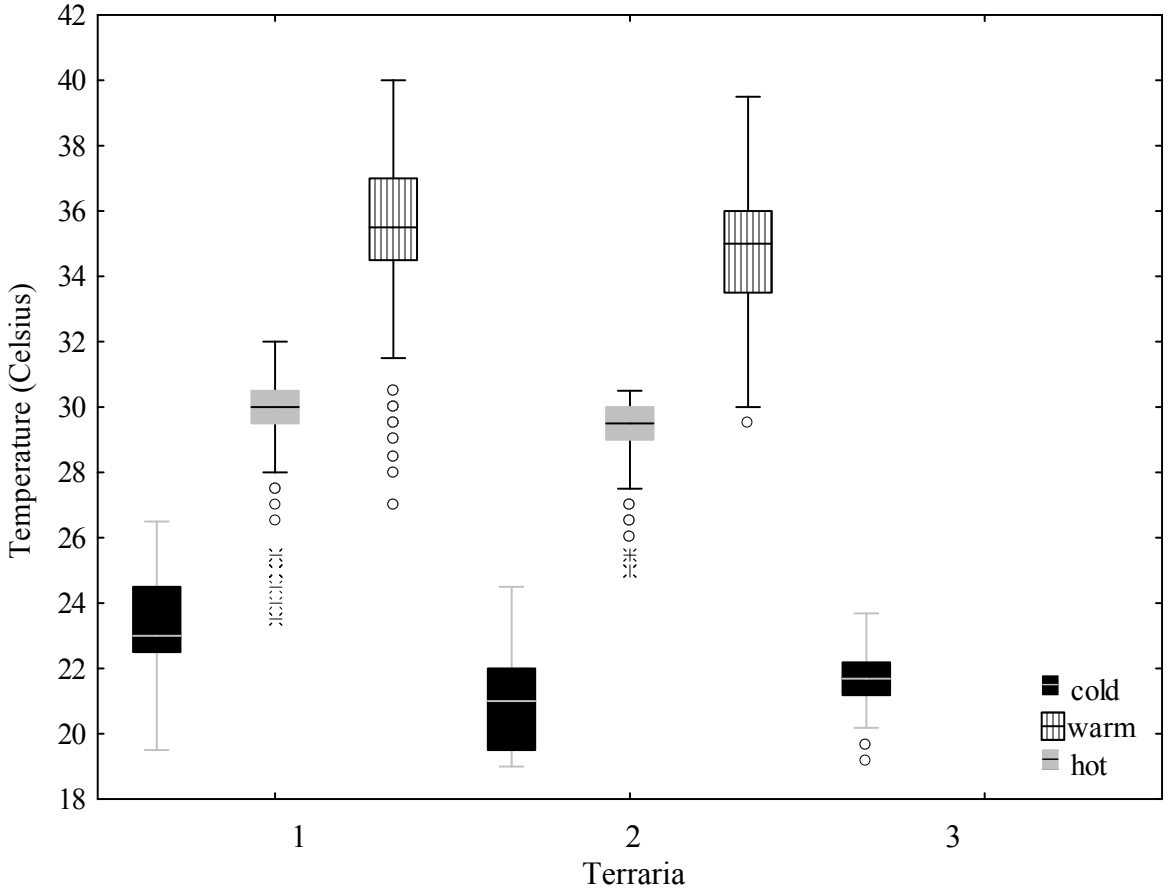

Figure 5.3 The three thermal microenvironments (cold, warm and hot) in the three different terraria designs. 1= larger variable microenvironment terraria (Type II), 2= smaller variable microenvironment terraria (Type II), 3= constant microenvironment terraria (Type I). In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

Table 5.2 Infection status at the beginning of each trial determined by qPCR assay, of *Litoria caerulea*, *L. wilcoxii* and *L. genimaculata* individuals that were housed either in terraria Type I (constant environment) or terraria Type II (variable environment) during the microenvironment selection study.

Species	**Terrarium type**	**Total *Bd* negative**	**Total *Bd* positive**
L. caerulea	I	7	5
	II	15	8
L. wilcoxii	I	3	3
	II	8	4
L. genimaculata	I	7	5
	II	8	4

Table 5.3 Changes in intensity of infection between the start and end of trials for each species in the microenvironment selection experiment. Frogs in Type I terraria were housed under constant environmental conditions, whereas frogs in Type II terraria had a choice of thermal and hydric environments.

Species	Terrarium type	Decrease of *Bd* infection	Increase of *Bd* infection	Fisher's exact test
All three species	I	6	10	P= 0.17
	II	11	6	
Litoria caerulea	I	2	7	P=0.38
	II	4	5	
Litoria wilcoxii	I	4	1	P= 0.33
	II	4	0	
Litoria genimaculata	I	3	2	P= 1.00
	II	3	1	

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Microenvironment selection during the day

A total of 15,522 day images were analysed. Within each species, thermal and hydric environment selection, substrate selection and movement pattern did not differ significantly between infected and uninfected individuals. *Litoria caerulea* was the only species that chose thermal microenvironments in a pattern significantly different from random (MRBP, $\delta = 0.29$, $p < 0.01$; $\delta = 0.37$, $p = 0.25$ for *L. wilcoxii*; $\delta = 0.29$, $p = 0.14$ for *L. genimaculata*). All three species, *L. caerulea*, *L. wilcoxii* and *L. genimaculata*, selected substrates in the less humid or high humid environment in a non-randomly pattern (MRBP, $\delta = 0.35$, $p < 0.01$; $\delta = 0.32$, $p < 0.01$; $\delta = 0.35$, $p = 0.01$, respectively; Figure 5.4).

The three species differed significantly in patterns of use of thermal microenvironments (MRPP, $\delta = 0.43$, $p < 0.01$). *Litoria caerulea* chose the warm and hot environments more often than *L. wilcoxii* and *L. genimaculata* (Figure 5.5). *Litoria wilcoxii* spent the least time in the warm and hot environments. Hydric environment selection did not significantly differ among the three species (MRPP, $\delta = 0.61$, $p = 0.20$), however, in combination with thermal microenvironments, *L. wilcoxii* spent more time in the high relative humidity environment at cold temperatures than did *L. genimaculata* and *L. caerulea* (MRPP, $\delta = 0.67$, $p < 0.01$; Figure 5.6).

Substrate selection differed significantly among the three frog species (MRPP, $\delta = 0.14$, $p < 0.01$). *Litoria wilcoxii* was found mostly on the ground, whereas *L. caerulea* and *L. genimaculata* were mostly observed on the wall (Figure 5.7). The overall tendency to move differed significantly among species (MRPP, $\delta = 0.25$, $p < 0.01$): *L. genimaculata* moved (more than its body length) most frequently, and *L. caerulea* remained in the same location across the highest proportion of images (Figure 5.8).

Microenvironment selection during the night

A total of 13,511 night images were analysed. No thermal gradient was present at night. Infected and uninfected *L. caerulea* did not differ significantly in hydric environment selection (MRPP, $\delta = 0.55$, $p = 0.09$), substrate selection (MRPP, $\delta = 0.48$,

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p=0.37) and movement pattern (MRPP, δ = 0.34, p=0.26). Infected *L. wilcoxii* moved less than uninfected individuals (MRPP, δ = 0.23, p< 0.01) (Figure 5.9) but their hydric environment (MRPP, δ = 0.71, p= 0.45) and substrate (MRPP, δ = 0.44, p= 0.61) selection did not differ significantly. Infected *L. genimaculata* preferred different substrates at night compared to uninfected individuals (MRPP, δ = 0.26, p< 0.01) (Figure 5.10) but their hydric environment (MRPP, δ = 0.59, p= 0.49) and movement pattern (MRPP, δ = 0.22, p= 0.09) did not differ significantly.

Substrate selection differed significantly among uninfected *L. caerulea*, *L. wilcoxii* and *L. genimaculata* (MRPP, δ = 0.38, p<0.01). *Litoria wilcoxii* spent most of its time at night on the ground (Figure 5.11). *L. caerulea* spent more time in the pond than did the other two species. A similar pattern was observed in the data for infected *L. caerulea*, *L. wilcoxii* and *L. genimaculata* (Figure 5.12); this pattern was also significant (MRPP, δ = 0.45, p<0.01).

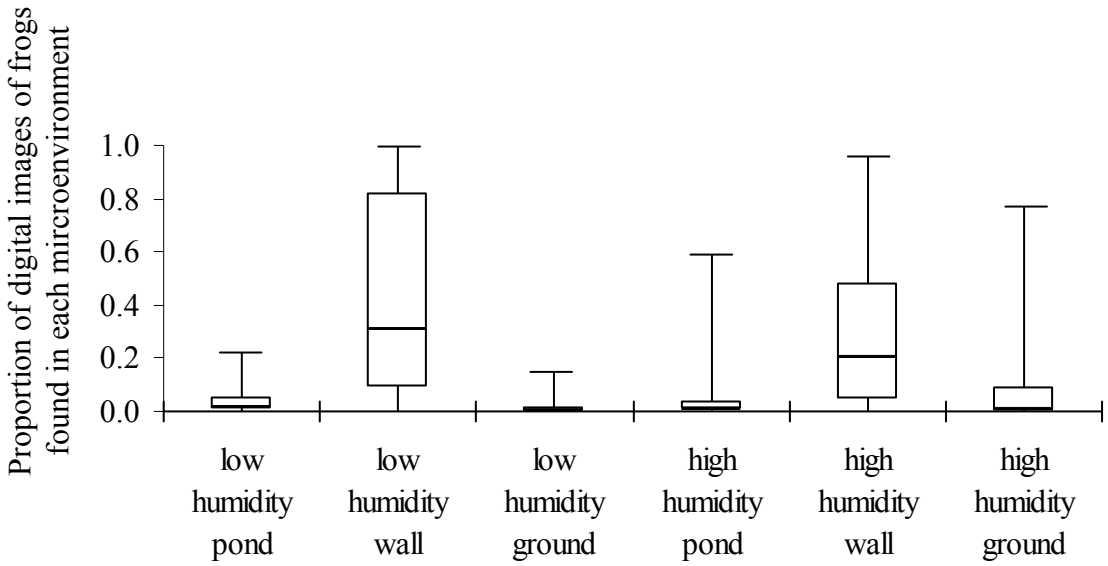

Figure 5.4 Hydric environment and substrate selection by *Litoria caerulea* during the day. In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

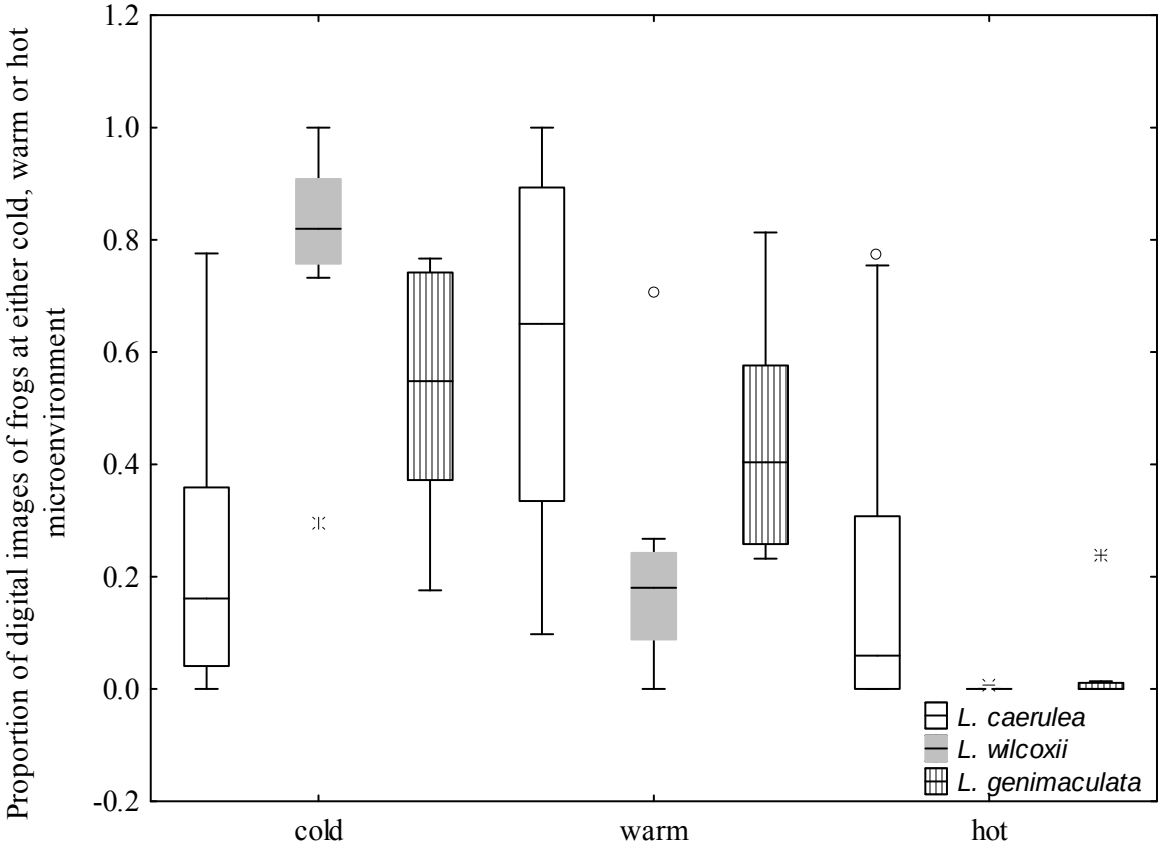

Figure 5.5 Proportion of digital images in which *Litoria caerulea*, *L. wilcoxii* and *L. genimaculata* were observed in each thermal environment (cold, warm and hot) during the day. In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

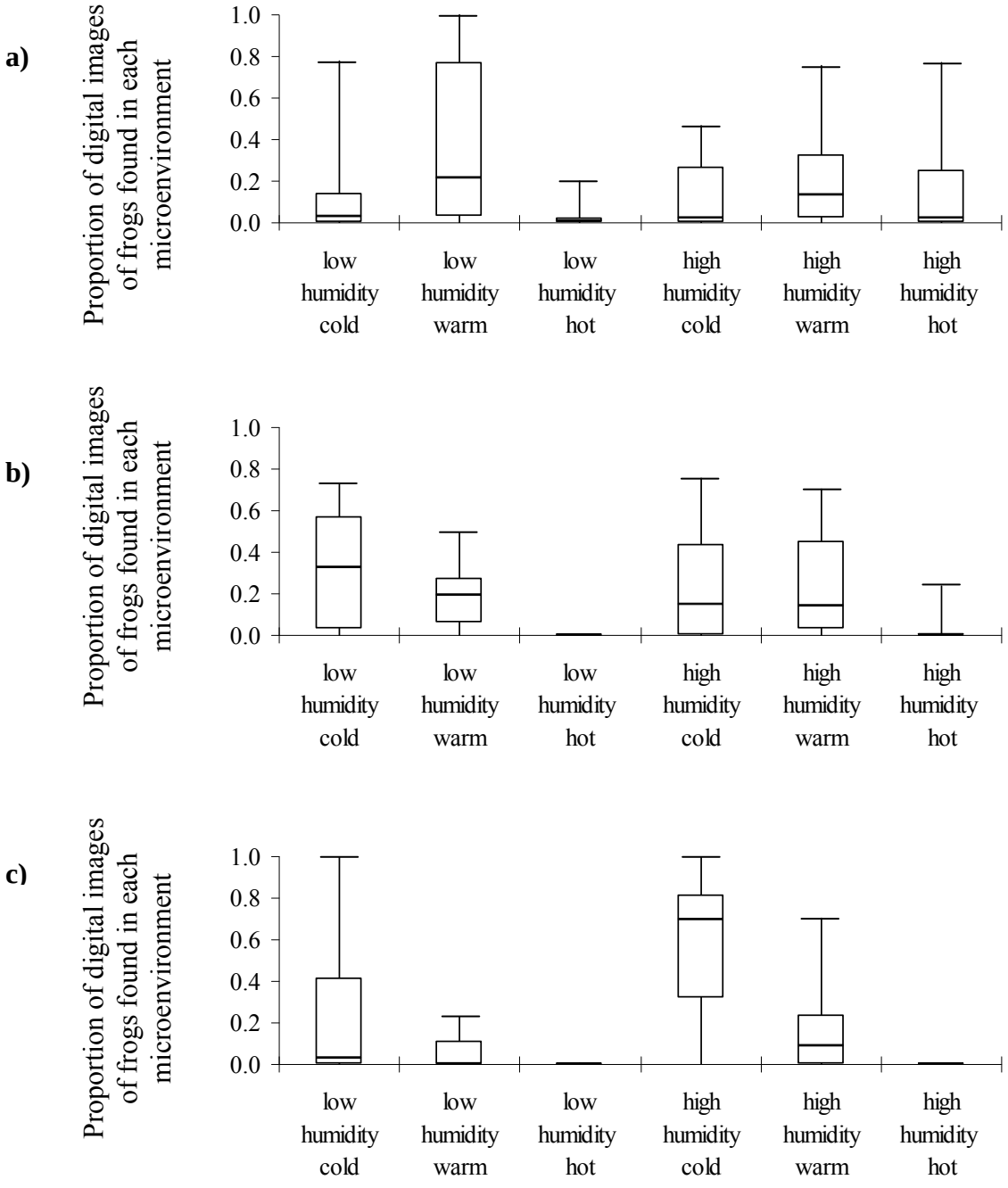

Figure 5.6 Proportion of digital images in which each species was observed in each combination of thermal and hydric environments for a) *Litoria caerulea*, b) *L. wilcoxii* and *L. genimaculata.* In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

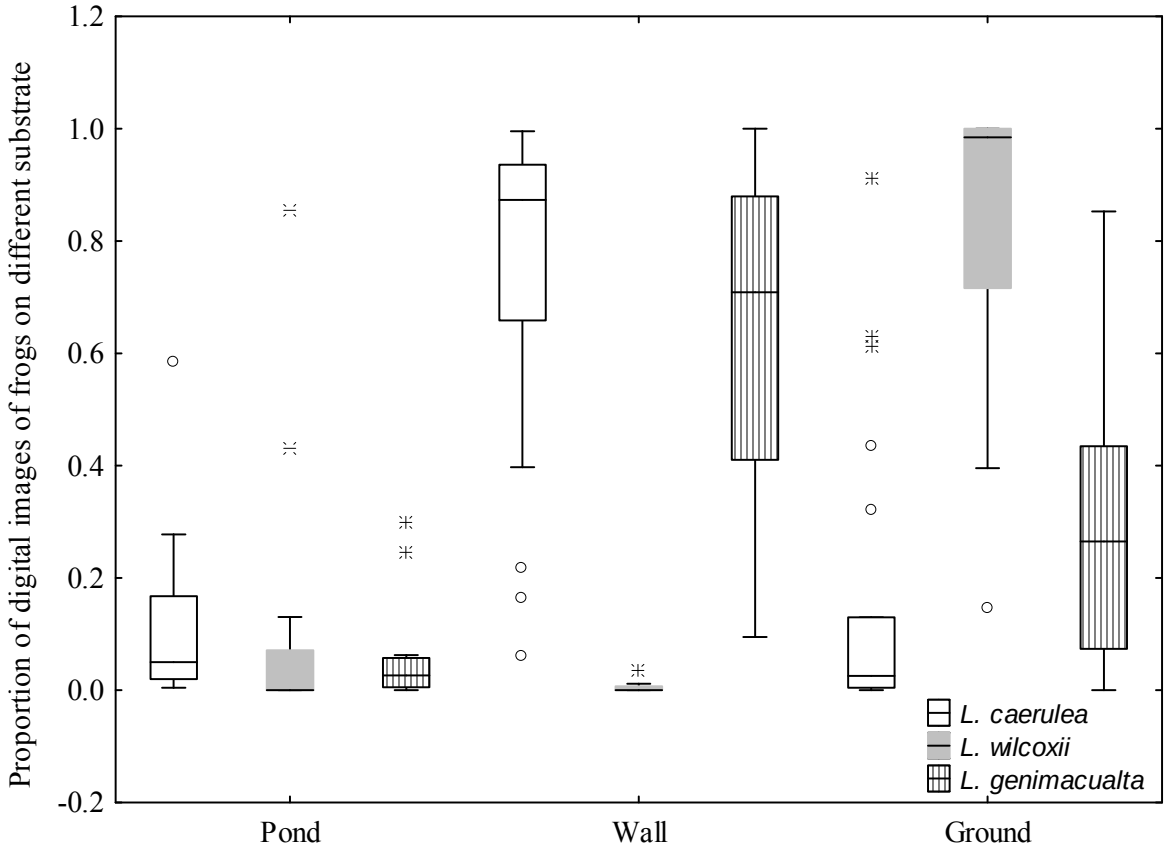

Figure 5.7 Proportion of digital images in which *Litoria caerulea*, *L. wilcoxii* and *L. genimaculata* were located on each substrate (in the pond, on the wall or on the ground) during the day. In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

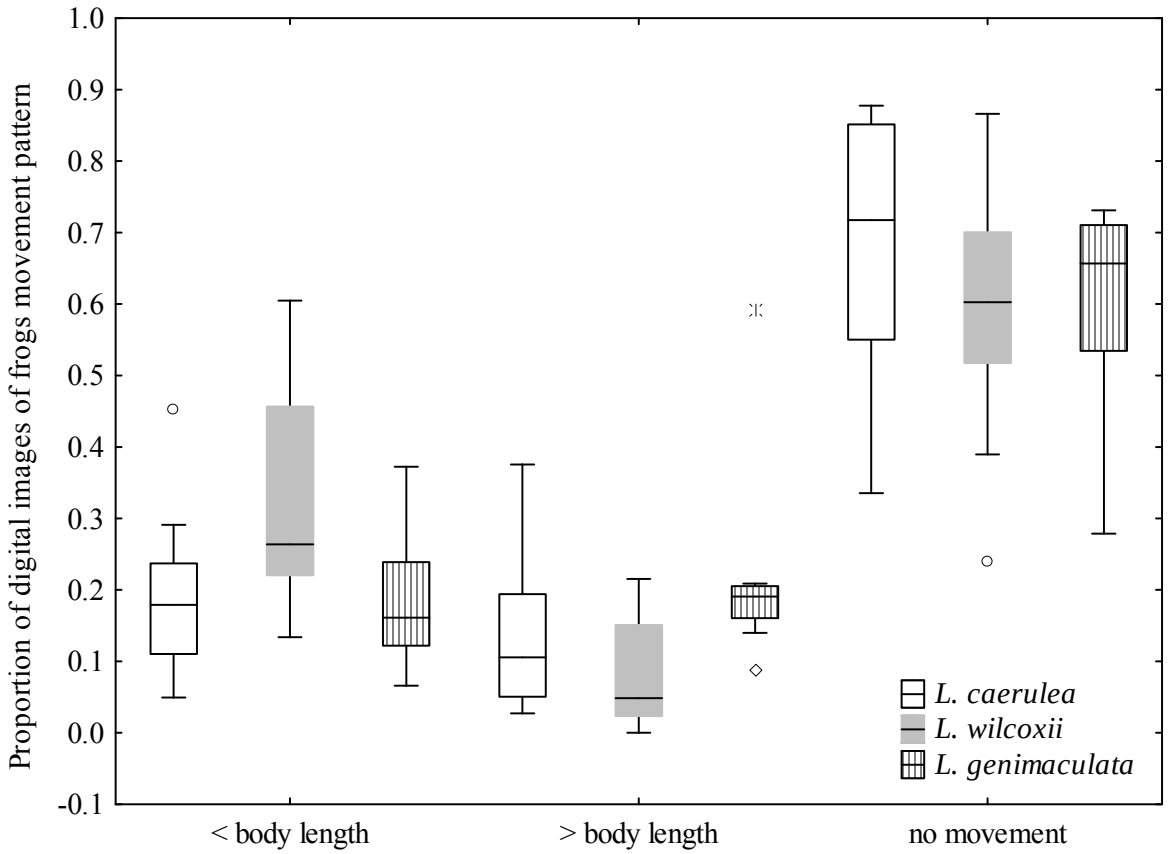

Figure 5.8 Proportion of digital images in which *Litoria caerulea*, *L. wilcoxii* and *L. genimaculata* had moved (< body length, > body length or no movement) within the terraria during the day. In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

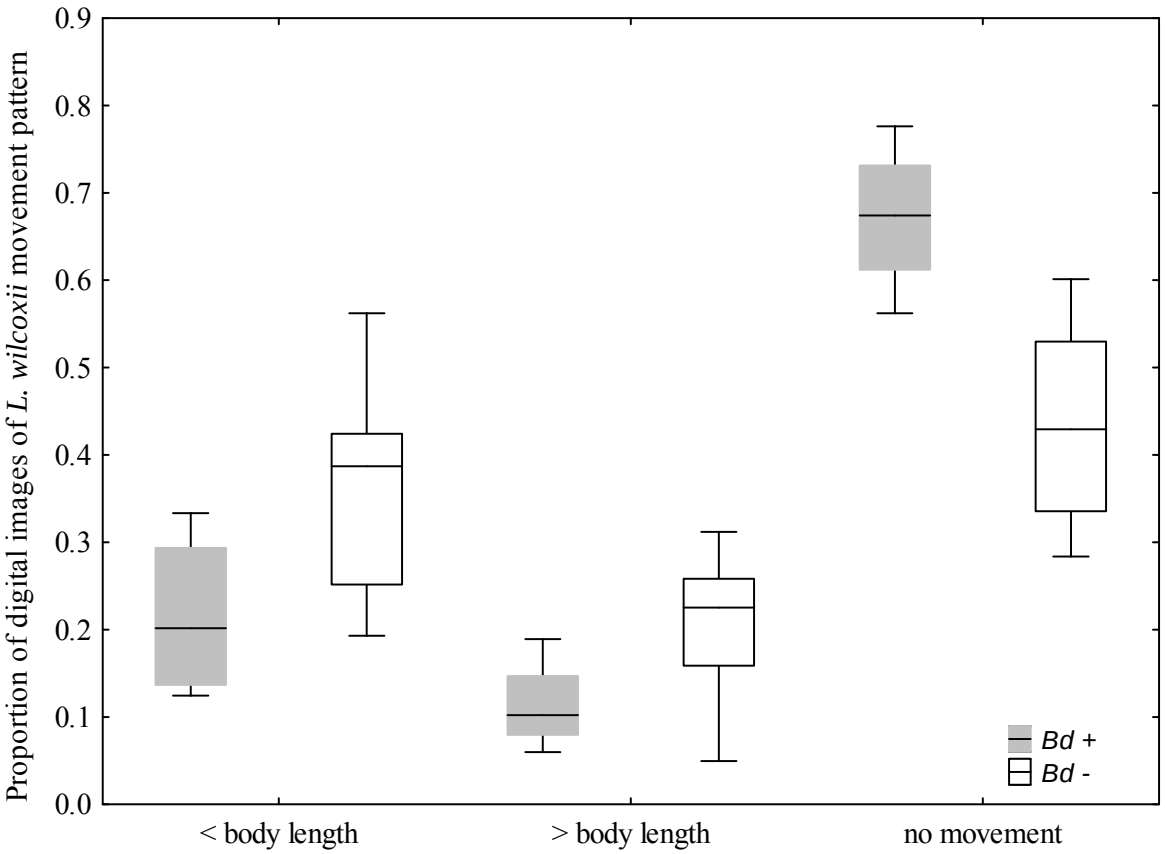

Figure 5.9 Proportion of digital images in which infected (*Bd* +) and uninfected (*Bd* -) *Litoria wilcoxii* movement pattern (< body length, > body length or no movement) during the night were observed. In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

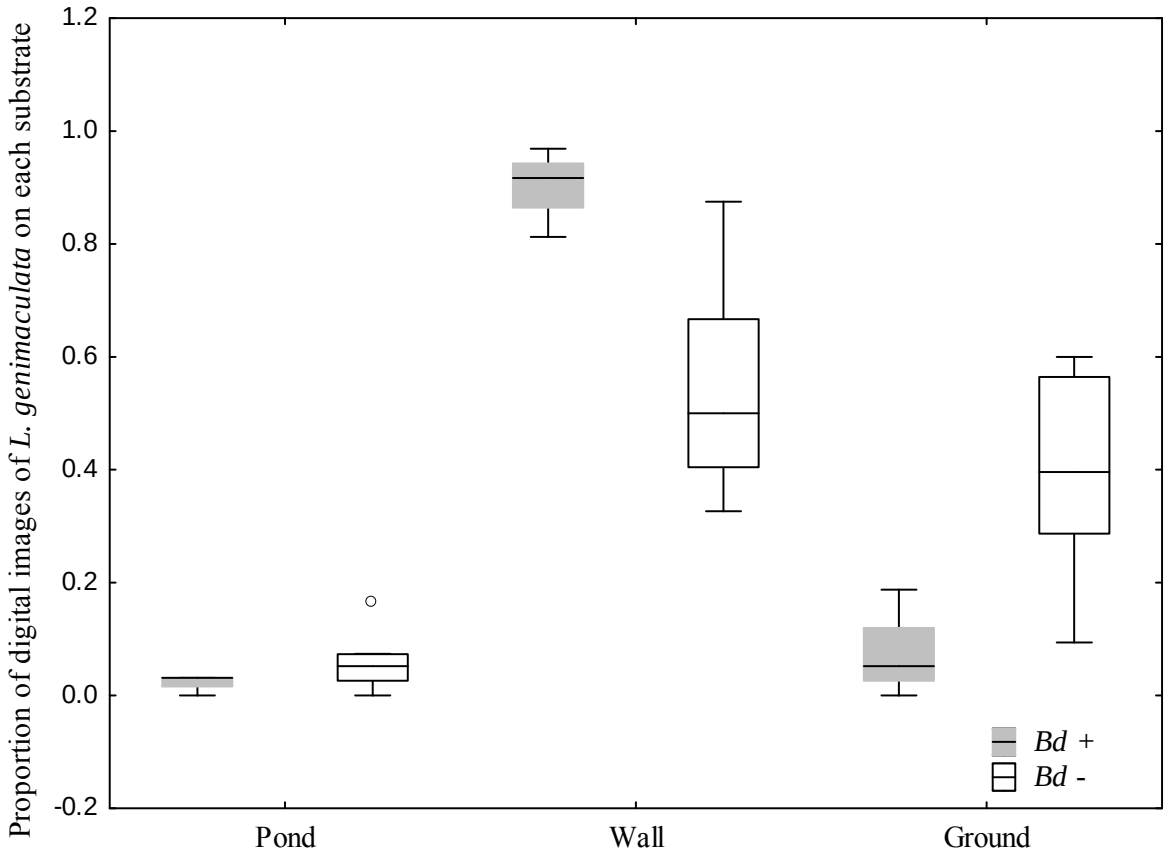

Figure 5.10 Proportion of digital images in which infected (*Bd* +) and uninfected (*Bd* -) *Litoria genimaculata* were observed on each substrate (pond, wall or ground) during the night. In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

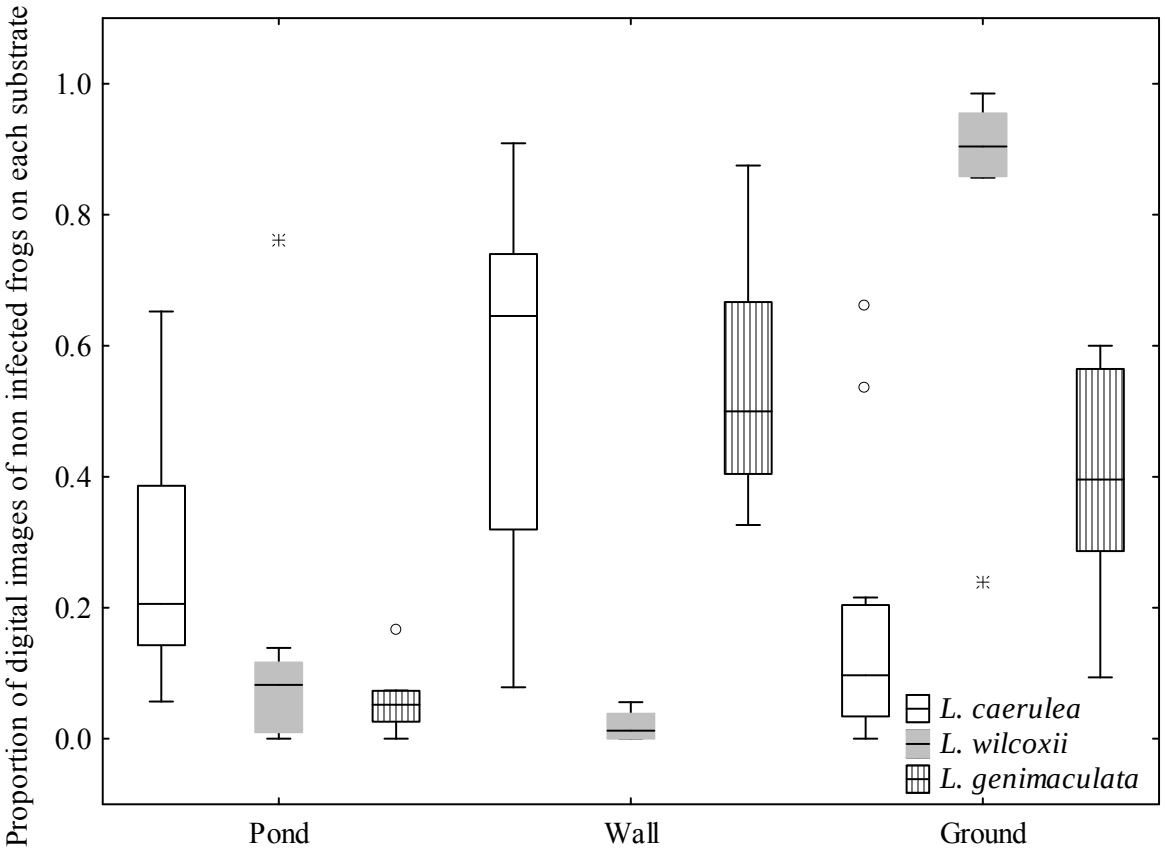

Figure 5.11 Proportion of digital images in which uninfected *Litoria caerulea*, *L. wilcoxii* and *L. genimaculata* were observed on each substrate (pond, wall or ground) during the night. In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

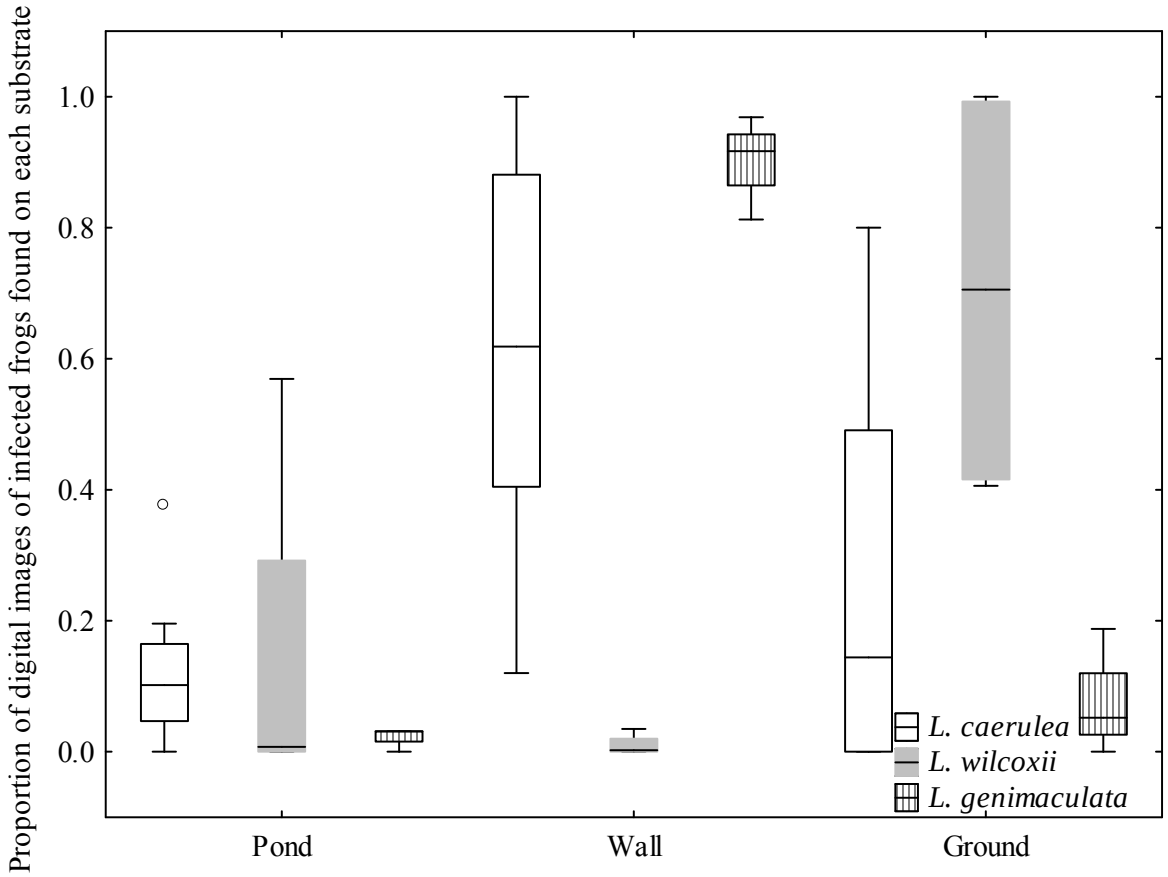

Figure 5.12 Proportion of digital images in which infected *Litoria caerulea*, *L. wilcoxii* and *L. genimaculata* were observed on each substrate (pond, wall or ground) during the night. In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

Discussion

Six *L. genimaculata* that tested positive for *Bd* at the time of capture tested negative in the laboratory 18 days later. Kriger and Herman (2006) observed recovery from *Bd* infection in wild *L. wilcoxii*. The mechanism that enables a frog to clear itself of *Bd* infection is unclear. The innate immune defences of frogs, predominantly antimicrobial peptides (AMPs), can kill *Bd* zoospores *in vitro* (Rollins-Smith et al., 2002b; Woodhams et al., 2007). This may result in a reduced rate of self infection that possibly reduces the progress of chytridiomycosis sufficiently to prevent mortality. Elevated body temperature, which can be achieved through warmer microenvironment selection, can also help frogs to clear infection with *Bd* (Woodhams et al., 2003). In the present study, intensity of infection in all three species was more likely to decrease over time in individuals that were able to choose their thermal and hydric microenvironments, although small sample sizes did not allow this trend to reach statistical significance. In addition, seven of the eleven individuals that cleared themselves of *Bd* infection during the experiment had a choice of thermal and microenvironments. On the other hand, several of the *L. genimaculata* in this study that were housed in stable climatic conditions (high humidity and an average temperature of 23ºC) that was suitable for *Bd* growth, also cleared infections, suggesting that other mechanisms, such as antimicrobial peptides, may have been involved in this process. Quantitative PCR uses rDNA, which does not decompose as quickly as RNA (Matsuo et al., 1999), to determine the infection status of a frog. It is possible that the number of zoospore equivalents detected at capture of the frogs may not necessarily indicate an active infection as *Bd* zoospores do not have to be viable to result in a positive qPCR assay. This aspect has been largely ignored in recent discussions on qPCR as a technique to determine the infection status of frogs with *Bd* (Kriger et al., 2007a; Smith, 2007) but needs to be taken into consideration in studies where infection status is a factor in the design.

Thermal environment selection differed significantly amongst the three species and can be linked to some extent to the decline pattern observed in the wild. *Litoria caerulea*, which has not experienced any population declines due to chytridiomycosis, spent more time in the hot and warm environments compared to *L. wilcoxii* and *L. genimaculata*.

Litoria genimaculata, which has experienced chytridiomycosis-associated declines, spent little time in the hot thermal environment. However, this environment was selected even less often by *L. wilcoxii*, even though this species has not declined due to chytridiomycosis. A radio-tracking study by Rowley (2007) found that *L. lesueuri* (which could either be *L. wilcoxii* or *L. jungguy* as the two are morphologically indistinguishable; Donnellan and Mahony, 2004) chose warmer temperatures than *L. genimaculata*. The average summer body temperature of *L. lesueuri* was around 25ºC (Rowley, 2007), lower than both the warm and hot thermal environments we provided. It appears, however, that *L. lesueuri* and *L. wilcoxii* can select microenvironments that are suitable for *Bd* growth, and studies have shown high *Bd* prevalence in the wild (Retallick et al., 2004; Kriger and Hero, 2007b). Because this species has not shown population declines attributable to chytridiomycosis, this suggests that it has other mechanisms to survive *Bd* infection, possibly more effective antimicrobial peptides as shown by Woodhams et al. (2005), avoidance behaviour, or low risk of transmission that reduces the time being exposed to *Bd* (Rowley and Alford, 2007b; Rowley et al., 2007).

Litoria wilcoxii spent long periods of time on the ground (the floors of the terraria), whereas *L. caerulea* and *L. genimaculata* spent more time on the walls of the terraria. This is similar to the results of Rowley and Alford (2007b), who found that *L. lesueuri* (possibly *L. wilcoxii*) spent substantial amounts of time on the ground and on leaf litter, while *L. genimaculata* was more arboreal. Being above ground in a less sheltered habitat increases a frog's opportunity to increase its body temperature by basking. It also, assuming *Bd* does not exist outside water bodies, reduces time of exposure to *Bd*. At this time there is no published evidence of the existence of viable or saprobic *Bd* outside of the stream environment, such as in the soil or forest leaf litter.

Individuals of all three species, *L. caerulea*, *L. wilcoxii* and *L. genimaculata*, regardless of their infection status, chose thermal environments that were above 32ºC at some time during the experiment. Berger (2001) observed *Bd* zoospore mortality *in vitro* at 32 ºC. Laboratory experiments have shown a reduction of infection with *Bd* at 27 ºC in

M. fasciolatus (Berger et al., 2004) and elimination of *Bd* infection in *L. chloris* at 37ºC (Woodhams et al., 2003). Therefore, it is possible that all three species may eliminate infection with *Bd* by temperature selection, not in response to infection but simply because they normally choose to spend some of their time at elevated body temperatures.

Most frog species, including *L. caerulea* (pers. obs.), *L. wilcoxii* and *L. genimaculata*, are predominantly nocturnal (Rowley and Alford, 2007b). At night, uninfected *L. wilcoxii* moved significantly more often than individuals that tested positive for *Bd*. Infected *L. genimaculata* spent significantly less time on the ground than uninfected individuals. This is the first study showing behavioural differences between individuals with differing infection status. These differences may not reflect adaptive responses to infection; they could both result from generally lower tendencies of infected frogs to move substantial distances, for example. It is unclear whether these changes in behaviour would affect the course of *Bd* infection in either captive or wild frogs. Radio-tracking of frogs of these species did not demonstrate such differences in behaviour (Rowley, 2007). Further studies comparing nocturnal behaviour in species, that have and have not suffered declines due to chytridiomycosis, are needed to understand the possible role of microenvironment selection in the progress of chytridiomycosis.

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Do frogs avoid water that contains *Batrachochytrium dendrobatidis* zoospores?

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Abstract

Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), has been implicated as a proximate cause of many amphibian declines around the globe. However, its effects vary widely among species; some species can persist despite infection while others are driven to local extinction. Species specific variation may be partly caused by factors such as immune function and microenvironment. Less is known about factors determining the vulnerability of species to initial infection. Because infection is caused by contact of waterborne zoospores with the skin, one possible source of variation in infection risk is an ability to detect and avoid water containing *Bd* zoospores. We examined whether frogs of three species, *Litoria caerulea, L. genimaculata* and *L. wilcoxii,* which have suffered to different degrees from chytridiomycosis, avoid water contaminated with *Bd* zoospores. Individual frogs were presented with a choice of *Bd*-contaminated and non-contaminated water bodies (ponds) to use for hydration. The initial choice of all three species appeared not to be influenced by pond contamination, however, significantly more *L. caerulea* subsequently chose non-contaminated ponds more often, and *L. genimaculata* showed a similar but not statistically significant trend. Overall, *L. caerulea* spent significantly more time submerged in water than *L. genimaculata* and *L. wilcoxii* and was the only species in which some individuals became infected by *Bd* during the experiment.

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Introduction

Chytridiomycosis is an amphibian skin disease caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*). It has been implicated as a proximate cause of many amphibian declines, many in relatively pristine habitats around the globe (Berger et al., 1998; Mutschmann et al., 2000; Ron and Merino, 2000; Bosch et al., 2001; Bradley et al., 2002; Garner et al., 2005; Lips et al., 2006; Skerratt et al., 2007). In Australia, chytridiomycosis was listed in 2002 as a key threatening process under the Environment Protection and Biodiversity Conservation Act (EPBC) because approximately 57 native anuran species had been found with *Bd*, of which approximately 30% have suffered declines (Speare and Berger, 2005).

Frog populations in northern Queensland, Australia were monitored before, during and after outbreaks of *Bd* in the early 1990's (McDonald and Alford, 1999). This revealed that the impact of *Bd* on populations varied among species. Some species, such as *Litoria caerulea, L. xanthomera*, *L. wilcoxii* and *L. jungguy* suffered no detectable ill effects, while at elevations above 400m in the Wet Tropics, *L. genimaculata* declined substantially and *L. nannotis* declined to local extinction (Richards et al., 1993; McDonald and Alford, 1999; McDonald et al., 2005). Several years after the declines, upland populations of *L. genimaculata* recovered, whilst other species, such as *L. nyakalensis* and *L. lorica,* failed to reappear (Ingram and McDonald, 1993; McDonald et al., 2005). Similar patterns of variation in susceptibility have also been reported from North and South America (Hale et al., 2005; Lips et al., 2005b; Garcia et al., 2006) and New Zealand, where the terrestrial *Leiopelma archeyi* appeared to decline due to *Bd*, while a sympatric semi-aquatic species, *L. hochstetteri,* showed no dramatic declines (Bell et al., 2004).

Variation among amphibian species in their susceptibility to declines caused by chytridiomycosis is often thought of as being related to differences in the fates of infected animals (Daszak et al., 1999; Lips et al., 2003; Lips et al., 2005b). For example, species may have stronger or weaker innate immune responses that affect progression of the disease (Rollins-Smith and Conlon, 2005; Woodhams et al., 2006), or

may inhabit microenvironments more or less favourable to the development of chytridiomycosis (Rowley and Alford, 2007b). Less thought has been devoted to another source of variation; differences in rates of exposure to *Bd*. Richards et al. (1993) first noted that declining Wet Tropics frog species, such as *L. nyakalensis, L. nannotis* or *L. genimaculata*, were associated with streams, and many authors since have suggested that stream-breeders may experience higher rates of exposure to *Bd* compared to terrestrial species (McDonald and Alford, 1999; Lips et al., 2003). However, there is substantial variability even among stream-breeding species (McDonald and Alford, 1999); some of this may be caused by differences in transmission rates caused by effects of behavior on exposure (Rowley and Alford, 2007a).

A variety of mechanisms may affect the exposure of frogs to *Bd* zoospores and thus their risk of infection. These include general levels of interaction with other frogs (Rowley and Alford, 2007a) and possible avoidance of contact with infected frogs or contaminated water bodies. It is known that *Bd* can be transmitted to frogs and tadpoles via contaminated water (Parris, 2004; Rachowicz and Vredenburg, 2004). If infection is often via contaminated water bodies, it is possible that anurans could reduce the probability of acquiring an infection by detecting and avoiding water bodies containing *Bd* zoospores. Parasite avoidance behavior has been observed in several mammal species (Lozano, 1991; Gilbert, 1997; Hutchings et al., 2001a); Hutchings et al. (1998) observed an increase in such avoidance behavior in parasitized animals.
Several studies (Resetarits and Wilbur, 1989; Egan and Paton, 2004; Rieger et al., 2004) have demonstrated that anurans can detect predator cues and use them to avoid ovipositing in water bodies containing predators. This detection is generally assumed to be via olfaction (Duchamp-Viret et al., 1996; Jorgensen, 2000). Given that frogs may detect the presence of predators in water through olfaction, it is possible that at least some frog species may detect *Bd* metabolites and use them as cues to discriminate between contaminated and non-contaminated water bodies. Additionally, it is possible that frogs may experience some degree of skin irritation as *Bd* encyst within the epidermis (Berger et al., 2005a) and use this cue in a similar way.

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Our aim was to determine whether frogs of three species (*L. wilcoxii, L. genimaculata* and *L. caerulea*) that are susceptible to *Bd* but have suffered from chytridiomycosis to different extents, discriminate among water bodies used for rehydration based on whether they contain *Bd* zoospores.

Materials and Methods

In order to minimize impact on frog population dynamics, our permit conditions required us to collect *L. wilcoxii* and *L. genimaculata* from aquatic life stages, as these two species were not available from captive breeders. *Litoria wilcoxii* were raised from tadpoles (no eggs were found) collected at Crystal Creek, Paluma Range National Park, Queensland, Australia (S18°58′54″ E146°12′01″). This species is part of the *L. lesueuri* group, which was recently split into three species (*L. wilcoxii*, *L. jungguy* and *L. lesueuri*) and occurs in rainforest habitat along the east coast of Australia (Donnellan and Mahony, 2004). Individuals of the sympatric species, *L. genimaculata*, were raised from eggs collected at Birthday Creek, Paluma Range National Park (S18°58′54″ E146°10′02″). *Litoria caerulea* is a widespread Australian pond breeder (Barker, et al. 1995) and subadults were supplied from a captive bred population (Brendan Tiernan, Morphett Vale, South Australia). All frogs were swabbed using a sterile tubed dry swab (Medical Wire and Equipment, Corsham, Wiltshire UK) that was run across frog's hands, feet, thighs and ventral surface twice, before and after each trial for diagnostic quantitative PCR analysis, which was carried out at the School of Veterinary and Biomedical Sciences, James Cook University.

Isolates of *Bd* (Tully-*L. rheocola* 06-LB-1) were cultured on agar plates in the School of Veterinary and Biomedical Science, James Cook University, following the protocol of Longcore et al. (1999), but using half of the recommended nutrients as this appeared to increase culture growth.

The experiment was designed to determine whether the presence of *Bd* zoospores affected the use of ponds by frogs and each trial ran for 8 days. During each trial, frogs were individually housed in glass terraria, each provided with two containers ("ponds";

115 mm diameter × 60 mm high), one at each end of the long axis and centered on the short axis. Each pond contained 120-200 mL for smaller sized species (*L. genimaculata* and *L. wilcoxii*) or 500-600 mL for larger sized species (*L. caerulea*) of rainwater. One pond was denoted the contaminated pond and the other the non-contaminated pond. The infectious pond was inoculated with 8,000-12,000 *Bd* zoospores per mL of rainwater. Zoospores were harvested by flooding plates with 2 mL of dilute salt solution (DS) (Boyle et al., 2003) for 10 minutes. The concentration of zoospores was determined by counting live and moving zoospores in three subsamples using a haemocytometer. The concentration of *Bd* zoospores in Australian rainforest streams is unknown. We chose to inoculate ponds with this concentration of zoospores because exposure to zoospores at this concentration has been used successfully to infect Australian frogs with *Bd* (Woodhams et al., 2003; Woodhams et al., 2007). Dilute salt solution with *Bd* zoospores was added to the rainwater to create the contaminated pond whilst an equal volume of DS solution without *Bd* zoospores was added to the same volume of rainwater to create the non-contaminated pond. For the control ponds, plates without *Bd* zoospores were flooded with the same amount of DS. The amount of DS added varied each time ponds were inoculated, as it depended on the number of zoospores per mL harvested from the agar plates. To exclude biases of frogs preferring particular sides of terraria, location of the infectious pond (either right or left side of terrarium) was determined randomly at the beginning of each trial. To minimize cross-contamination and ensure the presence of viable *Bd* zoospores, the water (with or without fresh *Bd* zoospores) and pond container were changed, and the positions of experimental and control ponds were reversed, every second day. Terraria and pond containers were disinfected between trials using F10 Veterinary Disinfectant which has been shown to be highly effective at killing *Bd* (Webb et al., 2007). Three digital cameras (Pentax Optio 33WR) were used to photograph the locations of frogs within the terraria at 30 minute intervals. A pilot study indicated that this was sufficient to capture most frog movements (Kenyon and Alford, unpublished). The first three trials on *L. caerulea* were conducted using six large terraria (750 × 350 × 500 mm) with one frog per terrarium. In order to increase sample size per experiment, we used twelve smaller (600 × 200 × 300 mm) terraria for all other trials. All trials were conducted in an air-

conditioned room at 23°C. A total of eight trials were run; four (three using larger terraria, one using smaller terraria) on *L. caerulea* (n=30, average SVL 61 mm, range 43-70 mm; mass 21 g, range 10.5-28.5 g), two on *L. genimaculata* (n=24, average SVL 28 mm, range 21-33 mm; mass 2 g, range 1-3.3 g), and two on *L. wilcoxii* (n=24, average SVL 39 mm, range 35-44 mm; mass 4 g, range 3.4-4.9 g).

Statistical analysis

We analyzed data combined across all trials of the experiment for each species. Any effects of the change in the size of the terraria during the trials with *L. caerulea* should contribute to the error variation in these analyses, so our results are conservative. We also performed additional analyses, where sample size permitted, within species in which we separately examined the data from individuals with different *Bd* infection histories. The analysis proceeded through several stages.

1) Each photograph was examined and the location of the frog was noted. Frogs were scored as having chosen a pond if they made contact with the interior of the pond container (either submerged or not submerged in water) and if they were not in contact with that pond during the previous digital image. In most analyses we included data only for frogs for which we recorded five or more choices during the eight days of the trial; this is the minimum number needed for any bias to be detectable if the null hypothesis is that pond choice by individuals is a binomial random variable with $p = 0.5$.

2) There is no simple way to simultaneously test the hypothesis across all frogs that individuals show biases towards one end of the terrarium. We therefore carried out independent binomial tests on the data for each frog to determine whether it showed a significant bias. We then used Fisher's combined probability test (Sokal and Rohlf, 1995) to evaluate the overall hypothesis that there is a tendency for frogs to exhibit a bias.

3) We next carried out a similar test to determine whether the presence of *Bd* affected frog pond choice throughout the trial, calculating the binomial probability of the observed number of choices of ponds with *Bd* for each frog that made more than five choices, and using a combined probability test to produce a chi-squared statistic for each species. We did not use data for frogs that made less than five choices because the individual binomial probabilities for such frogs could never be less than 0.05.

4) We next tested the hypothesis that the presence of *Bd* might affect each frog's initial choice of pond. We did this to definitively eliminate the influence of any possible bias that might have influenced subsequent choices (e.g., after initially choosing the pond at one end of the terrarium, based on the presence or absence of *Bd*, the frog might have continued to choose the pond at that end of the terrarium regardless of the presence of *Bd*), using a simple chi-squared goodness-of-fit test. All frogs that made at least one choice were included in this analysis.

5) For the next three analyses we included data where frogs were observed to be submerged in the pond water rather than simply making contact with the pond container. First, we used Wilcoxon's matched-pairs tests on the data for each species to determine whether the time spent in water with and without *Bd* zoospores differed consistently within individuals. We illustrated this graphically by subtracting the total number of images of each individual frog submerged in water without *Bd* from total images of each individual frog submerged in water with *Bd*. Second, we used Kruskal-Wallis tests to determine , whether *L. caerulea* with different infection status, spent different amount of times in ponds without *Bd*. Third, we determined whether individuals that initially chose a pond without *Bd* zoospores spent more time in the pond with or without *Bd*, compared to individuals that individuals that initially chose ponds with *Bd* zoospores, using Mann-Whitney U tests.

6) Finally we determined whether the total time spent in water of any sort (log transformed) differed among the three frog species using a Kruskal-Wallis test.

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Results

The qPCR assays showed that no *L. genimaculata* or *L. wilcoxii* were infected with *Bd* before or after the trials (Table 5.1). Seven *L. caerulea* tested positive for *Bd* infection before the trial; of these two tested negative at the end. Twenty-three *L. caerulea* were initially uninfected; of these three tested positive at the end of the trial.

1) No *L. wilcoxii* made five or more choices of ponds, so data for this species were excluded from most analyses.

2) Individuals of *L. caerulea* and *L. genimaculata* that chose a pond more than five times showed a tendency to choose the pond at one end of the terrarium (preferred pond) more frequently, regardless of whether *Bd* zoospores were present, than would be expected by chance (*L. caerulea*, n= 12, χ^2= 57.63, df= 24, p≤ 0.001; *L. genimaculata*, n=4, χ^2= 25.70, df= 8, p≤ 0.002; Figure 6.1).

3) Despite tending to prefer one end of the terrarium, seven *L. caerulea* individuals chose ponds that did not contain *Bd* significantly more frequently, two chose both types with equal frequency and three chose contaminated ponds more often (n= 12, χ^2=37.47, df= 24, p= 0.04; Figure 6.2). Although *L. genimaculata* did not show a significant tendency to avoid ponds containing *Bd* (n= 4, χ^2=4.00, df= 8, p= 0.64), in both species more than twice as many individuals chose non-contaminated ponds more frequently than ponds that contained *Bd* (Figure 6.2).

4) Two *L.caerulea* were not observed to visit the pond throughout the experiment. The initial pond choice of each species was not significantly influenced by the presence of *Bd* (*L. caerulea*, n= 28, χ^2=0.03, df= 2, p= 0.99; *L. genimaculata* n= 24, χ^2= 0.75, df= 2, p= 0.69; *L. wilcoxii*, n= 19, χ^2= 0.05, df= 2, p= 0.98).

			After trial	
			+ for *Bd*	- for *Bd*
Litoria genimaculata	Before trial	+ for *Bd*	0	0
		- for *Bd*	0	24
Litoria wilcoxii	Before trial	+ for *Bd*	0	0
		- for *Bd*	0	24
Litoria caerulea	Before trial	+ for *Bd*	5	2
		- for *Bd*	3	20

Table 6.1 Infection status of *Litoria genimaculata*, *L. wilcoxii* and *L. caerulea*, as determined by qPCR assay before and after a trial.

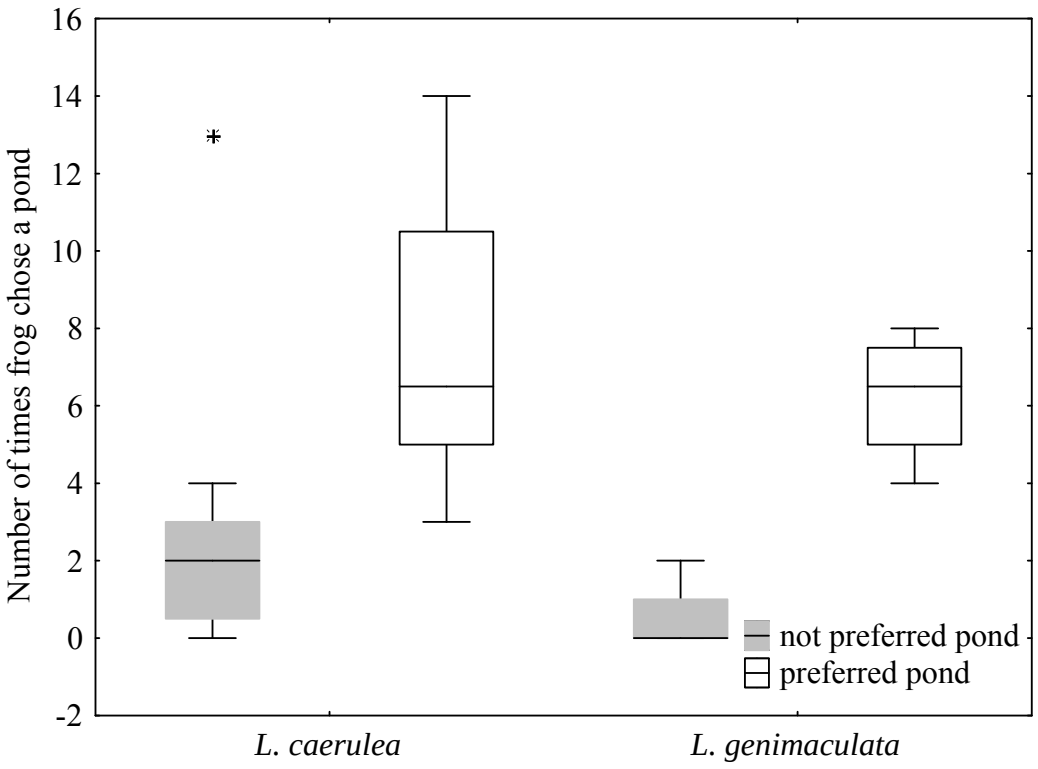

Figure 6.1 Number of digital images of individuals (*Litoria caerulea* and *L. genimaculata*) that chose a pond (frog being either submerged or not submerged in water) more than five times. The preferred pond was the pond that was chosen by an individual more frequently compared to the other pond (not preferred pond). In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

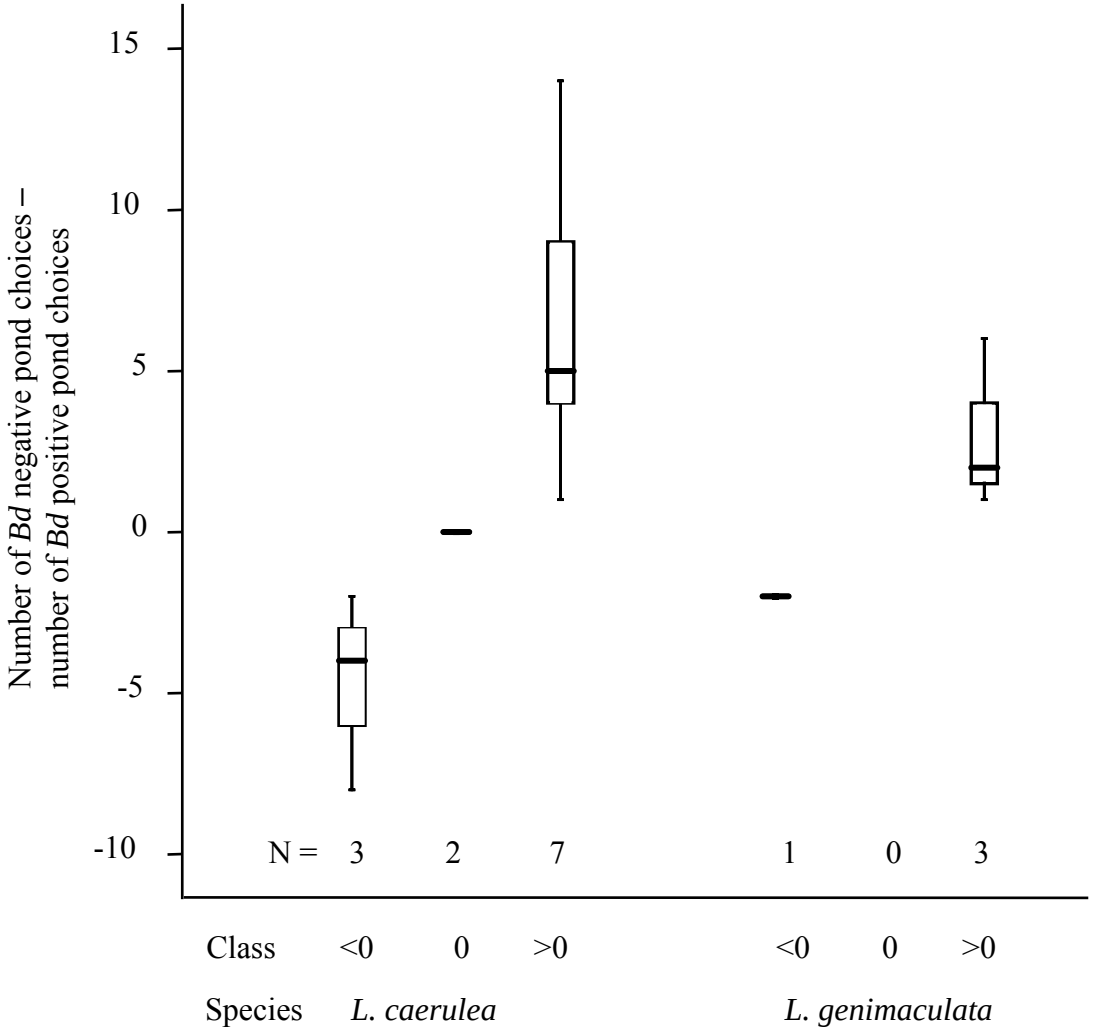

Figure 6.2 Distribution of the differences between the numbers of times individual *Litoria caerulea* and *L. genimaculata* chose ponds without and with *Bd* zoospores. Separate distributions (classes) are shown for frogs that chose ponds with *Bd* more often (<0), frogs that chose ponds without *Bd* more often (>0), and those that chose both types with equal frequency (=0). In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

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5) *Litoria caerulea*, *L. wilcoxii* and *L. genimaculata* individuals did not spend significantly more time in non-contaminated ponds than in ponds containing *Bd* zoospores (Wilcoxon's matched pairs tests, n=28, z=0.33, p=0.74; n=19, z=0.20, p=0.84; n=24, z=0.36, p=0.72, respectively). However, *L. caerulea* that were infected before the trial but not after (lost infection), spent significantly more time in ponds without *Bd*, than did *L. caerulea* that were infected before and after the trial (stayed infected) (Kruskal Wallis test, n=7, H= 3.75, p=0.05; Figure 6.3). The Mann-Whitney U tests indicated that the amount of time spent in water with or without *Bd* did not significantly depend on the initial experience of individual *L. caerulea* (n=28, z=-1.12, p=0.27), *L. genimaculata* (n=24, z=-0.90, p=0.39) and *L. wilcoxii* (n=19, z=0.20, p=0.84).

6) Overall, *L. caerulea* spent significantly more time in water than did *L. genimaculata* or *L. wilcoxii* (Kruskal Wallis test, n=71, H= 25.82, $p < 0.01$) (Figure 6.4).

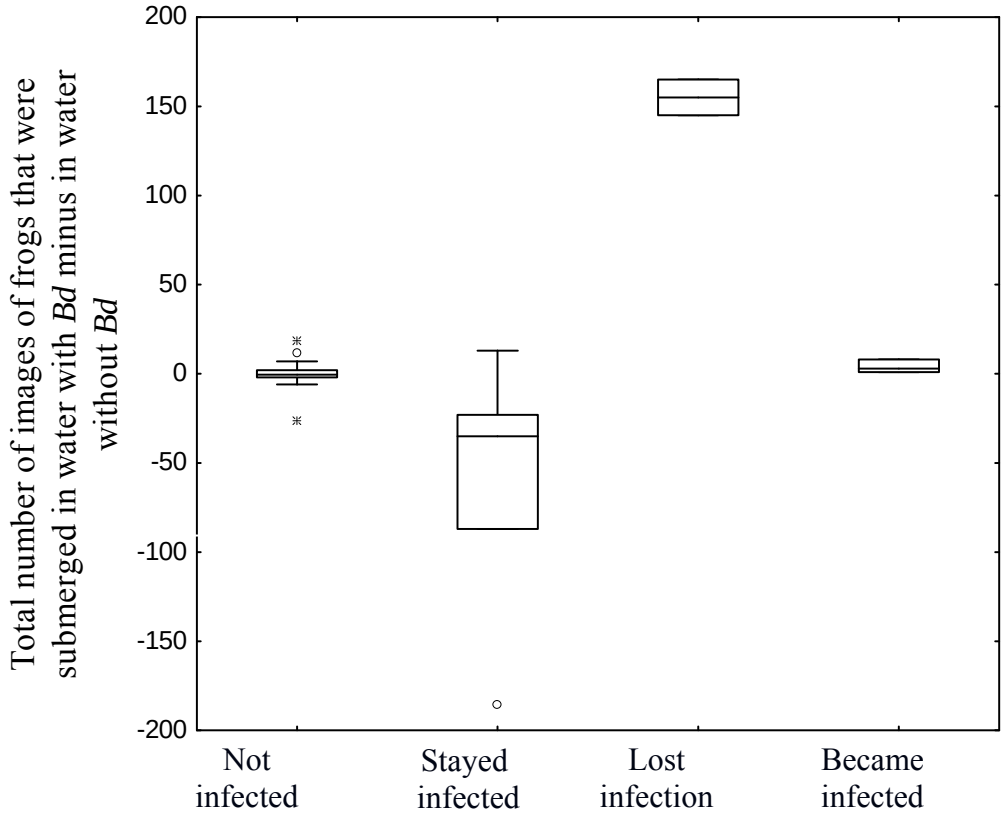

Figure 6.3 Differences between the number of images where a frog was submerged in water with *Bd* minus number of images where a frog was submerged in water without *Bd.* Consequently, if in the positive, frogs selected ponds without *Bd* more often. Individual *Litoria caerulea* were grouped according to their infection status before and after the trials as determined by qPCR assay. In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

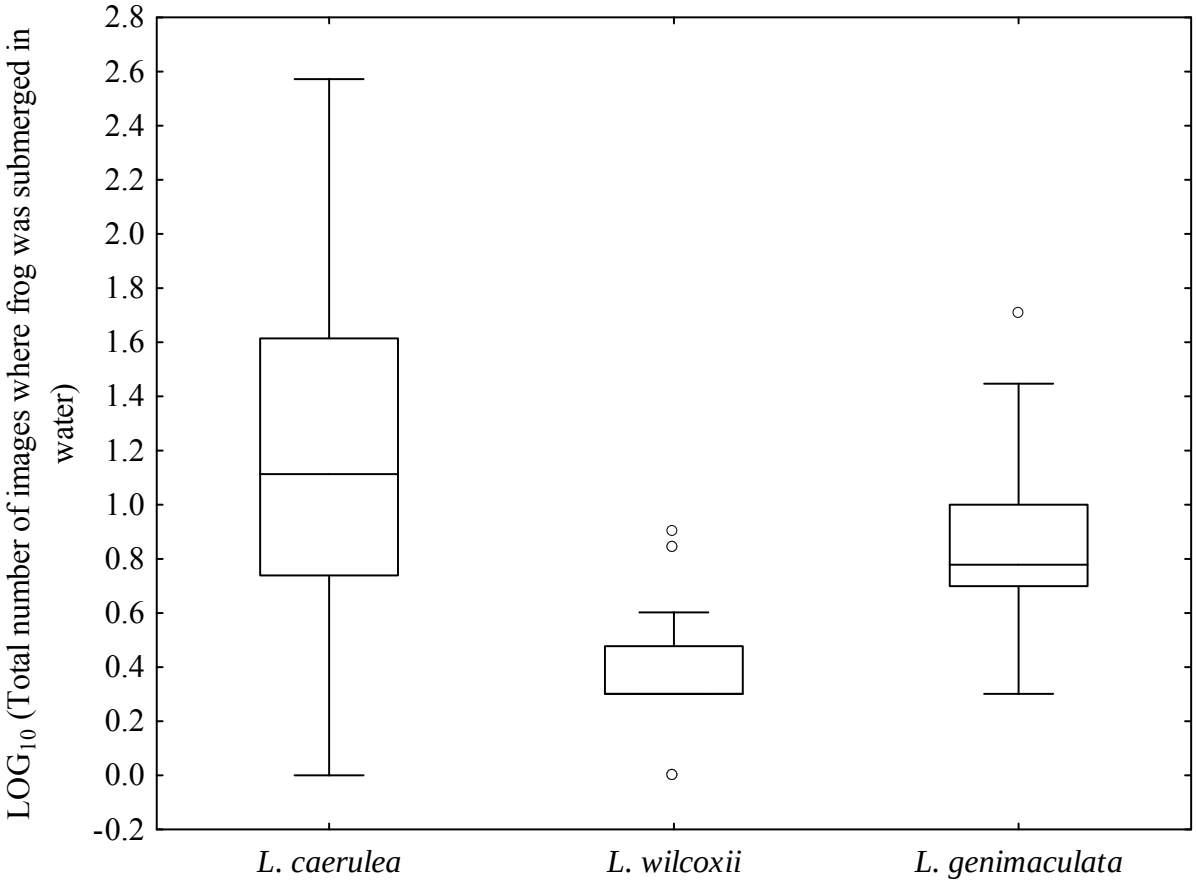

Figure 6.4 Number of images of frogs of the species *Litoria caerulea*, *L. wilcoxii* and *L. genimaculata* found submerged in water. In this Tukey (1977) boxplot, vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

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Discussion

Because we sampled the behavior of frogs by determining their locations using photographs taken every 30 minutes, our data may underestimate the number of times they visited water. We also cannot determine whether animals that avoided water containing *Bd* zoospores actually did not enter the water at all, or entered it for a short period only. Although our data do not show any *L. wilcoxii* making five or more decisions to become associated with ponds, it is possible that they visited ponds for short periods of time only. However, our pilot study on *L. wilcoxii*, using four minute intervals, recorded the same number of pond visits; this suggests that this species visits water infrequently. This has also been observed in the field; Rowley and Alford (2007b) found that individuals of the *Litoria lesueuri* complex (a mixture of *L. wilcoxii* or *L. jungguy*) only infrequently made contact with water.

Individuals of both of *L. caerulea* and *L. wilcoxii*, tended to select the pond at one end of the terrarium significantly more often than the other pond. Because the locations of ponds containing and not containing *Bd* zoospores were swapped each time water was changed, this bias would not have led to frogs selecting ponds with or without *Bd* at different rates. Selecting ponds at random or consistently choosing the pond at one end of the terrarium would both have led to equal numbers of choices for ponds with and without *Bd*. However, side fidelity did not prevent *L. caerulea* from significantly avoiding ponds containing *Bd* zoospores and *L. genimaculata* showed a similar but not statistically significant trend. In order for the avoidance to be genuinely advantageous to frogs, the detection of *Bd* would have to be rapid enough to reduce rates of colonization of zoospores.

Litoria caerulea was the only species that became infected with *Bd* during the study even though *L. genimaculata* and *L. wilcoxii* are susceptible in the wild (McDonald and Alford, 1999) and all individuals were at some point submerged in contaminated water containing a higher concentration of zoospores than is known to occur in natural water bodies (Kirshtein et al., 2007). It is possible that the availability of non-contaminated water may reduce the severity of infections. *Litoria caerulea* that were infected before

the trial, but not after, spent significantly more time in the non-contaminated pond, suggesting that sitting in *Bd*-free water may reduce the rate at which zoospores reinfect the host individual to such an extent that the final qPCR results were negative. None of the frogs had access to external heat sources and the room temperature was maintained at 23°C, a temperature where infection with *Bd* has been shown to be fatal to several frog species, including *L. caerulea* (Voyles et al., 2007).

Overall, *L. caerulea* spent significantly more time than other species sitting in water and was the only species becoming infected during the study. This is consistent with the hypothesis that species that spend more time in water are more likely to become infected. None of our species completely avoided water containing *Bd* zoospores, possibly in part because their pond choices were influenced by side fidelity, and possibly also because they needed to enter ponds to determine their contamination status. However, *L. caerulea* significantly avoided water containing *Bd* zoospores, suggesting that detection and avoidance of contaminated water may play a role in determining the vulnerability of some species to infection with the amphibian chytrid.

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Summary and Conclusion

The amphibian disease chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), provides an opportunity to gain insight into the interaction between an ectothermic host and a pathogen – a dynamic interaction that is less understood and differs substantially from that of pathogen and endothermic host (Delajara, 1991; Waldmann, 2003). A pathogen like *Bd* that invades the keratinised epidermal cells of adult amphibians and mouthparts of tadpoles (Berger et al., 2005a) is exposed to daily and sometimes hourly fluctuations of the host's water balance and body temperature (Lillywhite et al., 1998; Seebacher and Alford, 2002). The host-pathogen dynamic can therefore be influenced by features of the amphibian's behaviour (Myhre et al., 1977; Blanford et al., 1998; Blanford and Thomas, 1999; Elliot et al., 2002; Woodhams et al., 2003), such as microenvironment selection, as this changes the external environment of the pathogen but also the host's physical performance and innate immune defences (Huey and Berrigan, 2001).

Three different host-pathogen interactions in amphibians exposed to *Bd* have been recorded: the amphibian does not become infected even though it occurs in a habitat where *Bd* is present, or the amphibian becomes infected and either dies or survives. What factors contribute to the variable susceptibility to infection and mortality rates we observe in sympatric wild amphibian species? My project aimed to increase our understanding of the factors that contribute to interspecific variation in vulnerability to *Bd*. I investigated the hypotheses that such variation could be the result of differences in innate immune defences (antimicrobial peptides), innate or adaptive selection of microenvironment, or behavioural avoidance of infective water. I found evidence for all three mechanisms.

Innate immune defences - AMPs

AMPs may either prevent a frog from being colonised by *Bd* zoospores on the epidermis, or reduce the rate of self-infection and hence prevent the initial infection from becoming fatal (Rollins-Smith and Conlon, 2005). *In vitro*, AMPs from *L. genimaculata* and *L. rheocola* completely inhibited *Bd* growth, although some frogs

of these species tested positive for *Bd* by qPCR assay. Antimicrobial peptide defences did not differ significantly between infected and uninfected individuals, suggesting that infection with *Bd* may not alter antimicrobial peptide expression within individuals. However, at the population level, a strong correlation between *L. genimaculata* antimicrobial peptide defences, including volume of peptides secreted and overall protection afforded by AMPs of the frog against *Bd*, and prevalence of *Bd* suggests a link between antimicrobial peptide defences and anuran susceptibility to *Bd*.

The high seasonal variation I documented in antimicrobial peptide defences of *L. genimaculata* and *L. rheocola*, may result from physiological shifts driven by temperature, or may reflect adaptation to seasonal fluctuations in the risk of infection. I found evidence that the composition of AMPs secreted differs between high and low elevation populations. I did not find that the AMPs of individuals in high elevation populations, that produced AMPs effective against *Bd*, were more effective than those from low elevation populations. Combined, these findings suggest that although there is some differentiation between populations that have experienced differing levels of natural selection due to chytridiomycosis, there is little evidence for the appearance of new forms of AMPs with higher effectiveness against *Bd*. However, a significantly and substantially higher proportion of individuals of *L. genimaculata* in high elevation populations, produced AMPs with anti-*Bd* activity than in low elevation populations. This suggests that selection may have acted to increase the overall rates of production of anti-*Bd* AMPs in these populations, without affecting the chemistry of the compounds produced. Antimicrobial peptide defences did not differ significantly between high and low elevation population of *Litoria rheocola*, suggesting that this species may have recolonised upland areas. It is unclear under what selection pressure this species was, however, it had more effective and more successful antimicrobial peptide defences against *Bd* than *L. genimaculata*, suggesting a stronger selection pressure after the emergence of chytridiomycosis.

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Microenvironment selection

The effects of temperature and humidity on the progress of chytridiomycosis in anurans have been well documented (Woodhams, 2003; Woodhams et al., 2003; Berger et al., 2004; Drew et al., 2006). It is therefore likely that a frog's selection of microenvironment can alter and potentially inhibit the growth of *Bd*, and may eliminate established infections. Additionally, infected individuals may demonstrate behavioural fever, which has been observed in anurans at all life stages (Lefcort and Blaustein, 1995; Cabanac and Cabanac, 2004), although no previous study has investigated possible behaviour fever in association with *Bd* infection.

While I did not observe behavioural fever in the three study species (*L. caerulea*, *L. wilcoxii* and *L. genimaculata*), a higher proportion of infected frogs in each species decreased their intensity of *Bd* infection when housed in variable environment than frogs housed in constant environments. It is possible that in the wild, frogs infected with *Bd*, may not always have the option of selecting microenvironments that would decrease the intensity of *Bd* infection, supporting the strong link between habitat use and vulnerability to *Bd* observed in Australia (Rowley and Alford, 2007a). The thermal microenvironment selection observed in this study could be linked, to some extent, to decline patterns observed in the wild. *Litoria caerulea* (vulnerable to infection with *Bd* but no population declines due to chytridiomycosis have been detected) selected warm and hot environments significantly more often compared to *L. genimaculata* (vulnerable to infection with *Bd* with highland population declines followed by recovery). Two species, *L. wilcoxii* and *L. genimaculata,* significantly altered their nocturnal behaviour when infected with *Bd*, but in ways that would not be expected to affect the progress of chytridiomycosis. Nevertheless, it does indicate that infected individuals are able to change their behaviour and perhaps other species that have not been studied yet, may express behavioural changes that affect the progress of chytridiomycosis. Seven of the eleven individuals that cleared themselves of *Bd* infection during the experiment had a choice of microenvironments. However, while housed in the laboratory at constant, standard climatic conditions, six adult *L. genimaculata* lost their infection within 18

days, suggesting that other mechanisms than microenvironment selection, such as antimicrobial peptides, also influence the progress of chytridiomycosis.

Disease avoidance behaviour

Because infection results from contact with waterborne zoospores, one possible source of anuran interspecific variation in susceptibility to infection with chytridiomycosis is an ability to detect and avoid water containing *Bd* zoospores. I found evidence that frogs avoided, although not completely, ponds containing *Bd* zoospores. Significantly more *L. caerulea* chose un-contaminated ponds more often, and *L. genimaculata* showed a similar but not statistically significant trend. *Litoria caerulea* spent significantly more time submerged in water than *L. genimaculata* and *L. wilcoxii*, and was the only species in which some individuals became infected by *Bd* during the experiment.

None of the frog species were able to completely avoid water containing *Bd* zoospores, as their pond decisions appeared to be influenced in part by side fidelity within the terrarium. However, *L. caerulea* significantly selected uncontaminated water more often than water bodies containing *Bd* zoospores, suggesting that detection and avoidance of contaminated water may play a role in determining the vulnerability of species to infections with the amphibian chytrid fungus.

Future research

More than thirty percent of amphibian species are declining (IUCN, 2006; Smith et al., 2006). Globally, there is an effort to create rescue facilities and captive breed some of the most vulnerable species (Gewin, 2008). However, one of the causes of the decline cannot be removed: *Bd* would still be present in the environment. Consequently, in order for the conservation effort to be successful long term, we need to gain knowledge of the host-pathogen interaction and what factors influence this relationship.

My study has shown that the host-pathogen dynamic is a complex system and that multiple factors, such as innate immune defences and behaviour of anurans, can

contribute to variable susceptibility to chytridiomycosis. For example, captive breeding programs may be able to select individuals of one species with more effective AMPs in the hope of creating a population that may be able to exist in habitats where *Bd* occurs, as it is the case with *L. genimaculata* and *L. rheocola*. However, it is important to understand that the host-pathogen dynamic is complex and conservation efforts cannot only focus on AMPs alone but do have to incorporate the species' behaviour.

References

Adams M. J. (1999) Correlated factors in amphibian decline: exotic species and habitat change in western Washington. *Journal of Wildlife Management* **63**: 1162-1171.

Alexander D. J. (2000) A review of avian influenza in different bird species. *Veterinary Microbiology* **74**: 3-13.

Alford R. A., Richards S. J. and McDonald K. R. (2007) Biodiversity of amphibians. In: *Encyclopedia of Biodiversity* (ed. S. A. Levin) pp. 159-169. Academic Press, Sydney.

Ali M. F., Knoop F. C., Vaudry H. and Conlon J. M. (2003) Characterization of novel antimicrobial peptides from the skins of frogs of the R*ana esculenta complex*. *Peptides* **24**: 955-961.

Anholt B. R., Negovetic S. and Som C. (1998) Methods for anaesthetizing and marking larval anurans. *Herpetological Review* **29**: 153-154.

Annis S. L., Dastoor F. P., Ziel H., Daszak P. and Longcore J. E. (2004) A DNA-based assay identifies *Batrachochytrium dendrobatidis* in amphibians. *Journal of Wildlife Diseases* **40**: 420-428.

Apponyi M. A., Pukala T. L., Brinkworth C. S., Maselli V. M., Bowie J. H., Tyler M. J., Booker G. W., Wallace J. C., Carver J. A., Separovic F., Doyle J. and Llewellyn L. E. (2004) Host-defence peptides of Australian anurans: structure, mechanism of action and evolutionary significance. *Peptides* **25**: 1035-1054.

Barinaga M. (1990) Where have all the froggies gone? *Science* **247**: 1033-1034.

Barker J., Grigg G. and Tyler M. (1995) *A Field Guide to Australian Frogs*. Surrey Beatty and Sons, Chipping Norton NSW.

Batista C. V. F., Rosendo da Silva L., Sebben A., Scaloni A., Ferrara L., Paiva G. R., Olamendi-Portugal T., Possani L. D. and Bloch Jr C. (1999) Antimicrobial peptides from the Brazilian frog *Phyllomedusa distincta*. *Peptides* **20**: 679-686.

Beck D. D. and Jennings R. D. (2003) Habitat use by gila monster: the importance of shelters. *Herpetological Monographs* **17**: 111-129.

Begon M., Harper J. L. and Townsend C. R. (1996) *Ecology*. Blackwell Science Ltd, Oxford, England.

REFERENCES

****

Bell B. D., Carver S., Mitchell N. J. and Pledger S. (2004) The recent decline of a New Zealand endemic: how and why did populations of archey's frog *Leiopelma archeyi* crash over 1996-2001? *Biological Conservation* **120**: 189-199.

Berger L. (2001) Diseases in Australian Frogs. In: *School of Public Health and Tropical Medicine*. PhD Thesis. James Cook University, Townsville, Australia.

Berger L., Hyatt A. D., Speare R. and Longcore J. E. (2005a) Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms* **68**: 51-63.

Berger L., Marantelli G., Skerratt L. F. and Speare R. (2005b) Virulence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* varies with the strain. *Diseases of Aquatic Organisms* **68**: 47-50.

Berger L. and Speare R. (1998) Chytridiomycosis: a new disease of wild and captive amphibians. *ANZCCART Newsletter* **11**: 1-3.

Berger L., Speare R., Daszak P., Green D. E., Cunningham A. A., Goggin C. L., Slocombe R., Ragan M. A., Hyatt A. D., McDonald K. R., Hines H. B., Lips K. R., Marantelli G. and Parkes H. (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences of the United States of America* **95**: 9031-9036.

Berger L., Speare R., Hines H. B., Marantelli G., Hyatt A. D., McDonald K. R., Skerratt L. F., Olsen V., Clarke J. M., Gillespie G., Mahony M., Sheppard N., Williams C. and Tyler M. J. (2004) Effect of season and temperature on mortality in amphibians due to chytridiomycosis. *Australian Veterinary Journal* **82**: 434-439.

Berger L., Speare R. and Hyatt A. (1999) Chytrid fungi and amphibian declines: overview, implications and future directions. In: *Declines and disappearances of Australian frogs.* (ed. A. Campbell) pp. 23-33, Environment Australia, Canberra.

Berger L., Speare R. and Kent A. (2000) Diagnosis of chytridiomycosis in amphibians by histological examination. *Zoo's Print Journal* **15**: 184-190.

Bevins C. L. and Zasloff M. (1990) Peptides from frog skin. *Annual Review of Biochemestry* **59**: 395-414.

REFERENCES

****

Biotechnology P. (1997) Microplate BCA Protein Assay. In: *http://www.piercenet.com/files/1997as4.pdf*.

Blake J. T. (1968) Etiology of brisket disease cattle. *Cornell Veterinarian* **58**: 305-314.

Blanford S. and Thomas M. B. (1999) Host thermal biology: the key to understanding host-pathogen interactions and microbial pest control? *Agricultural and Forest Entomology* **1**: 195-202.

Blanford S., Thomas M. B. and Langewald J. (1998) Behavioural fever in the senegalese grasshopper, *Oedaleus senegalensis*, and its implications for biological control using pathogens. *Ecological Entomology* **23**: 9-14.

Blaustein A. R. (1994) Chicken little or nero's fiddle? A perspective on declining amphibian populations. *Herpetologica* **50**: 85-97.

Blaustein A. R., Romansic J. M., Kiesecker J. M. and Hatch A. C. (2003) Ultraviolet radiation, toxic chemicals and amphibian population declines. *Diversity and Distributions* **9**: 123-140.

Blaustein A. R., Romansic J. M., Scheessele E. A., Han B. A., Pessier A. P. and Longcore J. E. (2005) Interspecific variation in susceptibility of frog tadpoles to the pathogenic fungus *Batracbochytrium dendrobatidis*. *Conservation Biology* **19**: 1460-1468.

Blaustein A. R. and Wake D. B. (1990) Declining amphibian populations - a global phenomenon. *Trends in Ecology & Evolution* **5**: 203-204.

Blaustein A. R. and Wake D. B. (1995) The puzzle of declining amphibian populations. *Scientific American* **272**: 52-57.

Boland M. P. and Separovic F. (2006) Membrane interactions of antimicrobial peptides from Australian tree frogs. *Biochimica Et Biophysica Acta-Biomembranes* **1758**: 1178-1183.

Boman H. G. (1995) Peptide antibiotics and their role in innate immunity. *Annual Review of Immunology* **13**: 61-92.

Bonaccorso E., Guayasamin J. M., Mendez D. and Speare R. (2003) Chytridiomycosis as a possible cause of population declines in *Atelopus cruciger* (Anura: Bufonidae). *Herpetological Review* **34**: 331-334.

REFERENCES

****

Bosch J. and Martinez-Solano I. (2006) Chytrid fungus infection related to unusual mortalities of *Salamandra salamandra* and *Bufo bufo* in the Penalara Natural Park, Spain. *Oryx* **40**: 84-89.

Bosch J., Martinez-Solano I. and Garcia-Paris M. (2001) Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biological Conservation* **97**: 331-337.

Bowie J. H., Wegener K. L., Chia B. C. S., Wabnitz P. A., Carver J. A., Tyler M. J. and Wallace J. C. (1999) Host defence antibacterial peptides from skin secretions of Australian amphibians. The relationship between structure and activity. *Protein and Peptide Letters* **6**: 259-269.

Boyle D. G., Boyle D. B., Olsen V., Morgan J. A. T. and Hyatt A. D. (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms* **60**: 141-148.

Boyle D. G., Hyatt A. D., Daszak P., Berger L., Longcore J. E., Porter D., Hengstberger S. G. and Olsen V. (2003) Cryo-archiving of *Batrachochytrium dendrobatidis* and other chytridiomycetes. *Diseases of Aquatic Organisms* **56**: 59-64.

Bradfield K. S. (2004) Photographic identification of individual Archey's frogs, *Leiopelma archeyi*, from natural markings. *DOC Science Internal Series. Department of Conservation, Wellington.* **181**: 36.

Bradford D. F. (1991) Mass mortality and extinction in a high-elevation population of *Rana muscosa*. *Journal of Herpetology* **25**: 174-177.

Bradley G. A., Rosen P. C., Sredl M. J., Jones T. R. and Longcore J. E. (2002) Chytridiomycosis in native Arizona frogs. *Journal of Wildlife Diseases* **38**: 206-212.

Brinkworth C. S. and Bowie J. H. (2003) Negative ion electrospray mass spectra of the maculatin peptides from the tree frogs *Litoria genimaculata* and *Litoria eucnemis*. *Rapid Communications in Mass Spectrometry* **17**: 2215-2225.

Brinkworth C. S., Carver J. A., Wegener K. L., Doyle J., Llewellyn L. E. and Bowie J. H. (2003) The solution structure of frenatin 3, a neuronal nitric oxide synthase inhibitor from the giant tree frog, *Litoria infrafrenata*. *Biopolymers* **70**: 424-434.

REFERENCES

****

Brown L. J. (1997) An evaluation of some marking and trapping techniques currently used in the study of anuran population dynamics. *Journal of Herpetology* **31**: 410-419.

Burrowes P. A., Joglar R. L. and Green D. E. (2004) Potential causes for amphibian declines in Puerto Rico. *Herpetologica* **60**: 141-154.

Cabanac A. J. and Cabanac M. (2000) Heart rate response to gentle handling of frog and lizard. *Behavioral Processes* **52**: 89-95.

Cabanac A. J. and Cabanac M. (2004) No emotional fever in toads. *Journal of Thermal Biology* **29**: 669-673.

Caldow G., Gunn G., Crawshaw M., Rusbridge M. and CMcDiarmid J. (2004) Can test and cull be a part of Johne's disease control? *Cattle Practice* **12**: 249-253.

Carruthers R. I., Larkin T. S., Firstencel H. and Feng Z. D. (1992) Influence of thermal ecology on the mycosis of a rangeland grasshopper. *Ecology* **73**: 190-204.

Chan D. I., Prenner E. J. and Vogel H. J. (2006) Tryptophan- and arginine-rich antimicrobial peptides: structures and mechanisms of action. *Biochimica Et Biophysica Acta-Biomembranes* **1758**: 1184-1202.

Cheatwood J. L., Jacobson E. R., May P. G., Farrell T. M., Homer B. L., Samuelson D. A. and Kimbrough J. W. (2003) An outbreak of fungal dermatitis and stomatitis in a free-ranging population of pigmy rattlesnakes (*Sistrurus miliarius barbouri*) in Florida. *Journal of Wildlife Diseases* **39**: 329-337.

Chen L., Yuhong L., Jianxu L., Xueqing X., Ren L. and Quanming Z. (2007) An antimicrobial peptide with antimicrobial activity against *Helicobacter pylori*. *Peptides* **28**: 1527-1531.

Chen T., Zhou M., Rao P., Walker B. and Shaw C. (2006) The Chinese bamboo leaf odorous frog (*Rana* (*Odorrana*) *versabilis*) and north American rana frogs share the same families of skin antimicrobial peptides. *Peptides* **27**: 1738-1744.

Chia B. C. S., Carver J. A., Mulhern T. D. and Bowie J. H. (1999) The solution structure of uperin 3.6, an antibiotic peptide from the granular dorsal glands of the Australian toadlet, *Uperoleia mjobergii*. *Journal of Peptide Research* **54**: 137-145.

Chia B. C. S., Carver J. A., Mulhern T. D. and Bowie J. H. (2000) Maculatin 1.1, an anti-microbial peptide from the Australian tree frog, *Litoria genimaculata.* Solution structure and biological activity. *European Journal of Biochemistry* **267**: 1894-1908.

REFERENCES

****

Clarke R. D. (1972) The effect of toe clipping on survival in Fowler's toad (*Bufo woodhousei fowleri*). *Copeia* **1972**: 182-185.

Cleaveland S., Hess G. R., Dobson A. P., Laurenson M. K., McCallum H. I., Roberts M. G. and Woodroffe R. (2001) The role of pathogens in biological conservation. In: *The ecology of wildlife diseases* (eds. P. J. Hudson, A. Rizzoli, B. T. Grenfell, H. Heesterbeek & A. P. Dobson) pp. 139-150. Oxford University Press, Oxford, England.

Cogger H. G. (2000) *Reptiles and Amphibians of Australia*. Reed New Holland, Sydney, Australia.

Colizza V., Barrat A., Marthélemy M. and Vespigmani A. (2006) The modeling of global epidemics: stochastic dynamics and predictability. *Bulletin of Mathematical Biology* **68**: 1893-1921.

Csordas A. and Michl H. (1970) Isolierung und strukturaufklärung eines hämolytisch wirkenden polypeptides aus dem abwehrsekret europäischer unken. *Monatshefte für Chemie* **101**: 182-189.

Cunningham A. A. (2007) A walk on the wild side - emerging wildlife diseases. *British Medical Journal* **331**: 1214-1215.

Daszak P. (2006) Emerging infectious diseases and the socia-ecological dimension. *EcoHealth* **2**: 239-240.

Daszak P., Berger L., Cunningham A. A., Hyatt A. D., Green D. E. and Speare R. (1999) Emerging infectious diseases and amphibian population declines. *Emerging Infectious Diseases* **5**: 735-748.

Daszak P. and Cunningham A. A. (1999) Extinction by infection. *Trends in Ecology & Evolution* **14**: 279-279.

Daszak P., Cunningham A. A. and Hyatt A. D. (2001) Anthropogenic environmental change and the emergence of infectious diseases in wildlife. *Acta Tropica* **78**: 103-116.

Daszak P., Cunningham A. A. and Hyatt A. D. (2003) Infectious disease and amphibian population declines. *Diversity and Distributions* **9**: 141-150.

REFERENCES

****

Daszak P., Scott D. E., Kilpatrick A. M., Faggioni C., Gibbons J. W. and Porter D. (2005) Amphibian population declines at savannah river site are linked to climate, not chytridiomycosis. *Ecology* **86**: 3232-3237.

Davidson C. and Knapp R. A. (2007) Multiple stressors and amphibian declines: dual impacts of pesticides and fish on yellow-legged frogs. *Ecological Applications* **17**: 587-597.

Davidson H. W., Parris M., Copllins J. P., Longcore J. E., Pessier A. P. and Brunner J. (2003) Pathogenicity and transmission of chytridiomycosis in tiger salamanders (A*mbystoma tigrinum*). *Copeia* **3**: 601-607.

de Castro F. and Bolker B. (2005) Mechanisms of disease-induced extinction. *Ecology Letters* **8**: 117-126.

Delajara J. J. (1991) Promotion of health and prevention of disease - new orientations. *Revista Medica De Chile* **119**: 205-213.

Denton J. S. and Beebee T. J. C. (1993) Reproductive strategies in a female-biased population of natterjack toads, *Bufo calamita*. *Animal Behaviour* **46**: 1169-1175.

do Amaral J. P. S., Marvin G. A. and Hutchison V. H. (2002) The influence of bacterial lipopolysaccharide on the thermoregulation of the box turtle *Terrapene carolina*. *Physiological and Biochemical Zoology* **75**: 273-282.

Dobson A. and Foufopoulos J. (2001) Emerging infectious pathogens of wildlife. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* **356**: 1001-1012.

Donnellan S. C. and Mahony M. J. (2004) Allozyme, chromosomal and morphological variability in the *Litoria lesueuri* species group (Anura : Hylidae), including a description of a new species. *Australian Journal of Zoology* **52**: 1-28.

Doody J. S. (1995) A photographic mark-recapture method for patterned amphibians. *Herpetological Review* **26**: 19-21.

Drake D. L., Altig R., Grace J. B. and Walls S. C. (2007) Occurrence of oral deformities in larval anurans. *Copeia*: 449-458.

Drew A., Allen E. J. and Allen L. J. S. (2006) Analysis of climatic and geographic factors affecting the presence of chytridiomycosis in Australia. *Diseases of Aquatic Organisms* **68**: 245-250.

REFERENCES

****

Duchamp-Viret P., Palouzier-Paulignan B. and Duchamp A. (1996) Odor coding properties of frog olfactory cortical neurons. *Neuroscience* **74**: 885-895.

Duda T. F. J., Vanhoye D. and Nicolas P. (2002) Roles of diversifying selection and coordinated evolution in the evolution of amphibian antimicrobial peptides. *Molecular Biology and Evolution* **19**: 858-864.

Duellmann W. E. and Trueb L. (1994) *Biology of Amphibians*. The John Hopkins University Press, Baltimore.

Edgerton B. F., Henttonen P., Jussila J., Mannonen A., Paasonen P., Taugbol T., Edsman L. and Souty-Grosset C. (2004) Understanding the causes of disease in European freshwater crayfish. *Conservation Biology* **18**: 1466-1474.

Egan R. S. and Paton P. W. C. (2004) within-pond parameters affecting oviposition by wood frogs and spotted salamanders. *Wetlands* **24**: 1-13.

Elliot S. L., Blanford S. and Thomas M. B. (2002) Host-pathogen interactions in a varying environment: temperature, behavioural fever and fitness. *Proceedings of the Royal Society of London Series B-Biological Sciences* **269**: 1599-1607.

Fellers G. M. and Drost C. A. (1993) Disappearance of the cascades frog *Rana cascadae* at the southern end of its range, California, USA. *Biological Conservation* **65**: 177-181.

Fellers G. M., Green D. E. and Longcore J. E. (2001) Oral chytridiomycosis in the mountain yellow-legged frog (*Rana muscosa*). *Copeia* **2001**: 945-953.

Fisher M. C. and Garner T. W. J. (2007) The relationship between the emergence of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduced amphibian species. *British Mycological Society* **21**: 2-9.

Freidenburg L. K. and Skelly D. K. (2004) Microgeographical variation in thermal preference by an amphibian. *Ecology Letters* **7**: 369-373.

Frith C. and Frith D. (1991) *Australian tropical reptiles and frogs*. Frith and Frit Books, Malanda, Australia.

Frost-Mason S., Morrison R. and Mason K. (1994) Pigmentation. In: *Amphibian Biology. The Integument, Volume 1. Pp. 64-97* (ed. H. Heatwole (ed.)). Surrey Beatty & Sons, Chipping Norton, N.S.W, Australia.

REFERENCES

****

Frost D. R., Grant T., Faivovich J., Bain R. H., Haas A., Haddad C. F. B., De Sa R. O., Channing A., Wilkinson M., Donnellan S. C., Raxworthy C. J., Campbell J. A., Blotto B. L., Moler P., Drewes R. C., Nussbaum R. A., Lynch J. D., Green D. M. and Wheeler W. C. (2006) The amphibian tree of life. *Bulletin of the American Museum of Natural History*: 8-+.

Fuchs K., Deutz A. and Gressmann G. (2000) Detection of space–time clusters and epidemiological examinations of scabies in chamois. *Veterinary Parasitology* **92**: 63-73.

Funk W. C., Donnelly M. A. and Lips K. R. (2005) Alternative views of amphibian toe-clipping. *Nature* **433**: 193-193.

Gabre-Madhin E. Z. and Haggblade S. (2004) Successes in African agriculture: results of an expert survey. *World Development* **32**: 745-766.

Garcia T. S., Romansic J. M. and Blaustein A. R. (2006) Survival of three species of anuran metamorphs exposed to UV-B radiation and the pathogenic fungus *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms* **72**: 163-169.

Garner T. W. J., Walker S., Bosch J., Hyatt A. D., Cunningham A. A. and Fisher M. C. (2005) Chytrid fungus in Europe. *Emerging Infectious Diseases* **11**: 1639-1641.

Gewin V. (2008) Riders of a modern-day ark. *Plos Biology* **6**: e24.

Gherardi F. (2006) Crayfish invading Europe: the case study of *Procambarus clarkii*. *Marine and Freshwater Behaviour and Physiology* **39**: 175-191.

Gilbert K. A. (1997) Red howling monkey use of specific defecation sites as a parasite avoidance strategy. *Animal Behaviour* **54**: 451-455.

Glushakova A. M. and Chernov I. Y. (2007) Seasonal dynamic of the numbers of epiphytic yeasts. *Microbiology* **76**: 590-595.

Green D. E., Converse K. A. and Schrader A. K. (2002) Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996-2001. *Annals of the New York Academy of Sciences* **969**: 323-339.

Gubbins S. and Gilligan C. (1997) Persistence of host-parasite interactions in a disturbed environment. *Journal of Theoretical Biology* **188**: 241-258.

REFERENCES

****

Hagstrom T. (1973) Identification of newt specimens (Irodela, Triturus) by recording the belly pattern and a description of photographic equipment for such registrations. *British Journal of Herpetology* **4**: 321-326.

Hale S., Rosen P., Jarchow J. and Bradley G. (2005) Effects of the chytrid fungus on the tarahumara frog (*Rana tarahumarae*) in Arizona and Sonora, Mexico. *USDA Forest Service Proceedings RMRS-P-36*.

Hale W. G., Margham J. P. and Saunders V. A. (1995) *Collins Dictionary of Biology*. Harper Collins Publishers, Glasgow.

Halliday T. (1995) More on toe-clipping. *Froglog* **12**: 3.

Hawkins C. E., Baars C., Hesterman H., Hocking G. J., Jones M. E., Lazenby B., Mann D., Mooney N., Pemberton D., Pyecroft S., Restani M. and Wiersma J. (2006) Emerging disease and population decline of an island endemic, the Tasmanian devil *Sarcophilus harrisii*. *Biological Conservation* **131**: 307-324.

Hayes M. P. and Jennings M. R. (1986) Decline of ranid frog species in western north America: are bullfrogs (*Rana catesbeiana*) responsible? *Journal of Herpetology* **20**: 490-509.

Hays J. B., Blaustein A. R., Kiesecker J. M., Hoffman P. D., Pandelova I., Coyle D. and Richardson T. (1996) Developmental responses of amphibians to solar and artificial UVB sources: a comparative study. *Photochemistry and Photobiology* **64**: 449-456.

Healy W. R. (1975) Terrestrial activity and home range in efts of *Notophthalmus viridescens*. *American Midland Naturalist* **1975**: 131-138.

Hecnar S. J. and McLoskey R. T. (1997) The effects of predatory fish on amphibian species richness and distribution. *Biological Conservation* **79**: 123-131.

Heyer W. R., Rand A. S., Cruz C. A. G. d. and Peixoto O. L. (1988) Decimations, extinctions, and colonizations of frog populations in southeast Brazil and their evolutionary implications. *Biotropica* **20**: 230-235.

Hillyard S. D. (1999) Behavioral, molecular and integrative mechanisms of amphibian osmoregulation. *Journal of Experimental Zoology* **283**: 662-674.

Hoffman E. A. and Blouin M. S. (2000) A review of colour and pattern polymorphisms in anurans. *Biological Journal of the Linnean Society* **70**: 633–665.

****

Hoffman J. A., Kafatos F. C., Janeway C. A. and Ezekowitz R. A. B. (1999) Phylogenetic perspectives in innate immunity. *Science* **284**: 1313-1318.

Hoskin C. J. (2007) Description, biology and conservation of a new species of Australian tree frog (Amphibia : Anura : Hylidae : *Litoria*) and an assessment of the remaining populations of *Litoria genimaculata* Horst, 1883: systematic and conservation implications of an unusual speciation event. *Biological Journal of the Linnean Society* **91**: 549-563.

Hosseini P. R., Dhondt A. A. and Dobson A. (2004) Seasonality and wildlife disease: how seasonal birth, aggregation and variation in immunity affect the dynamics of *Mycoplasma gallisepticum* in house finches. *Proceedings of the Royal Society of London Series B-Biological Sciences* **271**: 2569-2577.

Huchzermeyer F. W., Gerdes G. H., Foggin C. M., Huchzermeyer K. D. A. and Limper L. C. (1994) Hepatitis in farmed hatchling nile crocodiles (*Crocodylus-niloticus*) due to chlamydial infection. *Journal of the South African Veterinary Association* **65**: 20-22.

Huey R. B. and Berrigan D. (2001) Temperature, demography, and ectotherm fitness. *American Naturalist* **158**: 204-210.

Huey R. B. and Kingsolver J. G. (1989) Evolution of thermal sensitivity of ectotherm performance. *Trends in Ecology & Evolution* **4**: 131-135.

Huey R. B. and Stevenson R. D. (1979) Intergrating thermal physiology and ecology of ectotherms: a discussion of approaches. *American Zoologist* **19**: 357-366.

Hutchings M. R., Gordon I. J., Kyriazakis I. and Jackson F. (2001a) Sheep avoidance of faeces-contaminated patches leads to a trade-off between intake rate of forage and parasitism in subsequent foraging decisions. *Animal Behaviour* **62**: 955-964.

Hutchings M. R., Kyriazakis I., Anderson D. H., Gordon I. J. and Coop R. L. (1998) Behavioural strategies used by parasitised and non-parasitised sheep to avoid ingestion of gastro-intestinal nematodes associated with faeces. *Animal Science* **67**: 97-107.

Hutchings M. R., Kyriazakis I. and Gordon I. J. (2001b) Herbivore physiological state affects foraging trade-off decisions between nutrient intake and parasite avoidance. *Ecology* **82**: 1138-1150.

REFERENCES

****

Hyatt A. D., Boyle D. G., Olsen V., Boyle D. B., Berger L., Obendorf D., Dalton A., Kriger K., Hero M., Hines H., Phillott R., Campbell R., Marantelli G., Gleason F. and Colling A. (2007) Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms* **73**: 175/192.

Ibelings B. W., De Bruin A., Kagami M., Rijkeboer M., Brehm M. and van Donk E. (2004) Host parasite interactions between freshwater phytoplankton and chytrid fungi (Chytridiomycota). *Journal of Phycology* **40**: 437-453.

Ingram G. J. and McDonald K. R. (1993) An update on the decline of Queensland's frogs. In: *Herpetology in Australia* (eds. D. Lunney & D. Ayers). Royal Zoological Society of New South Wales, Mosman.

IUCN (2006) Numbers of threatened species by major groups of organisms (1996–2006). International Union for Conservation of Nature and Natural Resources. http://www.iucnredlist.org/info/stats.

Jorgensen C. B. (2000) Amphibian respiration and olfaction and their relationships: from Robert Townson (1794) to the present. *Biological Reviews* **75**: 297-345.

Kim J. B., Iwamuro S., Knoop F. C. and Conlon J. M. (2001) Antimicrobial peptides from the skin of the Japanese mountain brown frog, *Rana ornativentris*. *Journal of Peptide Research* **58**: 349-356.

Kirshtein J. D., Anderson C. W., Wood J. S., Longcore J. E. and Voytek M. A. (2007) Quantitative PCR detection of *Batrachochytrium dendrobatidis* DNA from sediments and water. *Diseases of Aquatic Organisms* **77**: 11-15.

Kriger K. M., Ashton K. J., Hines H. B. and Hero J. M. (2007a) On the biological relevance of a single *Batrachochytrium dentrobatidis* zoospore: a reply to Smith (2007). *Diseases of Aquatic Organisms* **73**: 257-260.

Kriger K. M. and Herman J. R. (2006) Survivorship in wild frogs infected with chytridiomycosis. *EcoHealth* **3**: 171-177.

Kriger K. M. and Hero J. M. (2007a) The chytrid fungus *Batrachochytrium dendrobatidis* is non-randomly distributed across amphibian breeding habitats. *Diversity and Distributions* **13** (6): 781-788

Kriger K. M. and Hero J. M. (2007b) Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. *Journal of Zoology* **271**: 352-359.

REFERENCES

****

Kriger K. M., Pereoglou F. and Hero J. M. (2007b) Latitudinal variation in the prevalence and intensity of chytrid (*Batrachochytrium dendrobatidis*) infection in Eastern Australia. *Conservation Biology* **21**: 1280-1290.

La Marca E., Lips K. R., Lotters S., Puschendorf R., Ibanez R., Rueda-Almonacid J. V., Schulte R., Marty C., Castro F., Manzanilla-Puppo J., Garcia-Perez J. E., Bolanos F., Chaves G., Pounds J. A., Toral E. and Young B. E. (2005) Catastrophic population declines and extinctions in neotropical harlequin frogs (Bufonidae : Atelopus). *Biotropica* **37**: 190-201.

Lefcort H. and Blaustein A. R. (1995) Disease, predator avoidance, and vulnerability to predation in tadpoles. *Oikos* **74**: 469-474.

Lillywhite H. B., Mittal A. K., Garg T. K. and Das I. (1998) Basking behavior, sweating and thermal ecology of the Indian tree frog, *Polypedates maculatus*. *Journal of Herpetology* **32**: 169-175.

Lips K. R. (1998) Decline of a tropical montane amphibian fauna. *Conservation Biology* **12**: 106-117.

Lips K. R., Brem f., Brenes R., Reeve J. D., Alford R. A., Voyles J., Carey C., Livo L., Pessier A. P. and Collins J. P. (2006) Emerging infectious disease and the loss of biodiversity in a neotropical amphibian community. *PNAS* **103**: 3165-3170.

Lips K. R., Burrowes P. A., Mendelson J. R. and Parra-Olea G. (2005a) Amphibian declines in Latin America: widespread population declines, extinctions, and impacts. *Biotropica* **37**: 163-165.

Lips K. R., Burrowes P. A., Mendelson J. R. and Parra-Olea G. (2005b) Amphibian population declines in Latin America: a synthesis. *Biotropica* **37**: 222-226.

Lips K. R., Reeve J. D. and Witters L. R. (2003) Ecological traits predicting amphibian population declines in Central America. *Conservation Biology* **17**: 1078-1088.

Longcore J. E., Pessier A. P. and Nichols D. K. (1999) *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia* **91**: 219-227.

Lozano G. A. (1991) Optimal foraging theory - a possible role for parasites. *Oikos* **60**: 391-395.

REFERENCES

****

Mariner J. C., Jeggo M. H. and van't Klooster G. G. M. (2003) Rinderpest surveillance performance monitoring using quantifiable indicators. *Revue Scientifique Et Technique De L Office International Des Epizooties* **22**: 837-847.

Martinez-Solano I., Bosch J. and Garcia-Paris M. (2003) Demographic trends and community stability in a montane amphibian assemblage. *Conservation Biology* **17**: 238-244.

Matsuo S., Sugiyama T., Okuyama T., Yoshikawa K., Honda K., Takahashi R. and Maeda S. (1999) Preservation of pathological tissue specimens by freeze-drying for immunohistochemical staining and various molecular biological analyses. *Pathology International* **49**: 383-390.

Mazzoni R., Cunningham A. A., Daszak P., Apolo A., Perdomo E. and Speranza G. (2003) Emerging pathogen of wild amphibians in frogs (*Rana catesbeiana*) farmed for international trade. *Emerging Infectious Diseases* **9**: 995-998.

McCallum H. and Jones M. (2006) To lose both would look like carelessness: Tasmanian devil facial tumour disease. *Plos Biology* **4**: 1671-1674.

McClanahan L. J. and Baldwin R. (1969) Rate of water uptake through the integument of the desert toad, *Bufo punctatus*. *Comparative Biochemistry and Physiology* **28**: 381-389.

McDonald K. and Alford R. (1999) A review of declining frogs in northern Queensland. In: *Declines and Disappearances of Australian Frogs* pp. 14-22. Environment Australia, Canberra.

McDonald K. R., Mendez D., Mueller R., Freeman A. B. and Speare R. (2005) Decline in the prevalence of chytridiomycosis in frog populations in North Queensland, Australia. *Pacific Conservation Biology* **11**: 114-120.

Meyer A. H., Schmidt B. R. and Grossenbacher K. (1998) Analysis of three amphibian populations with quarter-century long time-series. *Proceedings of the Royal Society of London Series B-Biological Sciences* **265**: 523-528.

Miller M. W., Hobbs N. T. and Tavener S. J. (2006) Dynamics of prion disease transmission in mule deer. *Ecological Applications* **16**: 2208-2214.

****

Morehouse E. A., James T. Y., Ganley A. R. D., Vilgalys R., Berger L., Murphy P. J. and Longcore J. E. (2003) Multilocus sequence typing suggests the chytrid pathogen of amphibians is a recently emerged clone. *Molecular Ecology* **12**: 395-403.

Morens D. M., Folkers G. K. and Fauci A. S. (2004) The challenge of emerging and re-emerging infectious diseases. *Nature* **430**: 242-249.

Morgan J. A. T., Vredenburg V. T., Rachowicz L. J., Knapp R. A., Stice M. J., Tunstall T., Bingham R. E., Parker J. M., Longcore J. E., Moritz C., Briggs C. J. and Tayler J. W. (2007) Population genetics of the frog-killing fungus *Batrachochytrium dendrobatidis*. *PNAS* **104**: 13845-13850.

Morse S. S. (1995) Factors in the emergence of infectious Diseases. *Emerging Infectious Diseases* **1**: Perspective.

Muths E., Corn P. S., Pessier A. P. and Green D. E. (2003) Evidence for disease-related amphibian decline in Colorado. *Biological Conservation* **110**: 357-365.

Mutschmann F., Berger L., Zwart P. and Gaedicke C. (2000) Chytridiomycosis on amphibians - first report from Europe. *Berliner Und Munchener Tierarztliche Wochenschrift* **113**: 380-383.

Myhre K., Cabanac M. and MYhre G. (1977) Fever and behavioral temperature regulation in the frog *Rana esculenta*. *Acta Physiosology Scandinavia* **101**: 219-229.

Nichols D. K., Lamirande E. W., Pessier A. P. and Longcore J. E. (2001) Experimental transmission of cutaneous chytridiomycosis in dendrobatid frogs. *Journal of Wildlife Diseases* **37**: 1-11.

Nicolas P. and Mor A. (1995) Peptides as weapons against microorganisms in the chemical defense system of vertebrates. *Annual Review of Microbiology* **49**: 277-304.

Nicolas P., Vanhoye D. and Amiche M. (2003) Molecular strategies in biological evolution of antimicrobial peptides. *Peptides* **24**: 1669-1680.

Niidome T., Kobayashi K., Arakawa H., Hatakeyama T. and Aoyagi H. (2004) Structure-activity relationship of an antibacterial peptide, maculatin 1.1, from the skin glands of the tree frog, *Litoria genimaculata*. *Journal of Peptide Science* **10**: 414-422.

Oner A. F., Bay A., Arslan S., Akdeniz H., Sahin H. A., Cesur Y., Epcacan S., Yilmaz N., Deger I., Kizilyildiz B., Karsen H. and Ceyhan M. (2006) Avian influenza A

****

(H5N1) infection in eastern Turkey in 2006. *New England Journal of Medicine* **355**: 2179-2185.

Ouellet M., Mikaelian I., Pauli B. D., Rodrigue J. and Green D. M. (2005) Historical evidence of widespread chytrid infection in North American amphibian populations. *Conservation Biology* **19**: 1431-1440.

Papagianni M. (2003) Ribosomally synthesized peptides with antimicrobial properties: biosynthesis, structure, function, and applications. *Biotechnology Adv.* **21**: 465-499.

Parker J. M., Mikaelian I., Hahn N. and Diggs H. E. (2002) Clinical diagnosis and treatment of epidermal chytridiomycosis in African clawed frogs (*Xenopus tropicalis*). *Comparative Medicine* **52**: 265-268.

Parris M. J. (2004) Hybrid response to pathogen infection in interspecific crosses between two amphibian species (Anura: Ranidae). *Evolutionary Ecology Research* **6**: 457-471.

Paulus B. C., Kanowski J., Gadek P. A. and Hyde K. D. (2006) Diversity and distribution of saprobic microfungi in leaf litter of an Australian tropical rainforest. *Mycological Research* **110**: 1441-1454.

Pechmann J. H. K., Scott D. E., Semlitsch R. D., Caldwell J. P., Vitt L. J. and Gibbons J. W. (1991) Declining amphibian populations: The problem of separating human impacts from natural fluctuations. *Science* **253**: 892-896.

Pessier A. P. (2002) An overview of amphibian skin disease. *Seminars in Avian and Exotic Pet Medicine* **11**: 162-174.

Phillott A. D., Skerratt L. F., McDonald K., Lemckert F. L., Hines H. B., Clark J. M., Alford R. A. and Speare R. (2007) Toe-clipping as an acceptable method of identifying individual anurans in mark recapture studies. *Herpetological Review* **38**: 305-308.

Pounds J. A., Bustamante M. R., Coloma L. A., Consuegra J. A., Fogden M. P. L., Foster P. N., La Marca E., Masters K. L., Merino-Viteri A., Puschendorf R., Ron S. R., Sanchez-Azofeifa G. A., Still C. J. and Young B. E. (2006) Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature* **439**: 161-167.

Rachowicz L. J., Hero J., Alford R. A., Taylor J. W., Morgan J. A. T., Vredenburg V. T., Collins J. P. and Briggs C. J. (2005) The novel and endemic pathogen hypotheses:

****

Competing explanations for the origin of emerging infectious diseases of wildlife. *Conservation Biology* **19**: 1441-1448.

Rachowicz L. J., Knapp R. A., Morgan J. A. T., Stice M. J., Vredenburg V. T., Parker J. M. and Briggs C. J. (2006) Emerging infectious disease as a proximate cause of amphibian mass mortality. *Ecology* **87**: 1671-1683.

Rachowicz L. J. and Vredenburg V. T. (2004) Transmission of *Batrachochytrium dendrobatidis* within and between amphibian life stages. *Diseases of Aquatic Organisms* **61**: 75-83.

Reading C. J. (2007) Linking global warming to amphibian declines through its effects on female body condition and survivorship. *Oecologia* **151**: 125-131.

Reaser J. (1995) Marking amphibians by toe-clipping: a response to Halliday. *Froglog* **12**: 2.

Resetarits W. J. and Wilbur H. M. (1989) Choice of Oviposition Site by Hyla-Chrysoscelis - Role of Predators and Competitors. *Ecology* **70**: 220-228.

Retallick R. W., McCallum H. and Spear R. (2004) Endemic infection of the amphibian chytrid fungus in a frog community post-decline. *Plos Biology* **2**: 1965-1971.

Richards S. J. and Alford R. A. (2005) Structure and dynamics of a rainforest frog (*Litoria genimaculata*) population in northern Queensland. *Australian Journal of Zoology* **53**: 229-236.

Richards S. J., McDonald K. R. and Alford R. A. (1993) Declines in populations of Australia's endemic tropical rainforest frogs. *Pacific Conservation Biology* **1**: 66-77.

Rieger J. F., Binckley C. A. and Resetarits W. J. (2004) Larval performance and oviposition site preference along a predation gradient. *Ecology* **85**: 2094-2099.

Rolff J. and Siva-Jothy M. T. (2003) Invertebrate ecological immunology. *Science* **301**: 472-475.

Rollins-Smith L. A. (2001) Neuroendocrine-immune system interactions in amphibians - implications for understanding global amphibian declines. *Immunologic Research* **23**: 273-280.

Rollins-Smith L. A., Carey C., Conlon J. M., Reinert L. K., Doersam J. K., Bergman T., Silberring J., Lankinen H. and Wade D. (2003) Activities of temporin family peptides

****
against the chytrid fungus (*Batrachochytrium dendrobatidis*) associated with global amphibian declines. *Antimicrobial Agents and Chemotherapy* **47**: 1157-1160.

Rollins-Smith L. A., Carey C., Longcore J., Doersam J. K., Boutte A., Bruzgal J. E. and Conlon J. M. (2002a) Activity of antimicrobial skin peptides from ranid frogs against *Batrachochytrium dendrobatidis*, the chytrid fungus associated with global amphibian declines. *Developmental and Comparative Immunology* **26**: 471-479.

Rollins-Smith L. A. and Conlon J. M. (2005) Antimicrobial peptide defenses against chytridiomycosis, an emerging infectious disease of amphibian populations. *Developmental and Comparative Immunology* **29**: 589-598.

Rollins-Smith L. A., Reinert L. K., Miera V. and Conlon J. M. (2002b) Antimicrobial peptide defenses of the Tarahumara frog, *Rana tarahumarae*. *Biochemical and Biophysical Research Communications* **297**: 361-367.

Rollins-Smith L. A., Reinert L. K., O'Leary C. J., Houston L. E. and Woodhams D. C. (2005) Antimicrobial peptide defenses in amphibian skin. *Integrative and Comparative Biology* **45**: 137-142.

Rollins-Smith L. A., Woodhams D. C., Reinert L. K., Vredenburg V. T., Briggs C. J., Nielsen P. F. and Conlon J. M. (2006) Antimicrobial peptide defenses of the mountain yellow-legged frog (*Rana muscosa*). *Developmental and Comparative Immunology* **30**: 831-842.

Ron S. R. and Merino A. (2000) Amphibian declines in Ecuador: Overview and first report of chytridiomycosis from South America. *Froglog* **42**.

Rossi L., Fraquelli C., Vesco U., Permunian R., Sommavilla G. M., Carmignola G., Da Pozzo R. and Meneguz P. G. (2007) Descriptive epidemiology of a scabies epidemic in chamois in the Dolomite Alps, Italy. *European Journal of Wildlife Research* **53**: 131-141.

Rossifer P. B., Taylor W. P. and Bwangamoi B. (1987) Continuing presence of rinderpest virus as a threat in East-Africa, 1983-1985. *Veterinary Record* **120**: 59-62.

Rowley J. J. L. (2007) Why does chytridiomycosis drive some frog populations to extinction and not others? The effects of interspecific variation in host behaviour. In: *Marine and Tropical Biology Department*. James Cook University, Townsville.

REFERENCES

****

Rowley J. J. L. and Alford R. A. (2007a) Behaviour of Australian rainforest stream frogs may affect the transmission of chytridiomycosis. *Diseases of Aquatic Organisms* **77**: 1-9.

Rowley J. J. L. and Alford R. A. (2007b) Movement patterns and habitat use of rainforest stream frogs in northern Queensland, Australia: implications for extinction vulnerability. *Wildlife Research* **34**: 371-378.

Rowley J. J. L., Skerratt L. F., Alford R. A. and Campbell R. (2007) Retreat sites of rain forest stream frogs are not a reservoir for *Batrachochytrium dendrobatidis* in northern Queensland, Australia. *Diseases of Aquatic Organisms* **74**: 7-12.

Rozek T., Bowie J. H., Wallace J. C. and Tyler M. J. (2000) The antibiotic and anticancer active aurein peptides from the Australian Bell Frogs *Litoria aurea* and *Litoria raniformis*. Part 2. Sequence determination using electrospray mass spectrometry. *Rapid Communications in Mass Spectrometry* **14**: 2002-2011.

Rozek T., Waugh R. J., Steinborner S. T., Bowie J. H., Tyler M. J. and Wallace J. C. (1998) The maculatin peptides from the skin glands of the tree frog *Litoria genimaculata*: A comparison of the structures and antibacterial activities of maculatin 1.1 and caerin 1.1. *Journal of Peptide Science* **4**: 111-115.

Sanchez-Bayo F. and Goka K. (2007) Simplified models to analyse time- and dose-dependent responses of populations to toxicants. *Ecotoxicology* **16**: 511-523.

Schlaepfer M. A. (1998) Use of a fluorescent marking technique on small terrestrial anurans. *Herpetological Review* **29**: 25-26.

Seebacher F. and Alford R. A. (2002) Shelter microhabitats determine body temperature and dehydration rates of a terrestrial amphibian (*Bufo marinus*). *Journal of Herpetology* **36**: 69-75.

Simmaco M., Mignogna G. and Barra D. (1998) Antimicrobial peptides from amphibian skin: what do they tell us? *Peptide Science* **47**: 435-450.

Simoncelli F., Fragotti A., Dall'Olio R., Vagnetti D., Pascolini R. and Di Rosa I. (2005) Evidence of *Batrachochytrium dendrobatidis* infection in water frogs of the *Rana esculenta* complex in Central Italy. *EcoHealth* **2**: 307-312.

****

Singh J. S. and Kashyap A. K. (2007) Contrasting pattern of methanotrophs in dry tropical forest soils: Effect of soil nitrogen, carbon and moisture. *Microbiological Research* **162**: 276-283.

Skerratt L. F., Berger L., Speare R., Cashins S., McDonald K. R., Phillott A. D., Hines H. B. and Kenyon N. (2007) Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth* **4**: 125-134.

Smith K. F., Sax D. F. and Lafferty K. D. (2006) Evidence for the role of infectious disease in species extinction and endangerment. *Conservation Biology* **20**: 1349-1357.

Smith K. G. (2007) Use of quantitative PCR assay for amphibian chytrid detection: comment on Kriger et al. (2006a,b). *Diseases of Aquatic Organisms* **73**: 253-255.

Sokal R. R. and Rohlf F. J. (1995) *Biometry: the principles and practice of statistics in biological research.* W.H. Freeman and Co., New York.

Sparling D. W., Fellers G. M. and McConnell L. L. (2001) Pesticides and amphibian population declines in California, USA. *Environmental Toxicology and Chemistry* **20**: 1591-1595.

Speare R. and Berger L. (2005) Chytridiomycosis in amphibians in Australia. http://www.jcu.edu.au/school/phtm/PHTM/frogs/chyspec.htm.

Stallard R. F. (2001) Possible environmental factors underlying amphibian decline in eastern Puerto Rico: Analysis of US government data archives. *Conservation Biology* **15**: 943-953.

Stephenson E. M. and Stephenson N. G. (1957) Field observations on the New Zealand frog *Leiopelma* Fitzinger. *transactions of the Royal Society of New Zealand* **84**: 867-882.

Stolley P. and Lasky T. (1995) *Investigating disease patterns. The science of epidemiology*. Scientific American Library, New York.

Tattersall G. J., Eterovick P. C. and de Andrade D. V. (2006) Tribute to R.G. Boutilier: Skin colour and body temperature changes in basking *Bokermannohyla alvarengai* (Bokermann 1956). *Journal of Experimental Biology* **209**: 1185-1196.

Tennessen J. A. (2005) Molecular evolution of animal antimicrobial peptides: widespread moderate positive selection. *Journal of Evolutionary Biology* **18**: 1387/1394.

****

Tennessen J. A. and Blouin M. S. (2007) Selection for antimicrobial peptide diversity in frogs leads to gene duplication and low allelic variation. *Journal of Molecular Evolution* **65**: 605-615.

Tompkins D. M., White A. R. and Boots M. (2003) Ecological replacement of native red squirrels by invasive greys driven by disease. *Ecology Letters* **6**: 189-196.

van Riper C., van Riper S. G., Goff M. L. and Laird M. (1986) The epizootiology and ecological significance of Malaria in Hawaiian land birds. *Ecological Monographs* **56**: 327-344.

Van Uitregt V. O., Wilson R. S. and Franklin C. E. (2007) Cooler temperatures increase sensitivity to ultraviolet B radiation in embryos and larvae of the frog *Limnodynastes peronii*. *Global Change Biology* **13**: 1114-1121.

Vanhoye D., Bruston F., Nicolas P. and Amiche M. (2003) Antimicrobial peptides from hylid and ranin frogs originated from a 150-million-year-old ancestral precursor with a conserved signal peptide but a hypermutable antimicrobial domain. *European Journal of Biochemistry* **270**: 2068-2081.

Voyles J., Berger L., Young S., Speare R., Webb R., Warner J., Rudd D., Campbell R. and Skerratt L. F. (2007) Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis. *Diseases of Aquatic Organisms* **77**: 113-118.

Waichman A. V. (1992) An alphanumeric code for toe-clipping amphibians and reptiles. *Herpetological Review* **23**: 19-21.

Wake D. B. (1991) Declining amphibian populations. *Science* **253**: 860.

Wake D. B. (2007) climate change implicated in amphibian and lizard declines. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 8201-8202.

Waldmann T. A. (2003) Immunotherapy: past, present and future. *Nature Medicine* **9**: 269-277.

Walker B. L., Naugle D. E., Doherty K. E. and Cornish T. E. (2004) From the field: outbreak of West Nile virus in greater sage-grouse and guidelines for monitoring, handling, and submitting dead birds. *Wildlife Society Bulletin* **32**: 1000-1006.

REFERENCES

****

Walker S. F., Baldi Salas M., Jenkins D., Garner T. W. J., Cunningham A. A., Hyatt A. D., Bosch J. and Fisher M. C. (2007) Environmental detection of *Batrachochytrium dendrobatidis* in a temperate climate. *Diseases of Aquatic Organisms* **77**: 105-112.

Warner R. E. (1968) The role of introduced diseases in the extinction of the endemic Hawaiian avifauna. *The Condor* **70**: 101-120.

Webb R., Mendez D., Berger L. and Speare R. (2007) Additional disinfectants effective against the amphibian chytrid fungus Batrachochytrium dendrobatidis. *Diseases of Aquatic Organisms* **74**: 13-16.

Weldon C., du Preez L. H., Hyatt A. D., Muller R. and Speare R. (2004) Origin of the amphibian chytrid fungus. *Emerging Infectious Diseases* **10**: 2100-2105.

Wente W. H. and Phillips J. B. (2005) Seasonal color change in a population of Pacific Treefrogs (*Pseudacris regilla*). *Journal of Herpetology* **39**: 161-165.

Whitfield S. M., Bell K. e., Phillippi T., Sasa M., Bolanos F., Chaves G., Savage J. M. and Donnelly M. A. (2007) Amphibian and reptile declines over 35 years at La Selva, Costa Rica. *PNAS* **104**: 8352-8356.

Wilcove D. S., Rothstein D., Dubow J., Phillips A. and Losos E. (1998) Quantifying threats to imperiled species in the United States. *Bioscience* **48**: 607-615.

Wolcott T. G. and Wolcott D. L. (2001) Role of behavior in meeting osmotic challenges. *American Zoologist* **41**: 795-805.

Woodhams D. C. (2003) The Ecology of Chytridiomycosis and the Emerging Infectious Disease of Australian Rainforest Frogs. PhD Thesis. James Cook University, Townsville, QLD, Australia.

Woodhams D. C. and Alford R. A. (2005) Ecology of chytridiomycosis in rainforest stream frog assemblages of tropical Queensland. *Conservation Biology* **19**: 1449-1459.

Woodhams D. C., Alford R. A., Briggs C. J., Johnson M. and Rollins-Smith L. A. (2008) Life-history trade-offs influence disease in changing climates: strategies of an amphibian pathogen. *Ecology* **89**: 1627-1639.

Woodhams D. C., Alford R. A. and Marantelli G. (2003) Emerging disease of amphibians cured by elevated body temperature. *Diseases of Aquatic Organisms* **55**: 65-67.

REFERENCES

****

Woodhams D. C., Ardipradja K., Alford R. A., Marantelli G., Reinert L. K. and Rollins-Smith L. A. (2007) Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. *Animal Conservation* **10**: 409-417.

Woodhams D. C., Rollins-Smith L. A., Carey C., Reinert L., Tyler M. J. and Alford R. A. (2005) Population trends associated with skin peptide defenses against chytridiomycosis in Australia. *Oecologia* **146**: 531-540.

Woodhams D. C., Voyles J., Lips K. R., Carey C. and Rollins-Smith L. A. (2006) Predicted disease susceptibility in a panamanian amphibian assemblage based on skin peptide defenses. *Journal of Wildlife Diseases* **42**: 207-218.

Woodroffe R. (1999) Managing disease threats to wild mammals. *Animal Conservation* **2**: 185-193.

World Health Organization (2004) The world health report 2004. World Health Organization (WHO), www.who.int/en/.

Zasloff M. (1987) Magainins, a class of antimicrobial peptides from *Xenopus* skin: Isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *PNAS* **84**: 5449-5453.

Zasloff M. (2002) Antimicrobial peptides of multicellular organisms. *Nature* **415**: 389-395.

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Appendix A. Wild amphibian species known to be infected with *Batrachochytrium dendrobatidis* as of 30 June 2008.

Order	Family *	Species *	Area	Reference
Anura	Bufonidae	*Bufo funereus*	Africa	Goldberg et al., 2007
Anura	Hyperoliidae	*Hyperolius kivuensis*	Africa	Goldberg et al., 2007
Anura	Hyperoliidae	*Leptopelis christyi*	Africa	Goldberg et al., 2007
Anura	Hyperoliidae	*Leptopelis kivuensis*	Africa	Goldberg et al., 2007
Anura	Pipidae	*Xenopus gilli*	Africa	Weldon et al., 2004
Anura	Pipidae	*Xenopus laevis*	Africa	Weldon et al., 2004
Anura	Pipidae	*Xenopus meulleri*	Africa	Weldon et al., 2004
Anura	Pipidae	*Xenopus wittei*	Africa	Goldberg et al., 2007
Anura	Ranidae	*Afrana fuscigula*	Africa	Lane et al., 2003
Anura	Ranidae	*Phrynobatrachus graueri*	Africa	Goldberg et al., 2007
Anura	Ranidae	*Ptychadena anchietae (Rana angolensis)*	Africa	Parker et al., 2002
Anura	Ranidae	*Ptychadena mascareniensis*	Africa	Goldberg et al., 2007
Anura	Bufonidae	*Rhinella (Chaunus, Bufo) marinus*	Australia	Berger, 2001
Anura	Hylidae	*Cyclorana platycephala*	Australia	Berger et al., 2004
Anura	Hylidae	*Litoria adelaidensis*	Australia	Berger et al., 1999
Anura	Hylidae	*Litoria aurea*	Australia	Berger et al., 2004
Anura	Hylidae	*Litoria barringtonensis*	Australia	Berger et al., 2004
Anura	Hylidae	*Litoria booroolongensis*	Australia	Berger, 2001
Anura	Hylidae	*Litoria burrowsae*	Australia	Pauza and Driessen, 2008
Anura	Hylidae	*Litoria caerulea*	Australia	Hunter, pers. comm.

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Anura	Hylidae	*Litoria chloris*	Australia	Berger et al., 1999
Anura	Hylidae	*Litoria ewingii*	Australia	Berger et al., 1999
Anura	Hylidae	*Litoria genimaculata*	Australia	McDonald et al., 2005
Anura	Hylidae	*Litoria gracilenta*	Australia	Mendez, pers. comm.
Anura	Hylidae	*Litoria infrafrenata*	Australia	Speare and Berger, 2005
Anura	Hylidae	*Litoria lesueuri*	Australia	Berger, 2001
Anura	Hylidae	*Litoria moorei*	Australia	Berger et al., 1999
Anura	Hylidae	*Litoria nannotis*	Australia	Berger et al., 1998
Anura	Hylidae	*Litoria pearsoniana*	Australia	Berger et al., 1999
Anura	Hylidae	*Litoria peronii*	Australia	Berger, 2001
Anura	Hylidae	*Litoria raniformis*	Australia	Berger, 2001
Anura	Hylidae	*Litoria rheocola*	Australia	Berger et al., 1998
Anura	Hylidae	*Litoria spenceri*	Australia	Berger et al., 1998
Anura	Hylidae	*Litoria verreauxii*	Australia	Berger et al., 2004
Anura	Hylidae	*Litoria wilcoxii*	Australia	Kriger and Hero, 2007
Anura	Hylidae	*Litoria wilcoxii* or *jungguy*	Australia	Berger et al., 1998
Anura	Hylidae	*Nyctimystes dayi*	Australia	McDonald et al., 2005
Anura	Microhylidae	*Cophixalus ornatus*	Australia	Kriger and Hero, 2006
Anura	Myobatrachidae	*Adelotus brevis*	Australia	Berger et al., 1999
Anura	Myobatrachidae	*Crinia georgiana*	Australia	Aplin, pers. comm.
Anura	Myobatrachidae	*Crinia glauerti*	Australia	Aplin, pers. comm.
Anura	Myobatrachidae	*Crinia insignifera*	Australia	Aplin, pers. comm.
Anura	Myobatrachidae	*Crinia pseudinsignifera*	Australia	Berger et al., 1999
Anura	Myobatrachidae	*Crinia signifera*	Australia	Pauza and Driessen, 2008

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Anura	Myobatrachidae	*Crinia subinsignifera*	Australia	Aplin, pers. comm.
Anura	Myobatrachidae	*Crinia tasmaniensis*	Australia	Pauza and Driessen, 2008
Anura	Myobatrachidae	*Geocrinia rosea*	Australia	Aplin, pers. comm.
Anura	Myobatrachidae	*Geocrinia vitellina*	Australia	Aplin, pers. comm.
Anura	Myobatrachidae	*Heleioporus australiacus*	Australia	Berger et al., 1999
Anura	Myobatrachidae	*Heleioporus barycragus*	Australia	Aplin, pers. comm.
Anura	Myobatrachidae	*Heleioporus eyrei*	Australia	Aplin, pers. comm.
Anura	Myobatrachidae	*Lechriodus fletcheri*	Australia	Berger et al., 2004
Anura	Myobatrachidae	*Limnodynastes fletcheri*	Australia	Berger et al., 1999
Anura	Myobatrachidae	*Limnodynastes dorsalis*	Australia	Berger et al., 1999
Anura	Myobatrachidae	*Limnodynastes dumerili*	Australia	Berger et al., 1998
Anura	Myobatrachidae	*Limnodynastes peronii*	Australia	Berger et al., 2004
Anura	Myobatrachidae	*Limnodynastes tasmaniensis*	Australia	Berger et al., 1998
Anura	Myobatrachidae	*Limnodynastes terraereginae*	Australia	Berger, 2001
Anura	Myobatrachidae	*Mixophyes carbinensis*	Australia	Bell, pers. comm.
Anura	Myobatrachidae	*Mixophyes fasciolatus*	Australia	Symonds et al., 2007
Anura	Myobatrachidae	*Mixophyes fleayi*	Australia	Symonds et al., 2007
Anura	Myobatrachidae	*Mixophyes iteratus*	Australia	Kriger et al., 2006
Anura	Myobatrachidae	*Mixophyes schevilli*	Australia	Bell, pers. comm.
Anura	Myobatrachidae	*Myobatrachus gouldii*	Australia	Aplin, pers. comm.
Anura	Myobatrachidae	*Neobatrachus pelobatoides*	Australia	Spear and Berger, 2005
Anura	Myobatrachidae	*Pseudophryne corroboree*	Australia	Spear and Berger, 2005
Anura	Myobatrachidae	*Pseudophryne pengilleyi*	Australia	Spear and Berger, 2005
Anura	Myobatrachidae	*Taudactylus acutirostris*	Australia	Berger et al., 1998

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Anura	Myobatrachidae	*Taudactylus eungellensis*	Australia	Berger et al., 1998
Anura	Alytidae (Discoglossidae)	*Alytes muletensis*	Europe	Fisher and Garner, 2007
Anura	Alytidae (Discoglossidae)	*Alytes obstetricans*	Europe	Bosch et al., 2001
Anura	Bombinatoridae	*Bombina pachypus*	Europe	Stagni et al., 2002
Anura	Bufonidae	*Bufo bufo*	Europe	Bosch and Martinez-Solano, 2006
Anura	Ranidae	*Lithobates (Rana) catesbeianus*	Europe	Garner et al., 2006
Anura	Ranidae	*Pelophylax lessonae*	Europe	Fisher and Garner, 2007
Anura	Ranidae	*Rana arvalis*	Europe	Mutschmann et al., 2000
Anura	Ranidae	*Rana esculenta*	Europe	Simoncelli et al., 2005
Caudata	Salamandridae	Mesotriton alpestris	Europe	Fisher and Garner, 2007
Caudata	Salamandridae	*Salamandra salamandra*	Europe	Bosch and Martinez-Solano, 2006
Caudata	Salamandridae	*Euproctus platycephalus*	Europe	Fisher and Garner, 2007
Garner et al. (2005) stated that 20 European amphibian species have been found positive for *Bd*, however, the complete list of all species has not been published as of 30 June 2008.				
Anura	Amphignathodontidae (Leptodactylidae)	*Gastrotheca cornuta*	Latin America	Lips et al., 2006
Anura	Aromobatidae (Dendrobatidae)	*Allobates (Colostethus) olfersioides*	Latin America	De Queiroz Carnaval et al., 2006
Anura	Aromobatidae (Dendrobatidae)	*Allobates (Colostethus) talamancae*	Latin America	Lips et al., 2006
Anura	Aromobatidae (Dendrobatidae)	*Mannophryne olmonae*	Latin America	Alemu et al., 2008
Anura	Brachycephalidae	*Craugastor (Eleutherodactylus)*	Latin America	Puschendorf et al., 2006

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	(Leptodactylidae)	*aurilegulus*		
Anura	Brachycephalidae (Leptodactylidae)	*Craugastor azueroensis*	Latin America	Lips et al., 2006
Anura	Brachycephalidae (Leptodactylidae)	*Craugastor bransfordii*	Latin America	Lips et al., 2006
Anura	Brachycephalidae (Leptodactylidae)	*Craugastor crassidigitus*	Latin America	Lips et al., 2006
Anura	Brachycephalidae (Leptodactylidae)	*Craugastor (Eleutherodactylus) emcelae*	Latin America	Berger et al., 1998
Anura	Brachycephalidae (Leptodactylidae)	*Craugastor (Eleutherodactylus) fitzingeri*	Latin America	Puschendorf and Bolanos, 2006
Anura	Brachycephalidae (Leptodactylidae)	*Craugastor gollmeri*	Latin America	Lips et al., 2006
Anura	Brachycephalidae (Leptodactylidae)	*Craugastor megacephalus*	Latin America	Lips et al., 2006
Anura	Brachycephalidae (Leptodactylidae)	*Craugastor noblei*	Latin America	Lips et al., 2006
Anura	Brachycephalidae (Leptodactylidae)	*Craugastor podiciferus*	Latin America	Lips et al., 2006
Anura	Brachycephalidae (Leptodactylidae)	Craugastor punctariolus	Latin America	Lips et al., 2006
Anura	Brachycephalidae (Leptodactylidae)	*Craugastor tabasarae*	Latin America	Lips et al., 2006
Anura	Brachycephalidae (Leptodactylidae)	*Craugastor talamancae*	Latin America	Lips et al., 2006
Anura	Brachycephalidae (Leptodactylidae)	*Limnophys (Craugastor) bufoniformis*	Latin America	Lips et al., 2006
Anura	Brachycephalidae	*Pristimantis achatinus*	Latin America	Cashins, pers. comm..

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Anura	Brachycephalidae (Leptodactylidae)	*Pristimantis (Craugastor) cerasinus*	Latin America	Lips et al., 2006
Anura	Brachycephalidae (Leptodactylidae)	*Pristimantis (Eleutherodactylus) caryophyllceus*	Latin America	Lips et al., 2006
Anura	Brachycephalidae	*Pristimantis crenunguis*	Latin America	Cashins, pers. comm.
Anura	Brachycephalidae (Leptodactylidae)	*Pristimantis (Eleutherodactylus) cruentus*	Latin America	Berger et al., 1998; Lips et al., 2006
Anura	Brachycephalidae (Leptodactylidae)	*Pristimantis (Eleutherodactylus) diastema*	Latin America	Lips et al., 2006
Anura	Brachycephalidae (Leptodactylidae)	*Pristimantis (Eleutherodactylus) elegans*	Latin America	Ruiz and Rueda-Almonacid, 2008
Anura	Brachycephalidae	*Pristimantis latidiscus*	Latin America	Cashins, pers. comm.
Anura	Brachycephalidae (Leptodactylidae)	*Pristimantis (Eleutherodactylus) museosus*	Latin America	Lips et al., 2006
Anura	Brachycephalidae (Leptodactylidae)	*Pristimantis (Eleutherodactylus) ridens*	Latin America	Lips et al., 2006
Anura	Brachycephalidae (Leptodactylidae)	*Pristimantis (Eleutherodactylus) vocator*	Latin America	Lips et al., 2006
Anura	Bufonidae	*Atelopus varius*	Latin America	Puschendorf and Bolanos, 2006
Anura	Bufonidae	*Atelopus bomolochos*	Latin America	Berger et al., 1999; Ron and Merino, 2000
Anura	Bufonidae	*Atelopus cruciger*	Latin America	Bonaccorso et al., 2003
Anura	Bufonidae	*Atelopus ignescens*	Latin America	Ron and Merino, 2000
Anura	Bufonidae	*Atelopus mittermeieri*	Latin America	Ruiz and Rueda-Almonacid, 2008
Anura	Bufonidae	*Atelopus mucubajiensis*	Latin America	Lampo et al., 2007
Anura	Bufonidae	*Atelopus zeteki*	Latin America	Lips et al., 2006

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Anura	Bufonidae	*Bufo haematiticus*	Latin America	Berger et al., 1998; Lips et al., 2006
Anura	Bufonidae	*Ollitis coniferus*	Latin America	Lips et al., 2006
Anura	Bufonidae	*Peltophryne (Bufo) longinasus*	Latin America	Lips et al., 2006
Anura	Bufonidae	*Rhinella (Chaunus, Bufo) marinus*	Latin America	Lips et al., 2006
Anura	Centrolenidae	*Centrolene ilex*	Latin America	Lips et al., 2006
Anura	Centrolenidae	*Centrolene prosoblepon*	Latin America	Lips et al., 2006
Anura	Centrolenidae	*Cochranella albomaculata*	Latin America	Berger et al., 1998; Lips et al., 2006
Anura	Centrolenidae	*Cochranella euknemos*	Latin America	Berger et al., 1998
Anura	Centrolenidae	*Cochranella prosoblepon*	Latin America	Berger et al., 1998
Anura	Centrolenidae	*Hyalinobatrachium colymbiphyllum*	Latin America	Berger et al., 1998
Anura	Ceratophryidae	*Telmatobius atacamensis*	Latin America	Barrionuevo and Mangione, 2006
Anura	Ceratophryidae	*Telmatobius marmoratus*	Latin America	Seimon et al., 2007
Anura	Ceratophryidae	*Telmatobius pisanoi*	Latin America	Barrionuevo and Mangione, 2006
Anura	Ceratophryidae (Leptodactylidae)	*Telmatobius niger*	Latin America	Ron and Merino, 2000
Anura	Cycloramphidae (Leptodactylidae)	*Thoropa miliaris*	Latin America	De Queiroz Carnaval et al., 2006
Anura	Dendrobatidae	*Colostethus panamensis*	Latin America	Lips et al., 2006
Anura	Dendrobatidae	*Colostethus pratti*	Latin America	Lips et al., 2006
Anura	Dendrobatidae	*Dendrobates auratus*	Latin America	Lips et al., 2006
Anura	Dendrobatidae	*Hyloxalus (Colostethus) awa*	Latin America	Cashins, pers. comm.
Anura	Dendrobatidae	*Mannophryne olmonae*	Latin America	Alemu et al., 2008
Anura	Dendrobatidae	*Oophaga (Dendrobates) vicentei*	Latin America	Lips et al., 2006

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Anura	Dendrobatidae	*Phyllobates lugubris*	Latin America	Lips et al., 2006
Anura	Dendrobatidae	*Silverstoneia (Colostethus) flotator*	Latin America	Lips et al., 2006
Anura	Dendrobatidae	*Silverstoneia (Colostethus) nubicola*	Latin America	Lips et al., 2006
Anura	Hylidae	*Agalychnis callidryas*	Latin America	Lips et al., 2006
Anura	Hylidae	*Agalychnis litodryas*	Latin America	Cashins, pers com
Anura	Hylidae	*Agalychnis moreletii*	Latin America	Felger et al., 2007
Anura	Hylidae	*Bokermannohyla gouveai*	Latin America	De Queiroz Carnaval et al., 2006
Anura	Hylidae	*Ecnomiohyla miliaria*	Latin America	Lips et al., 2006
Anura	Hylidae	*Hyla microcephala,*	Latin America	Lips et al., 2006
Anura	Hylidae	*Hylomantis lemur*	Latin America	Lips et al., 2006
Anura	Hylidae	*Hyloscirtus bogotensis*	Latin America	Ruiz and Rueda-Almonacid, 2008
Anura	Hylidae	*Hyloscirtus colymba*	Latin America	Lips et al., 2006
Anura	Hylidae	*Hyloscirtus palmeri*	Latin America	Lips et al., 2006
Anura	Hylidae	*Hyloscirtus (Hyla) psarolaima*	Latin America	Ron and Merino, 2000
Anura	Hylidae	*Hypsiboas freicanecae*	Latin America	De Queiroz Carnaval et al., 2006
Anura	Hylidae	*Smilisca phaeota*	Latin America	Lips et al., 2006
Anura	Hylodidae (Leptodactylidae)	*Crossodactylus caramaschii*	Latin America	De Queiroz Carnaval et al., 2006
Anura	Leiuperidae	*Pleurodema marmoratum*	Latin America	Seimon et al., 2007
Anura	Leiuperidae	*Physalaemus pustulosus*	Latin America	Lips et al., 2006
Anura	Leptodactylidae	*Leptodactylus pentadactylus*	Latin America	Lips et al., 2006
Anura	Leptodactylidae	*Leptodactylus ocellatus*	Latin America	Herrera et al., 2005
Anura	Leptodactylidae	*Leptodactylus pentadactylus*	Latin America	Lips et al., 2006

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Anura	Microhylidae	*Nelsonophryne aterrima*	Latin America	Lips et al., 2006
Anura	Plethodontidae (Microhylidae)	*Bolitoglossa colonnea*	Latin America	Lips et al., 2006
Anura	Plethodontidae (Microhylidae)	*Bolitoglossa schizodactyla*	Latin America	Lips et al., 2006
Anura	Plethodontidae (Microhylidae)	*Oedipina collaris*	Latin America	Lips et al., 2006
Anura	Plethodontidae (Microhylidae)	*Oedipina cf. parvipes*	Latin America	Lips et al., 2006
Anura	Ranidae	*Lithobates catesbeianus (Rana catesbiana)*	Latin America	Mazzoni et al., 2003
Anura	Ranidae	*Lithobates (Rana) maculata*	Latin America	Felger et al., 2007
Anura	Ranidae	*Lithobates (Rana) pipiens*	Latin America	Lips et al., 2006
Anura	Ranidae	*Lithobates (Rana) tarahumarae*	Latin America	Rollins-Smith et al., 2002
Anura	Ranidae	*Lithobates (Rana) warszewitschii*	Latin America	Lips et al., 2006
Caudata	Plethodontidae	*Plethodon neomexicanus*	Latin America	Cummer et al., 2005
Anuran	Hylidae	*Litoria raniformis*	New Zealand	Waldman et al., 2001
Anuran	Hylidae	*Litoria ewingii*	New Zealand	Bishop, 2000
Anuran	Leopelmatidae	*Leiopelma archeyi*	New Zealand	Bell et al., 2004
Anura	Bufonidae	*Anaxyrus (Bufo) americanus*	North America	Longcore et al., 2007
Anura	Bufonidae	*Anaxyrus (Bufo) baxteri*	North America	Green et al., 2002
Anura	Bufonidae	*Anaxyrus (Bufo) boreas*	North America	Green et al., 2002
Anura	Bufonidae	*Anaxyrus (Bufo) californicus*	North America	CCADC, 2007
Anura	Bufonidae	*Anaxyrus (Bufo) canorus*	North America	Carey et al., 1999

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Anura	Bufonidae	*Bufo boreashalophilus*	North America	CCADC, 2007
Anura	Hylidae	*Acris crepitans*	North America	Pessier et al., 1999
Anura	Hylidae	*Exerodonta melanomma*	North America	Frias-Alvarez et al., 2008
Anura	Hylidae	*Hyla arenicolor*	North America	Bradley et al., 2002
Anura	Hylidae	*Hyla euphorbiacea*	North America	Frias-Alvarez et al., 2008
Anura	Hylidae	*Hyla eximia*	North America	Frias-Alvarez et al., 2008
Anura	Hylidae	*Hyla versicolor*	North America	Ouellet et al., 2005
Anura	Hylidae	*Hyloscirtus (Hyla) psarolaima*	North America	Ron and Merino, 2000
Anura	Hylidae	*Pseudacris triseriata*	North America	Ouellet et al., 2005
Anura	Hylidae	*Pseudacris (Hyla) regilla*	North America	Green et al., 2002
Anura	Brachycephalidae (Leptodactylidae)	*Eleutherodactylus coqui*	North America	Beard and O'Neill, 2005
Anura	Ceratophryidae (Leptodactylidae)	*Telmatobius niger*	North America	Berger et al., 1999
Anura	Ranidae	*Lithobates (Rana) palustris*	North America	Ouellet et al., 2005
Anura	Ranidae	*Lithobates (Rana) berlandieri*	North America	Sredl and Cardwell, 2002
Anura	Ranidae	*Lithobates (Rana) blairi*	North America	Sredl and Cardwell, 2002
Anura	Ranidae	*Lithobates (Rana) catesbeianus*	North America	Pearl et al., 2007
Anura	Ranidae	*Lithobates (Rana) chiricahuensis*	North America	Morell, 1999
Anura	Ranidae	*Lithobates (Rana) clamitans*	North America	Ouellet et al., 2005
Anura	Ranidae	*Lithobates (Rana) megapoda*	North America	Frias-Alvarez et al., 2008
Anura	Ranidae	*Lithobates (Rana) montezumae*	North America	Frias-Alvarez et al., 2008
Anura	Ranidae	*Lithobates (Rana) neovolacanica*	North America	Frias-Alvarez et al., 2008

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Anura	Ranidae	*Lithobates (Rana) pipiens*	North America	Green et al., 2002
Anura	Ranidae	*Lithobates (Rana) septentrionalis*	North America	Ouellet et al., 2005
Anura	Ranidae	*Lithobates (Rana) spectabilis*	North America	Frias-Alvarez et al., 2008
Anura	Ranidae	*Lithobates (Rana) sphenocephala*	North America	Green et al., 2002
Anura	Ranidae	*Lithobates (Rana) sylvatica*	North America	Ouellet et al., 2005
Anura	Ranidae	*Lithobates (Rana) tarahumarae*	North America	Cashins, pers. comm
Anura	Ranidae	*Lithobates (Rana) yavapiensis*	North America	Green et al., 2002
Anura	Ranidae	*Rana aurora*	North America	Pearl et al., 2007
Anura	Ranidae	*Rana boylii*	North America	CCADC, 2007
Anura	Ranidae	*Rana cascadae*	North America	CCADC, 2007
Anura	Ranidae	*Rana draytonii*	North America	CCADC, 2007
Anura	Ranidae	*Rana luteiventris*	North America	Pearl et al., 2007
Anura	Ranidae	*Rana muscosa*	North America	Green et al., 2002
Anura	Ranidae	*Rana pretiosa*	North America	Pearl et al., 2007
Caudata	Ambystomatidae	*Ambystoma altamirani*	North America	Frias-Alvarez et al., 2008
Caudata	Ambystomatidae	*Ambystoma californiense*	North America	CCADC, 2007
Caudata	Ambystomatidae	*Ambystoma granulosum*	North America	Frias-Alvarez et al., 2008
Caudata	Ambystomatidae	*Ambystoma macrodactylum croceum*	North America	Green et al., 2002
Caudata	Ambystomatidae	*Ambystoma maculatum*	North America	Ouellet et al., 2005)
Caudata	Ambystomatidae	*Ambystoma rivulare*	North America	Frias-Alvarez et al., 2008
Caudata	Ambystomatidae	*Ambystoma tigrinum*	North America	Davidson et al., 2000
Caudata	Ambystomatidae	*Ambystoma velasci*	North America	Frias-Alvarez et al., 2008

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Caudata	Salamandridae	*Notophthalmus viridescens*	North America	Ouellet et al., 2005
Caudata	Salamandridae	*Pseudotriton ruber*	North America	Speare and Berger, 2005
Caudata	Salamandridae	*Taricha torosa torosa*	North America	CCADC, 2007

* Names in parentheses are those recognized previously

References for Appendix A

Alemu J. B., Cazabon N. E., Dempewolf L., Hailey A., Lehtinen R. M., Mannette R. P., Naranjit K. T. and Roach A. C. J. (2008) Presence of the chytrid fungus *Batrachochytrium dendrobatidis* in populations of the critically endangered frog *Mannophryne olmonae* in Tobago, West Indies. *EcoHealth* **5**: 34-39.

Barrionuevo S. and Mangione S. (2006) Chytridiomycosis in two species of *Telmatobius* (Anura : Leptodactylidae) from Argentina. *Diseases of Aquatic Organisms* **73**: 171-174.

Beard K. H. and O'Neill E. M. (2005) Infection of an invasive frog Eleutherodactylus coqui by the chytrid fungus *Batrachochytrium dendrobatidis* in Hawaii. *Biological Conservation* **126**: 591-595.

Bell B. D., Carver S., Mitchell N. J. and Pledger S. (2004) The recent decline of a New Zealand endemic: how and why did populations of archey's frog *Leiopelma archeyi* crash over 1996-2001? *Biological Conservation* **120**: 189-199.

Berger L. (2001) Diseases in Australian Frogs. In: *School of Public Health and Tropical Medicine*. PhD Thesis. James Cook University, Townsville, Australia.

Berger L., Speare R., Daszak P., Green D. E., Cunningham A. A., Goggin C. L., Slocombe R., Ragan M. A., Hyatt A. D., McDonald K. R., Hines H. B., Lips K. R., Marantelli G. and Parkes H. (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences of the United States of America* **95**: 9031-9036.

Berger L., Speare R., Hines H. B., Marantelli G., Hyatt A. D., McDonald K. R., Skerratt L. F., Olsen V., Clarke J. M., Gillespie G., Mahony M., Sheppard N., Williams C. and Tyler M. J. (2004) Effect of season and temperature on mortality in amphibians due to chytridiomycosis. *Australian Veterinary Journal* **82**: 434-439.

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Berger L., Speare R. and Hyatt A. (1999) Chytrid fungi and amphibian declines: overview, implications and future directions. In: *Declines and disappearances of Australian frogs.* (ed. A. Campbell) pp. 23-33, Environment Australia, Canberra.

Bishop P. (2000) chytrid fungi identified from dying frogs in New Zealand. *DAPTF Working Group New Zealand*.

Bonaccorso E., Guayasamin J. M., Mendez D. and Speare R. (2003) Chytridiomycosis as a possible cause of population declines in *Atelopus cruciger* (Anura: Bufonidae). *Herpetological Review* **34**: 331-334.

Bosch J. and Martinez-Solano I. (2006) Chytrid fungus infection related to unusual mortalities of *Salamandra salamandra* and *Bufo bufo* in the Penalara Natural Park, Spain. *Oryx* **40**: 84-89.

Bosch J., Martinez-Solano I. and Garcia-Paris M. (2001) Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biological Conservation* **97**: 331-337.

Bradley G. A., Rosen P. C., Sredl M. J., Jones T. R. and Longcore J. E. (2002) Chytridiomycosis in native Arizona frogs. *Journal of Wildlife Diseases* **38**: 206-212.

Carey C., Cohen N. and Rollins-Smith L. (1999) Amphibian declines: an immunological perspective. *Developmental and Comparative Immunology* **23**: 459-472.

CCADC (2007) California species affected. http://www.ccadc.us/docs/AffectedSpecies.pdf.

Cummer M. R., Green D. E. and O'Neill E. M. (2005) Aquatic chytrid pathogen detected in terrestrial plethodontid salamander. *Herpetological Review* **36**: 248-249.

Davidson E. W., Pessier A. P., Longcore J. E., Parris M., Jancovich J., Brunner J., Schock D. and Collins J. P. (2000) Abstract: Chytridiomycosis in Arizona (USA) Tiger Salamanders. In: *Conference Proceedings: Getting the Jump on Amphibian Disease, Cairns, Australia, 26-30 August, 2000*, available at http://www.jcu.edu.au/school/phtm/PHTM/frogs/gjoad.htm.

De Queiroz Carnaval A. C. O., Puschendorf R., Peixoto O. L., Verdade V. K. and Rodrigues M. T. (2006) Amphibian chytrid fungus broadly distributed in the Brazilian Atlantic Rain Forest. *EcoHealth* **3**: 41-48.

Felger J., Enssle J., Mendez D. and Spear R. (2007) Chytridiomycosis in El Salvador. *Salmandra* **43**: 122-127.

Fisher M. C. and Garner T. W. J. (2007) The relationship between the emergence of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduced amphibian species. *British Mycological Society* **21**: 2-9.

Frias-Alvarez P., Vredenburg V. T., Familiar-Lopez M., Longcore J. E., Gonzalez-Bernal E., Santos-Berrera G., Zambrano L. and Parra-Olea G. (2008) Chytridiomycosis survey in wild and captive mexican amphibians. *EcoHealth* **5**: 18-26.

Garner T. W. J., Perkins M. W., Govindarajulu P., Seglie D., Walker S., Cunningham A. A. and Fisher M. C. (2006) The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana*. *Biological Letters* **2**: 455-459.

****

Garner T. W. J., Walker S., Bosch J., Hyatt A. D., Cunningham A. A. and Fisher M. C. (2005) Chytrid fungus in Europe. *Emerging Infectious Diseases* **11**: 1639-1641.

Goldberg T. L., Readel A. M. and Lee M. H. (2007) Chytrid Fungus in Frogs from an Equatorial African Montane Forest in Western Uganda. *Journal of Wildlife Diseases* **43**: 521-524.

Green D. E., Converse K. A. and Schrader A. K. (2002) Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996-2001. *Annals of the New York Academy of Sciences* **969**: 323-339.

Herrera R. A., Steciow M. M. and Natale G. S. (2005) Chytrid fungus parasitizing the wild amphibian Leptodactylus ocellatus (Anura : Leptodactylidae) in Argentina. *Diseases of Aquatic Organisms* **64**: 247-252.

Kriger K. M., Hero J. and Ashton K. J. (2006) Cost efficiency in the detection of chytridiomycosis using PCR assay. *Diseases of Aquatic Organisms* **71**: 419-154.

Kriger K. M. and Hero J. M. (2006) *Cophixalus ornatus* (Ornate nursery frog). Chytridiomycosis. *Herpetological Review* **37**: 443.

Kriger K. M. and Hero J. M. (2007) The chytrid fungus *Batrachochytrium dendrobatidis* is non-randomly distributed across amphibian breeding habitats. *Diversity and Distributions*.

Lampo M., Barrio-Amoros C. and Han B. A. (2007) *Batrachochytrium dendrobatidis* infection in the recently rediscovered Ateloopus mucubajiensis (Anura, Bufonidae), a critically endangered frog from the VenezuelanAndes. *EcoHealth* **3**: 299-302.

Lane E. P., Weldon C. and Bingham J. (2003) Histological evidence of chytridiomycete fungal infection in a free-ranging amphibian, *Afrana fuscigula (*Anura: Ranidae). *Journal of the South African Veterinary Association* **74**: 20-21.

Lips K. R., Brem f., Brenes R., Reeve J. D., Alford R. A., Voyles J., Carey C., Livo L., Pessier A. P. and Collins J. P. (2006) Emerging infectious disease and the loss of biodiversity in a neotropical amphibian community. *PNAS* **103**: 3165-3170.

Longcore J. R., Longcore J. E., Pessier A. P. and Halteman W. A. (2007) Chytridiomycosis widespread in anurans of northeastern United States. *Journal of Wildlife Management* **71**: 435-444.

Mazzoni R., Cunningham A. A., Daszak P., Apolo A., Perdomo E. and Speranza G. (2003) Emerging pathogen of wild amphibians in frogs (*Rana catesbeiana*) farmed for international trade. *Emerging Infectious Diseases* **9**: 995-998.

McDonald K. R., Mendez D., Mueller R., Freeman A. B. and Speare R. (2005) Decline in the prevalence of chytridiomycosis in frog populations in North Queensland, Australia. *Pacific Conservation Biology* **11**: 114-120.

Morell V. (1999) Are pathogens felling frogs? *Science* **284**: 728-731.

Mutschmann F., Berger L., Zwart P. and Gaedicke C. (2000) Chytridiomycosis on amphibians - first report from Europe. *Berliner Und Munchener Tierarztliche Wochenschrift* **113**: 380-383.

****

Ouellet M., Mikaelian I., Pauli B. D., Rodrigue J. and Green D. M. (2005) Historical evidence of widespread chytrid infection in North American amphibian populations. *Conservation Biology* **19**: 1431-1440.

Parker J. M., Mikaelian I., Hahn N. and Diggs H. E. (2002) Clinical diagnosis and treatment of epidermal chytridiomycosis in African clawed frogs (*Xenopus tropicalis*). *Comparative Medicine* **52**: 265-268.

Pauza M. and Driessen M. (2008) Distribution and potential spread of amphibian chytrid fungus Batrachochytrium dendrobatidis in the Tasmanian wilderness world heritage area. *online*.

Pearl C. A., Bull E. L., Green D. E., Bowerman J., Adams M. J., Hyatt A. and Wente W. H. (2007) Occurrence of the Amphibian Pathogen *Batrachochytrium dendrobatidis* in the Pacific Northwest. *Journal of Herpetology* **41**: 145-149.

Pessier A. P., Nichols D. K., Longcore J. E. and Fuller M. S. (1999) Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp.) and white's tree frogs (*Litoria caerulea*). *Journal of Veterinary Diagnostic Investigation* **11**: 194-199.

Puschendorf R. and Bolanos F. (2006) Detection of *Batrachochytrium dendrobatidis* in Eleutherodactylus fitzingeri: Effects of skin sample location and histologic stain. *Journal of Wildlife Diseases* **42**: 301-306.

Puschendorf R., Castaneda F. and McCranie J. R. (2006) Chytridiomycosis in wild frogs from Pico Bonito National Park, Honduras. *EcoHealth* **Online First**: 1-4.

Rollins-Smith L. A., Reinert L. K., Miera V. and Conlon J. M. (2002) Antimicrobial peptide defenses of the Tarahumara frog, *Rana tarahumarae*. *Biochemical and Biophysical Research Communications* **297**: 361-367.

Ron S. R. and Merino A. (2000) Amphibian declines in Ecuador: Overview and first report of chytridiomycosis from South America. *Froglog* **42**.

Ruiz A. and Rueda-Almonacid J. V. (2008) *Batrachochytrium dendrobatidis* and chytridiomycosis in anuran amphibians of Colombia. *EcoHealth*.

Seimon T. A., Seimon A., Daszak P., Halloy S. R. P., Schloegel L. M., Aguilar C. A., Sowell P., Hyatt A. D., Konecky B. and Simmons J. E. (2007) Upward range extension of Andean anurans and chytridiomycosis to extreme elevations in response to tropical deglaciation. *Global Change Biology* **13**: 288-299.

Simoncelli F., Fragotti A., Dall'Olio R., Vagnetti D., Pascolini R. and Di Rosa I. (2005) Evidence of *Batrachochytrium dendrobatidis* infection in water frogs of the *Rana esculenta* complex in Central Italy. *EcoHealth* **2**: 307-312.

Speare R. and Berger L. (2005) Chytridiomycosis in amphibians in Australia. http://www.jcu.edu.au/school/phtm/PHTM/frogs/chyspec.htm.

Sredl M. J. and Cardwell D. (2002) Update on chytrid surveys of frogs from Arizona. *Tucson Herpetological Newsletter* **13**: 1.

Stagni G., Scoccianti C. and Fusini R. (2002) Segnalazione di chytridiomicosi in popolazioni di *Bombina pachypus* (Anura, Bombinatoridae) dell'Appennino tosco-emiliano. In: *Abstracts IV; Congresso della Societas Herpetologica Italica; Napoli: Societas Herpetologica Italica*.

****

Symonds E. P., Hines H. B., Bird P. S., Morton J. M. and Mills P. C. (2007) Surveillance for *Batrachochytrium dendrobatidis* using *Mixophyes* (Anura: Myobatrachidae) larvae. *Journal of Wildlife Diseases* **43**: 48-60.

Waldman B., van de Wolfshaar K. E., Klena J. D., Andjic V., Bishop P. J., Norman R. and North P. (2001) Chytridiomycosis in New Zealand frogs. *Surveillance* **28**.

Weldon C., du Preez L. H., Hyatt A. D., Muller R. and Speare R. (2004) Origin of the amphibian chytrid fungus. *Emerging Infectious Diseases* **10**: 2100-2105.