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Ecology of microhylid frogs in the Australian Wet Tropics and implications for their vulnerability to chytridiomycosis.

Adult and juvenile *Copixalus ornatus*

Thesis submitted by

Kim Fiona Hauselberger BSc(Hons) Dip Ed

in July 2010

for the degree of Doctor of Philosophy

in the School of Marine & Tropical Biology

James Cook University

STATEMENT OF SOURCES DECLARATION

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 Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

_____ 15th July 2010_____

Signature Date

DECLARATION OF ETHICS AND SCIENTIFIC PERMITS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *Joint NHRMC/AVCC statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimentation Ethics: Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (approval numbers A931 and A960).
Research was carried out under Scientific Purposes permits issued by the Queensland Parks and Wildlife Service (WISP02464008 and WITK 02464108), and a Permit to Collect issued by the Queensland Environmental Protection Agency (ATH 08/005).

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STATEMENT OF CONTRIBUTION OF OTHERS

This thesis was co-supervised by Ross Alford, Lin Schwarzkopf and Stephen Williams. Professor Ross Alford provided input on experimental design, statistical assistance, and editorial advice on all chapters. Lin Schwarzkopf provided editorial assistance to several thesis chapters. Stephen Williams allowed access to transects that were established in the Atherton and Carbine uplands. Sara Bell offered advice on culturing *Batrachochytrium dendrobatidis* (*Bd*) and completing challenge assays, and provided editorial assistance on chapters three and four. PCR diagnostic tests for *Bd* were performed by Ruth Campbell, Stephen Garland and Andrea Phillot at the school of Veterinary and Biomedical Sciences, JCU; and Alex Hyatt at the Australian Animal Health Laboratory at CSIRO.

Chapters two, four, five, and six are in the process of being prepared for submission to a variety of journals. Professor Ross Alford will appear as co-author on all publications from this thesis, Sara Bell will appear as a co-author on publications derived from chapters three and four, and Lin Schwarzkopf will appear as a co-author on the publication derived from chapter two.

I was supported by an Australian Postgraduate Research Scholarship. Research was funded by the Australian Rainforest Co-operative Research Centre, and by tender 43/2004 from the Australian Department of Environment and Heritage (R. Alford, principal investigator).

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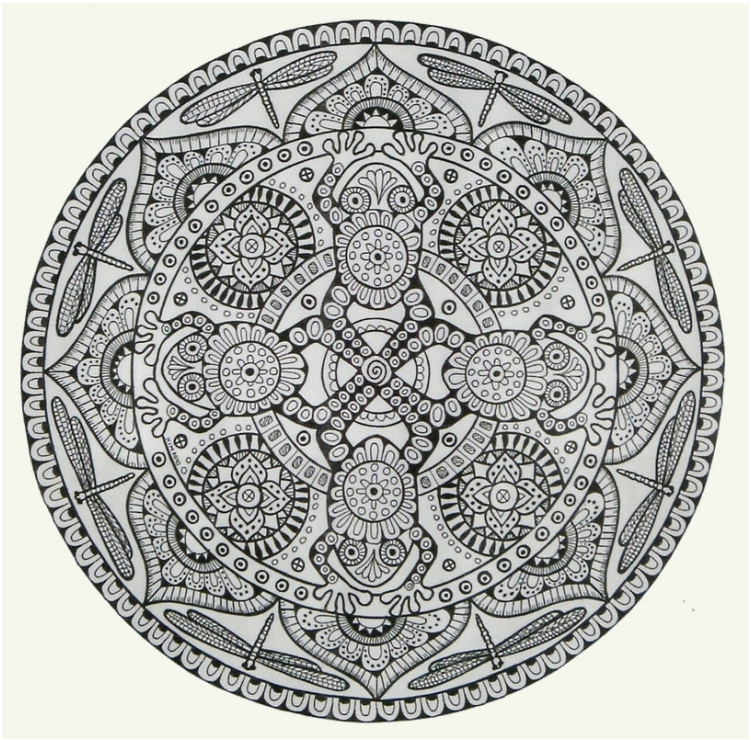
Lokah Samasta Sukhino Bavantu



ABSTRACT

Chytridiomycosis is an emerging infectious disease of amphibians caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), and is responsible for causing mass mortality, population declines and extinctions of amphibian species in much of the world. The extent of pathological effects of chytridiomycosis varies amongst hosts, and terrestrial frogs and salamanders that have parental care of direct-developing eggs may tend to be less susceptible. The frog family Microhylidae is characterised by terrestrially-breeding species that reach their highest diversity in Australia in the Wet Tropics (WT) bioregion of northern Queensland. In this region, frogs of other families suffered severe declines and extinctions in association with outbreaks of chytridiomycosis. There is a lack of information on the responses of Australian microhylids to this emerging disease, and this project was carried out to gain explicit information on the interactions of microhylids with *Bd*.

The temporal calling patterns of two microhylid species (*Austrochaperina robusta* and *Cophixalus ornatus*) were examined at one site within the WT, using automated recordings every night for five entire wet seasons, over an 11-year period. Environmental variables were also recorded, to examine correlations between calling and weather conditions. The calling activity of *C. ornatus* and *A. robusta* fluctuated only slightly from year to year; the observed fluctuations were less than two-fold. There was no downward trend in average and maximum calls for either species over time, suggesting that population densities of these species have not suffered from major losses, and that variation in calling is unlikely to be due to climate change or an external factor such as *Bd.*

To determine if microhylids could be infected with *Bb* in the wild, I examined 595 samples from nine species, and found that none showed evidence of infection by *Bd*. When this data were regarded as a single sample representative of Australian microhylids, the upper 95% binomial confidence limit for presence of infection was 0.0062 (less than 1%). This suggests that microhylids have a very low prevalence of *Bd* in nature, and are either not susceptible, or are only slightly susceptible, to chytridiomycosis under natural conditions.

The susceptibility of the most common species of microhylid (*Cophixalus ornatus*) to *Bd* was tested in a series of laboratory experiments. Seven *C. ornatus* and five *Litoria wilcoxii* (susceptible controls) were exposed to increasing numbers of *Bd* zoospores and tested for infection using quantitative PCR assays. All *C. ornatus*, and four of the five *L. wilcoxii*, became infected by *Bd* at some point during the experiment. The mean intensity of infection, as measured by number of zoospore equivalents in infected individuals, was significantly higher in *L. wilcoxii* than in *C. ornatus*. All *C. ornatus* individuals eliminated their infections by the end of the experimental trials, whereas *L. wilcoxii* individuals still retained relatively intense infections.

Innate immune defenses in the form of antimicrobial peptides (AMPs) may be particularly important in providing resistance to chytridiomycosis, and highly effective AMPs could be responsible for the extremely low prevalences of *Bd* infection I found in nature. To test this hypothesis, the AMPs of 81 microhylids from six species were examined. Secretions containing skin peptides were collected by norepinephrine induction, and used in growth inhibition assays to measure their effectiveness against *Bd*. Sixty two percent of samples contained AMPs with at least some activity against *Bd*, and 17% showed 100% inhibition of *Bd* growth at the levels tested. Microhylid species produced peptides in quantities similar to two sympatric hylid frogs (*Litoria genimaculata* and *L. rheocola*). Mean protein secretion of microhylids did not differ significantly from these species, however, the overall protection of microhylids provided by AMPs was significantly lower than that of hylids. This suggests that AMPs are not likely to be responsible for the low prevalence of infection by *Bd* in Australian microhylids.

Thermal and hydric environmental variables are known to affect the prevalence of *Bd* infections and the occurrence of epidemic outbreaks of chytridiomycosis, as *Bd* requires water to reproduce, and has a thermal optimum of 17-25ºC. Measurements of the body temperatures of frogs were combined with longer-term temperature and moisture data collected from permeable and impermeable agar models. These models were placed in frog retreat sites to document the thermal and hydric environments that microhylids experience in the field. Models never lost more than 20% of weight after periods exceeding 96hrs, suggesting that retreat sites provide insulation from dehydration. Models produced an accurate outline of the thermal envelope of microhylids as 90% of frogs had surface temperatures that were inside the range of the models. Surface temperature readings of 197 frogs were within the growth range for *Bd*, and model thermal data suggested that at two of the four transects (Carbine uplands and Paluma), temperature levels during the period of data collection were entirely within the optimal range for *Bd* growth. However, at Bellenden Ker, models were below the optimal range for *Bd* growth 70 percent of the time, and at the Atherton uplands, model temperatures reached levels that were above the range for optimal growth of *Bd* 24 percent of the time. Model temperatures also reached levels above 28ºC, which may cause the fungus to stop growing. Given that data were collected over a narrow time window, and that *Bd* has a narrow range of tolerance for temperature and moisture conditions, the data indicate that microenvironments used by microhylids are likely to affect the overall dynamics of the host-pathogen system. Some individuals and species may be less susceptible to *Bd* infection due to the environments they use.

In summary, the results of this study provide new levels of understanding of the interactions between Australian frogs of the family Microhylidae and the amphibian chytrid fungus. Despite inhabiting environments where *Bd* has caused declines in other frog species, they have not suffered population losses associated with *Bd*, and *Bd* infections are either at very

low prevalence or are entirely absent in nature. This apparent resistance to infection does not appear to be solely due to AMPs, as samples collected from microhylids in the field were no more effective against *Bd* in microhylids than are those of hylid species. Microhylids are not constitutively immune to infection by *Bd* from some other, unknown mechanism intrinsic to themselves, since *C. ornatus* readily became infected in laboratory experiments. Field data showed that the thermal environments experienced by microhylids may contribute to their near-immunity to *Bd* infection in nature, but are unlikely to explain it entirely. One potentially important source of resistance to *Bd* infection that was not evaluated in the present study is the contribution of symbiotic skin microbes, and future work should evaluate this, as it is possible that the Australian microhylids have a skin microbiota that is particularly effective at combating *Bd* infection in the field.

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CHAPTER 1: GENERAL INTRODUCTION

Amphibian declines

Amphibians (caecilians, frogs and salamanders) are a diverse class of animals that includes almost 6000 recognised species. They account for a large proportion of the vertebrate biomass in some systems (e.g. Burton and Likens 1975; Stewart and Woolbright 1996), and they are the most abundant vertebrate group in much of the tropics (Stebbins and Cohen 1995; McDiarmid and Altig 1999). In some communities, they occupy the highest trophic level, and have been described as keystone species due to their disproportionately large impacts on ecosystem structure (Wissinger et al. 1999).

Global amphibian declines currently rank among the most critical issues in conservation biology. Forty three percent of species are experiencing some form of population decline, and 32.5% of species are globally threatened (Stuart et al. 2004; Mendleson et al. 2005). The World Conservation Union Global Amphibian Assessment (GAA) estimated that between 9 and 122 amphibian species have become extinct since 1980, and that 427 species are listed as "critically endangered" (Stuart et al. 2004). Declines first became clearly obvious in the 1980's and many occurred in relatively pristine areas such as the tropical rainforests of northern Queensland and Central America (Richards et al. 1993; Mahony 1996; Lips 1998). These declines were characterised by Stuart et al. (2004) as "enigmatic" because it seemed unlikely they had been caused by common anthropogenic factors such as habitat loss or environmental contaminants. Various authors had speculated that some of the declines categorised as enigmatic may have been caused by disease, however at the time of those publications no definite cause had been identified (Laurance et al. 1996; Lips 1998; Carey et al. 1999).

Disease

In comparison with habitat loss, over harvesting, pollution, and climate change, the threat posed to species by disease may appear to represent a minor problem for conservation. However, disease is having a growing impact on wildlife populations as contact with human and domestic animals increases, and with alterations in climate and landscape ecology (Scott 1988; Cleaveland et al. 2001). In recent years, a number of pathogenic infectious diseases have emerged, causing sudden and unexpected declines in abundance of host species, and representing a substantial threat to global biodiversity (Daszak et al. 2000).

It is becoming better appreciated that disease is an important factor affecting survival, fecundity, age structure and interspecific interactions, including dispersal, competition, and predation, of host species (Tompkins and Begon 1999; Parris and Cornelius 2004). Pathogenic organisms are frequently cited as important drivers of community dynamics (Anderson and May

1986; McCallum and Dobson 1995; Cleaveland et al. 2001; Hudson et al. 2001), and emerging pathogens have been implicated in the declines and extinctions of a wide variety of species (Warner 1968; Berger et al. 1998; Cunningham and Daszak 1998; Daszak and Cunningham 1999). Pathogens can convert species-rich systems into depauperate communities that are dominated by a few resistant species. This phenomenon has been observed in island birds (Warner 1968; Van Riper III et al. 1986), forest trees (Burdon 1991), and rainforest frogs (Lips et al. 2006). In these scenarios, some species experience severe declines, others decline less severely, and others suffer no losses.

Disease dynamics differ greatly among, and even within, species (Wakelin 1978; Weinstein 2009), and are the result of complex processes involving factors related to the host, pathogen, and the environment (Warner 1968; Van Riper III et al. 1986; Lips et al. 2006). Ecological factors have a strong influence on disease patterns, and micro and macro-climatic variables can influence pathogens, vectors, and host defenses (Dobson and Carper 1993; Epstein 2001).

Emerging infectious disease

Emerging infectious diseases (EIDs) are caused by pathogens that have recently been discovered, have recently increased in incidence or geographic range, have recently moved into new host populations, or are newly evolved (Daszak et al. 2003). Pathogens may emerge by acquiring new capacities for initiating disease, or by altering the host's natural ability to mount an effective immune response. The key factor in the emergence of most EIDs is a change in host-parasite ecology that causes changes in host demography, behaviour or social structure (Garnett and Holmes 1996). These ecological changes allow increased contact with new host populations or species, greater transmission between hosts, and selection pressure leading to the dominance of pathogens which are adapted to these new environmental conditions (Daszak et al. 2001).

EIDs are hypothesised to originate in two ways. The "novel pathogen hypothesis" states that a pathogen can spread into a new geographic area, encountering naïve host individuals or species that are highly susceptible to it (Rachowicz et al. 2005; Skerratt et al. 2007). The "endemic pathogen hypothesis" suggests that a pathogen has been present in the environment for an extended period of time, but has entered new host species or increased in pathogenicity because of changes in the immunological, ecological, and/or behavioural parameters of the host or parasite (Rachowicz et al. 2005).

EIDs are an escalating threat to biodiversity, ecosystem integrity, and human health, and have been reported as increasing causes of death in humans and wildlife (Garnett and Holmes 1996; Carey 2000; Daszak et al. 2000). One of the most serious EIDs in recent history

is the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). The pattern of amphibian deaths and population declines in Australia and around the world is characteristic of an introduced virulent pathogen dispersing through naïve populations (Anderson and May 1986; Laurance et al. 1996; Lips et al. 2006). *Batrachochytrium dendrobatidis*' recent geographical expansion around the world, its increase in impact on amphibian populations, and its recent discovery, mark it as an EID.

Batrachochytrium dendrobatidis

The amphibian chytrid fungus, *Bd*, has been implicated as one of the causative agents of mass mortalities, population crashes and the extinction of amphibian species (Berger et al. 2000b; Rachowicz et al. 2005; Skerratt et al. 2007; Goka et al. 2009). In particular, it is now accepted as the cause of many of the declines that Stuart et al. (2004) categorised as "enigmatic". These declines have been characterised as the "most spectacular loss of vertebrate biodiversity due to disease in recorded history" (Skerratt et al. 2007). Chytridiomycosis, the disease caused by *Bd*, has been accepted as a "key threatening process" in Australia under the Commonwealth Environment Protection and Biodiversity Conservation Act (EBPC Act).

Bd (Phylum Chytridiomycota) was first described in 1998, and named for the blue poison dart frog (*Dendrobates auratus*) from which it was isolated (Berger et al. 1998; Longcore et al. 1999; Pessier et al. 1999). Members of the phylum Chytridiomycota are small spherical fungi with diverse lifecycles and requirements, and they are frequently found in soil and water, in a range of ecosystems (Powell 1993). *Batrachochytrium dendrobatidis* is the first and only member of the phylum Chytridiomycota known to cause disease and death in a vertebrate host (Berger et al. 1998).

Like all members of the Phylum Chytridiomycota, *Bd* is a heterotrophic fungus that is ubiquitous and cosmopolitan (Sparrow 1960; Karling 1977). It develops without hyphae, and has two life stages: the sessile growth stage, a thallus that lives within host cells and develops a spherical reproductive zoosporangium (10-40μm), and the infectious stage, an aquatic, motile, uniflagellate zoospore (1-5 μm), which is released from the zoosporangium when mature (Johnson and Speare 2003; Berger et al. 2005). No resting stage has been identified for *Bd* in laboratory culture, and it is unknown if one occurs in nature (Longcore et al. 1999), although Di Rosa et al. (2007) found evidence of spherical, unicellular organisms on the surface of frog skin that may be resting stages of the organism.

Bd infects keratinised, epidermal skin cells (Figure 1.1). The motile, reproductive zoospores attach to the keratinised outer layers of an amphibian host. They then encyst (cease movement and settle) by burying themselves and inserting cytoplasm into the epidermal skin cells. They then form a thallus, and mature to form a spherical zoosporangium (Pessier et al.

1999). The zoosporangium has thread-like rhizoids and a projecting, discharge tubule, and is the site of development of new zoospores. At maturity, the zoosporangium contains up to 300 infectious zoospores, which are released via the discharge tubule to the exterior of the skin (Berger et al. 2000a). These infective zoospores frequently reinfect nearby areas of the skin of the same host, but they can also pass into the external environment and infect new hosts (Berger et al. 1998; Longcore et al. 1999; Pessier et al. 1999). The life cycle takes about 4-5 days in culture at 22°C, and it is assumed that it is the same within the skin (Longcore et al. 1999; Berger et al. 2005). The fungus requires water to multiply and release zoospores, which are motile for only about 24 h before they encyst (attach and develop a cell wall) or die. In calm water they move less than 2 cm (Powell 1993; Pessier et al. 1999; Piotrowski et al. 2004). Zoosporangia are found in the keratinised epidermis of amphibians, but also may exist and proliferate in the environment (Lips et al. 2006; Richards-Zawacki 2009).

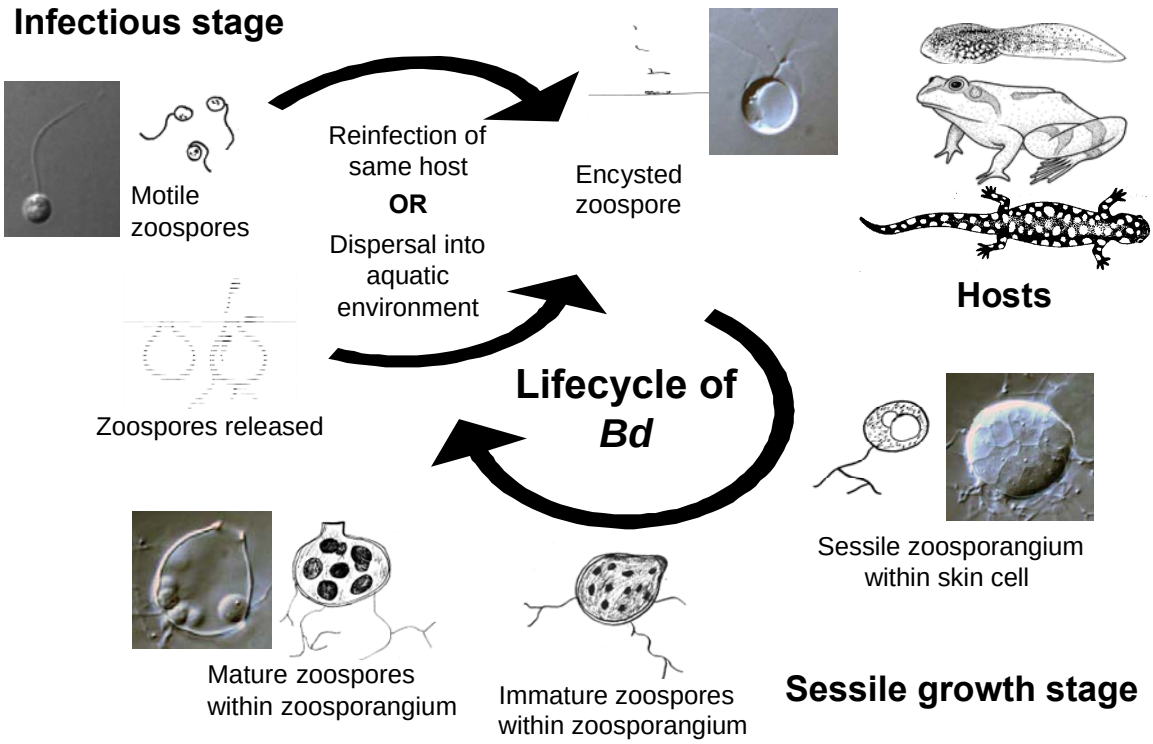

Figure 1.1 Life-cycle of *Bd* showing infectious and sessile growth stages. Photos from Rosenblum et al. (2010).

Persistence of *Bd* in the absence of a host is not well understood, but it may survive and reproduce as a saprophytic organism, as this is common in other chytrid species (Powell 1993; Daszak et al. 1999; Longcore et al. 1999). Zoospores can develop into sporangia in the absence of an amphibian host, as they survive and remain infectious in autoclaved lake water for up to seven weeks (Johnson and Speare 2003). Zoospores can also survive on a variety of

substrates such as dead amphibian and snake skin, dead algae, insect exoskeletons, soil and feathers (Johnson and Speare 2005), and *Bd* has been detected in the environment. In Central America, six out of seven samples taken from substrates associated with dead frogs tested positive for *Bd*, as was one of nine randomly selected stream boulders (Lips et al. 2006). Six percent of randomly selected moist environmental substrates such as sticks and leaves tested positive for *Bd* during an epidemic in Panama (Richards-Zawacki 2009), and *Bd* DNA was detected in 64% of ponds surveyed around the Sierra de Guadarrama in Spain, using filtration of water and quantitative PCR analysis (Walker et al. 2007). These results strongly suggest that *Bd* can survive and grow in the environment in the absence of amphibian hosts.

Chytridiomycosis

Amphibian chytridiomycosis is characterised by the colonisation of epidermal cells by *Bd* thalli, the development of sporangia, and the release of zoospores at the skin surface, often followed by recolonisation. Infections are not always fatal, but in susceptible species, post-metamorphic individuals are most vulnerable, and can die within as little as 18 days following exposure to zoospores (Carey et al. 1999). Mortality rates of 100% have been reported from a number of species of wild and captive frogs, and death usually occurs between 18 and 48 days after exposure (Berger et al. 1998; Longcore et al. 1999; Nichols et al. 2001; Weinstein 2009).

Individuals with chytridiomycosis may die, show a variety of symptoms, or appear clinically normal. Behavioural changes are the most obvious of the clinical signs, and include: convulsions, loss of righting reflex, slow response to tactile stimuli, abnormal posture, lethargy, and sudden death (Berger et al. 1999a). Physiological changes to the skin include: thickening and sloughing of the superficial epidermis, hyperplasia, hyperkeratosis and spongiosis of the *stratum corneum* and *stratum granulosum*, small ulcers or lesions on the skin, reddening of ventral skin, hyperemia of digital and ventral skin, and congestion of the viscera (Berger et al. 1999b; 2005).

*C*hytridiomycosis appears to cause death by disrupting the cutaneous osmoregulatory functioning of the skin (Voyles et al. 2009). In frogs suffering from chytridiomycosis, electrolyte transport across the epidermis is inhibited, causing an osmotic imbalance. Plasma sodium and potassium concentrations are reduced, and asystolic cardiac arrest occurs, leading to death (Voyles et al. 2009).

Chytridiomycosis causes mortality in a large range of amphibian species that live in a variety of habitats, however the intensity of infection and the extent of pathological effects appear to depend strongly on the species of the host. Some species have high susceptibility, and are extirpated in initial epidemic outbreaks of *Bd*, others have moderate susceptibility, resulting

in declines without the loss of all individuals, some can tolerate infections without developing clinical signs of the disease, and some appear resistant to infection (Berger et al. 1998; Daszak et al. 2003; Lips et al. 2006). Experimental studies have demonstrated that significant inherent differences exist between species infected with *Bd* in a laboratory setting. For example the Australian frog species *Litoria caerulea, L. chloris, Mixophyes fasciolatus* and *Limnodynastes tasmaniensis* showed differences in the number of deaths, post-exposure survival times, behavior, and pathogen loads after being exposed to the same amount of *Bd* zoospores (Ardipradja 2001). In addition to intrinsic factors that can be expressed in simplified laboratory environments, vulnerability to infection by *Bd* and morbidity and mortality caused by chytridiomycosis is likely to be affected by many, more complex factors in natural environments; these are discussed below.

Transmission of chytridiomycosis

Transmission is an essential component of the epidemic process, and can be the primary driver of the dynamics of infectious disease (Begon et al. 2002). The long-term spread and persistence of many diseases depends largely on the contact rate between susceptible hosts and infectious pathogens (Swinton 1998). *Batrachochytrium dendrobatidis* is transmitted among hosts by motile, water-borne zoospores (Longcore et al. 1999), and because zoospores swim less than 2 cm before they encyst (Piotrowski et al. 2004), the pathogen is likely to be spread by close contact during aggregation or mating (Berger et al. 1999a; Daszak et al. 2000; Speare 2000). However, zoospores have also been detected on contaminated environmental substrates during an epidemic, which suggests that abiotic reservoirs may play a role in transmission of the fungus (Lips et al. 2006; Richards-Zawacki 2009).

Tadpoles also play an important role in the transmission of disease as they stand a high chance of being exposed to the fungus' aquatic zoospores, and they may serve as carriers that maintain the pathogen in the environment (Waldman et al. 2001; Lamirande and Nichols 2002; Rachowicz and Vredenburg 2004). Tadpoles are not generally killed by chytridiomycosis, due to their lack of keratinised skin (Marantelli et al. 2004), and only show infections within the keratinised epidermis of their mouthparts (Berger et al. 1998; Berger et al. 2000b). These infections are not lethal, and infected tadpoles can carry the disease through metamorphosis. During this time, the skin becomes increasingly keratinised, allowing the fungus to become widespread on the body, and the infection may be spread to other individuals (Berger et al. 1998; Marantelli et al. 2004). Transmission to terrestrial individuals probably also occurs when they enter water contaminated with zoospores shed by infected tadpoles.

From their observations of a mass die-off event at El Cope, Panama, Lips et al. (2006) postulated that chytridiomycosis emerges at a site and spreads by a combination of frog-frog

and environment-frog transmission. These routes of transmission have been demonstrated in the laboratory (Davidson et al. 2003), and in field mesocosms (Parris and Cornelius 2004). As prevalence within a community increases, diseased frogs spread zoospores into the environment, or directly pass them to other amphibians through contact. The presence of *Bd* in the environment, and the long period of infectivity of many amphibian species promote saturation of the environment with zoospores, enabling transmission among species that partition the habitat spatially or temporally and producing a pattern where prevalence quickly changes from very low to high followed by widespread mortality.

Rowley and Alford (2007a) tracked three species of WT rainforest frogs of northern Queensland (*Litoria genimaculata*, *L. leseuri*, and L. *nannotis*) using radio-telemetry at sites where *Bd* is endemic. Behaviour and frequency of contact with other frogs, stream water and various environmental substrates differed significantly among the species, in a manner consistent with their relative levels of vulnerability to declines caused by chytridiomycosis. Thus, behaviour and microhabitat selection are likely to play major roles in determining species' susceptibility to chytridiomycosis.

Temperature

Numerous studies have explored the thermal requirements of *Bd*, and have found that it grows most rapidly in culture at 17-25°C (Berger et al. 1998; Longcore et al. 1999; Pessier et al. 1999; Piotrowski et al. 2004). Optimal growth of *Bd* occurs at 23°C in culture, with reversible cessation of growth at 28°C, and death at 29°C (Longcore et al. 1999). Experimental data also demonstrate that the rate of progress and outcome of chytridiomycosis depend very strongly on the environment experienced by the host (Woodhams et al. 2003; Berger et al. 2004). Woodhams et al. (2003) showed that thermal regime can strongly affect the progress of chytridiomycosis; when the body temperature of red-eyed treefrogs (*Litoria chloris*) was increased to 37°C for less than 16 hours, they were cleared of the pathogen, preventing further development of the disease.

These factors may have important implications for the host-parasite ecology of chytridiomycosis, and its effects in wild populations. The relatively narrow thermal optimum range of *Bd* is reflected in the epidemiology of disease outbreaks, which tend to occur at higher elevations (Speare 2000; Lips et al. 2005), increase in severity in the cooler months (Berger et al. 2004; McDonald et al. 2005; Woodhams and Alford 2005; Kriger and Hero 2007a), and increase in the wet season in tropical environments (Berger et al. 2004; Lips et al. 2006). Amphibians can alter their body temperatures through changes in behaviour, either by changing their use of microenvironments or by more active means such as basking (Bradford 1984), and they may even alter their body temperatures in response to infection via behavioural fever

(Woodhams et al. 2003; Richards-Zawacki 2009). Due to this, ecological and behavioural factors can have major affects on the susceptibility of species and individuals to chytridiomycosis.

Effects of ecology and life history

The extent to which *Bd* affects amphibian species is likely to be influenced by ecological and life-history traits (Blaustein and Wake 1990; Williams and Hero 1998; Lips et al. 2003; Retallick et al. 2004; Woodhams and Alford 2005). Environmental variables such as temperature and humidity; life-history traits such as reproductive mode and fecundity; ecological factors including interactions with other species, range size, habitat specialisation, and association with water; and complex ecological factors that affect the life-cycle of the chytrid, might all be important in determining the vulnerability of species to declines (Williams and Hero 1998; Lips et al. 2003; Woodhams et al. 2003; Retallick et al. 2004; Woodhams and Alford 2005).

The habitat or microhabitat used by individual species may alter the extent or likelihood of initial contact with *Bd* (Rowley and Alford 2007a), and thermal and hydric environments chosen by host species may influence disease development (Woodhams 2003; Woodhams et al. 2003; Richards-Zawacki 2009). Host behaviour is also likely to affect pathogen transmission, depending on the frequency of contact between individuals, contact with infected water bodies or contact with contaminated environmental substrates (Lips et al. 2006; Rowley and Alford 2007a). Frog species that are not susceptible to *Bd* in the wild may simply not be coming into contact with *Bd* zoospores, or may exist in environments that do not permit the growth of *Bd*.

Although most anurans mate and deposit eggs in water, many species reproduce terrestrially, depositing their eggs on land. Many of these species undergo direct development, which is characterised by the absence of a free-living, aquatic larval stage and the direct, embryonic formation of adult features (Jennings and Hanken 1998). World-wide, the presence of *Bd* infection in this group of amphibians is low, and very few examples of population crashes have been recorded in the literature. One possible explanation for this could be that their terrestrial life-style limits their exposure to, and reduces contact with, *Bd* zoospores.

Innate and adaptive immunity

In addition to the effects of environment, ecology, behaviour, and life-history, some species may have immune responses that increase their resistance to chytridiomycosis. Like other vertebrates, amphibians have well-developed immune defenses, including both adaptive and innate components (Duellman and Trueb 1994; Carey et al. 1999; Apponyi et al. 2004). Innate immunity functions rapidly, is not pathogen-specific, and includes: antimicrobial

peptides, phagocytic macrophage and neutrophil cells, cytokines, natural killer cells, and acute phase reactants (Carey et al. 1999). Adaptive immunity functions more slowly, improving with repeated exposures, is pathogen specific, and includes direct complement activity through the membrane attack complex or via antibody-antigen systems (Carey et al. 1999; Richmond et al. 2009).

Although limited lymphocytic infiltration in the chytrid-infected skin of frogs suggests that they have a poor cell-mediated innate immune response against *Bd* (Berger et al. 1998; Pessier et al. 1999), frogs with previous exposure to *Bd* can survive secondary infections better than immunologically naïve frogs (Richmond et al. 2009). This suggests that acquired immunity in the form of antibodies and cytokines may be of importance in maintaining protection from infection (Richmond et al. 2009).

Antimicrobial peptides

One mechanism of innate immunity that appears to function in reducing or preventing infection by *Bd* in amphibians is antimicrobial peptides (AMPs). AMPs are found in microorganisms, plants, insects, and in parts of animals including amphibian skin (Nicolas and Mor 1995; Zasloff 2002). The granular glands (also called serous glands or poison glands) in the dermal layer of amphibian skin produce and store an array of host-defensive, bioactive substances which form an integral part of their defense systems (Apponyi et al. 2004). These substances include peptides that have antimicrobial properties (Nicolas and Mor 1995; Rinaldi 2002; Apponyi et al. 2004; Pukala et al. 2006). AMPs are active against bacteria, yeast, fungi, protozoa, and viruses (reviewed in Nicolas and Mor 1995; Rinaldi 2002; Apponyi et al. 2004), and each amphibian species appears to produce its own unique suite of peptides that are effective against a variety of pathogens (Erspamer 1994; Simmaco et al. 1998; Rinaldi 2002; Apponyi et al. 2004; Pukala et al. 2006; Woodhams et al. 2006). Over 300 AMPs have been isolated and described, and *in vitro* experiments have shown that a range of AMPs are active against *Bd* (Rollins-Smith et al. 2002b; Woodhams et al. 2005).

Microhylids

The frog family Microhylidae has a global distribution centred on the tropics (Duellman and Trueb 1994). In Australia, they occur in the far northern periphery of the continent, being restricted to small pockets of rainforest between Townsville and Cape York in Northern Queensland, and at the very northern tip of the Northern Territory (Cogger 1996). In Australia they comprise a limited component of the frog fauna, representing just 9% of the species richness (Hoskin 2004); however, in the rainforests of the Wet Tropics (WT) in northern

Queensland, microhylids dominate frog richness, accounting for 54% of the endemic anuran fauna within the region (Hoskin 2004).

Microhylids of the WT have persisted for at least 10 million years (Hoskin 2004). Long-term stability of the habitats in which they occur best explains their patterns of spatial distribution; the highest species richness occurs in regions where rainforest has been continually available over very long time periods, persisting through historical expansions and contractions (Graham et al. 2006). They are represented in Australia by two genera in the subfamily Genyophryninae (Zweifel 1985): *Cophixalus* contains 14 species, of which 11 are endemic to the WT, and *Austrochaperina* contains five species, of which three are endemic to the WT (Zweifel 2000; Hoskin 2004).

Knowledge of the biology of *Austrochaperina* and *Cophixalus* has lagged behind that of most other Australian frog fauna, with little research conducted on their social and breeding behaviour (Zweifel 1985; Hoskin 2004; Williams et al. 2006). This is primarily due to their restriction to remote areas in the far north, into which access is limited during the wet season when males are calling. Additionally, the species are generally very small and cryptic (Hoskin 2004). It is known that they are terrestrial breeders that occur primarily in rainforest habitats. They require reliably high precipitation, comparatively low temperatures, and high diversity in the vegetation that they inhabit (Williams and Hero 2001). They are predominantly leaf-litter dwellers, but males call from a range of positions, including leaves and axils of palms, fallen epiphytes, and among boulders or rocky scree (Hoskin 2004).

The breeding biology of microhylids is poorly known. Like other genyophrynine microhylids, all breeding records of *Austrochaperina* and *Cophixalus* involve deposition of clutches of large eggs in terrestrial environments away from permanent water sources (Zweifel 1985). Their eggs are heavily yolked and undergo direct development without the production of tadpoles, and hatch as fully formed frogs on land (Price 1992). The majority of Australian microhylids inhabit the forest floor and trees away from permanent water sources, and place their eggs under rocks, logs, fallen debris, or in cricket holes associated with high levels of soil moisture (Dennis and Trenerry 1991). Clutch size across species is consistently small, ranging from 6-22 eggs and averaging ~12 eggs. Eggs are coiled or clumped and at least partially joined by a strong mucilaginous cord (Hoskin 2004).

Felton (1999) showed that *Cophixalus ornatus* females begin courtship after approaching a particular male on the basis of call characteristics. Males lead females to nest sites, and females choose males as mates on the basis of nest site characteristics. Similar courtship behaviour has also been observed in 5 other Australian microhylid species (*A. robusta, C. aenigma, C. bombiens, C. hosmeri,* and *C. neglectus*; Hoskin 2004; Y. Williams pers. comm.).

Microhylids, like other species of direct-developing amphibians, commonly exhibit parental care of the developing egg clutch (Duellman and Trueb 1994; Crump 1995). There is a general trend for an adult frog (particularly males) to be in close proximity to the eggs, and males of several species may brood eggs (Zweifel 1985; Olding 1998; Hoskin 2004). Egg brooding may prevent predation (Kluge 1981), desiccation (Forester 1984; Townsend et al. 1984), and fungal infestation (Forester 1979; Simon 1993) of the developing embryos. Felton (1999) found that male *C. ornatus* spent ~50% of their time tending nests, and that there was greater hatching success in nests guarded by males. This was hypothesised to be due to prevention of desiccation and predation. Simon (1993) found high mortality among microhylid embryos, usually due to fungal attack, when the brooding adult was removed. Amphibians that exhibit parental care in the form of brooding behaviour may possess bacterial flora that inhibit the growth of a range of bacteria and fungi, including *Bd* (Austin 2000; Harris et al. 2006). Egg brooding adults may reduce microbial attacks on eggs by providing either or both of antimicrobial skin secretions or antimicrobial substances produced by their skin microbiota (Harris et al. 2006).

Microhylids are an example of species that exist in cooler habitats at high elevations in the tropics that may be particularly vulnerable to changes in climate. Subtle climatic changes resulting in warmer, drier conditions are potential stressors for these frogs, as unusual weather patterns may alter reproductive behaviour (Beebee 1995), and may cause physiological stress, possibly leading to immunosuppression and increased susceptibility to disease (Carey 1993). Many species have restricted distributions and exist at high elevations, with some species being endemic to specific mountain ranges.

A study by Williams and Hilbert (2006) has emphasised the importance of understanding the effects of changes in climate on susceptible species of frogs. Of the 21 endemic WT vertebrates whose core habitat is expected to be reduced by at least 50% with 1°C of warming, five are microhylid frogs (*C. concinnus, C. hosmeri, Cneglectus, C. exiguus,* and *C. monticola*). Small changes in average climate could have serious effects on the distribution of species, resulting in massive reductions in geographical range, and increased range fragmentation. *Cophixalus concinnus* is undoubtedly one of the most restricted amphibian species in Australia, with a total distribution of only ~7.2km^2 (Hoskin 2004). Whilst *C. concinnus*' habitat is completely protected within the WT World Heritage Area, and the species is currently at high density (Hoskin pers. obs.), bioclimatic modeling of the effect of climate change of this species is alarming (Williams et al. 2003).

Microhylids exist in environments favourable to *Bd*, as they occur in cool, moist habitats, and they also have ecological traits that characterise susceptible species, including occurring at high altitudes, and restricted distributions (Laurance et al. 1996; Lips 1998; McDonald and Alford 1999; Murray and Hose 2005). Despite the absence of extensive

population data, it does not appear that this family has undergone population losses, as observational data suggest that population densities are high and that local extinctions have not occurred (Williams 2007). A lack of population declines in Australian microhylids caused by chytridiomycosis could have several causes. Firstly, the terrestrial environment may not be a competent reservoir for *Bd* because it is drier and warmer than riparian habitats (Brem and Lips 2008). Thus, as microhylids do not reproduce aquatically, and do not seem to enter stream water, they may not be exposed to infective zoospores (e.g., Rowley and Alford 2007a). It is also possible that the microenvironments occupied by microhylids might be too warm, dry, or both for infections to build up and lead to disease. Finally, microhylids as a group may be resistant to infection by *Bd* because they have highly effective innate immune defences against fungi, to reduce fungal infestations that would otherwise overwhelm terrestrial egg clutches.

There is a need for long-term population surveys and fine-scale, ecological analyses of the ecology of narrowly-distributed microhylids. There is also a need to survey the presence and prevalence of infection by *Bd* in nature. Ecological investigations will aid in refining our understanding of the possible consequences of climate change, and will increase the certainty of predictions regarding their future persistence. If this family is susceptible to chytridiomycosis, then the persistence of several species may be in doubt, because they are already at risk due to other features of their ecology. Small changes in environmental conditions may have greatly affected the prevalence and virulence of *Bd* in other species (Pounds et al. 2006); because many Australian microhylids have very limited geographical ranges, alterations in the ambient environment caused by climate change could affect entire species over a very short period of time. Given the recent, dire predictions regarding possible responses of narrowly-distributed species to climate change in the WT (Williams et al. 2003), the added pressure of chytridiomycosis outbreaks could result in the extinction of microhylid species. If this group cannot be infected by *Bd*, then understanding the mechanism of their resistance may aid in captive management and treatment of other species of frogs that are vulnerable to the pathogen. Until the status of *Bd* infection of microhylids in nature is understood, it is impossible to design or implement management strategies to ensure their persistence.

In summary, the family Microhylidae contains the greatest diversity of rainforest endemic frogs in the WT (Williams and Hero 1998), and population losses in this family would severely reduce the remaining amphibian diversity in the region. The apparent lack of susceptibility to declines caused by chytridiomycosis in Australian microhylids, despite severe declines of sympatric frogs of other families, makes them an excellent candidate group to determine how some amphibian species may have resisted the disease.

Study species

The microhylids that were chosen for this study (Table 1.1) are rainforest obligates that have prolonged breeding seasons during the summer wet season (November-March). Seven species were chosen as the study species for a number of reasons. Their abundances at the study sites and the accessibility of these sites for field investigations made them suitable species for ecological studies. The fact that historic data were available for analysis of *Bd* prevalence or population status and calling behaviour for some species increased their usefulness as study species, as comparisons across years could be made. Finally, the prolonged breeding season of these species provided opportunities to carry out numerous surveys at multiple sites.

Table 1.1 Distribution and conservation status of study species (GAA 2004).

Species	IUCN Status	Area (km^2)	Elevational range (m)	Distribution
Austrochaperina fryi Cricket chirper	Least concern	4661	600-1300	Lake Barrine (Atherton Tablelands) to south of Cooktown
Austrochaperina pluvialis White-browed chirper	Least concern	5969	0-900	Seaview range (inland of Ingham) to south of Cooktown
Austrochaperina robusta Pealing chirper	Least concern	5211	400-1590	Paluma range to the Atherton Tablelands to Lake Eacham
Cophixalus aenigma Tapping nursery frog	Vulnerable	930	750- 1300	Mt Lewis to south of Cooktown
Cophixalus hosmeri Pipping nursery frog	Vulnerable	298	900-1250	Carbine uplands - mainly Mt Lewis and Mt Spurgeon
Cophixalus neglectus Tangerine nursery frog	Endangered	37	1150-1600	Mt Bellenden Ker and Mt Bartle Frere
Cophixalus ornatus Common nursery frog	Least concern	6532	0-1590	Bluewater Range (inland of Townsville) to Carbine Uplands

Study Site - Wet Tropics

The WT bioregion of Queensland extends from Cooktown to Townsville along the north-eastern coast of Australia (15-19°S, 145-146°30'E; Figure 1.2). It encompasses a narrow band of tropical rainforest extending over a north-south distance of 450 km, and is largely confined to a series of disjunct mountain ranges with elevations varying between sea-level and 1615m. Although the area of rainforest is small on a global scale (*ca.*10 000 km^2), it is Australia's most extensive remaining area of tropical rainforest. The WT is the most biologically rich area in Australia, supporting over 400 rare or threatened species (Nix and Switzer 1991), and it contains 65 species of rainforest vertebrates that are regionally endemic (Nix and Switzer 1991; Williams and Pearson 1997). The WT has the highest richness of frogs in Australia, with around 50 species occurring within its boundaries. This is reflected in the conservation status of WT frogs, which include the largest number of rare and geographically

localised species. The WT has both the highest anuran endemism and the highest species richness of any area in Australia (Slatyer et al. 2007).

The WT region has been described as a "mesotherm archipelago" comprising a "chain of temperate mountain and tableland islands that rises from the shallow sea of tropical and sub-tropical lowlands" (Nix and Switzer 1991). Analysis of rainfall and temperature patterns of the region reveals a major cluster of mountainous mesotherm "islands" which are separated by the warmer, drier lowland barriers (Nix and Switzer 1991). Partly because of extensive clearing in the lowlands, most rainforest in the WT is above 300m elevation and most of the regionally endemic species are cool-adapted upland species. The present distributional structure of WT vertebrates has been strongly influenced by past climate changes which has led to fragmentation and speciation in a number of species, particularly relatively immobile species such as amphibians (House and Moritz 1991; Nix and Switzer 1991; Winter 1991). Consequently, the distributions of many species are concentrated in the cooler, wetter uplands, and comprise isolated populations (Winter et al. 1984; Winter 1997).

In the WT, eight species of stream-dwelling frogs experienced population declines during the late 1980s and early 1990s (McDonald and Alford 1999). One species (*Litoria genimaculata*) has recovered, but seven others are still endangered. The endangered frogs have a greater fidelity to stream habitats than do common species, and they also have lower fecundity and greater habitat specialisation compared to other rainforest frogs with aquatic larvae (Williams and Hero 1998; McDonald and Alford 1999). *Batrachochytrium dendrobatidis* has been found within the WT and is now endemic to the area (Berger et al. 2004; McDonald et al. 2005; Woodhams and Alford 2005). The earliest confirmed occurrence of *Bd* within the WT is from a *Litoria genimaculata* collected in 1989 (IUCN 2010). The WT is home to 14 species of terrestrial, leaf-litter dwelling, microhylid frogs whose response in the face of chytridiomycosis and population declines of sympatric species is unknown.

Twelve study sites were used in this study. They were situated in tropical rainforest habitat in four main areas within the WT bioregion of northern Queensland. These areas are: the Atherton Uplands (AU), Carbine Uplands (CU), Bellenden Ker Range (BK), and Paluma Range (P; Figure 1.2). Sites were chosen to provide an overview of the latitudinal and altitudinal ranges of the WT, and they encompassed latitudinal range of (15-19°S, 145-146°30'E) and an altitudinal gradient ranging from 200-1550m elevation.

Figure 1.2 Wet Tropics World Heritage Area of northern Queensland showing the four regions where field sampling was conducted. AU = Atherton uplands, BK = Mt. Bellenden Ker, CU = Carbine uplands, and Pal = Paluma.

All transects had been previously established by R. Alford or S. Williams (pers. comm.). Paluma was the most southerly site, located 85 km northwest of Townsville, and Julatten was the most northerly site, located 90km north-west of Cairns. All other sites were situated between these two locations (Table 1.2). The WT provides a rare opportunity to survey a terrestrial frog fauna that occurs sympatrically with species that have suffered drastic population declines in recent history, and with species that occur in areas where *Bd* is now endemic (Berger et al. 2004; McDonald et al. 2005; Woodhams and Alford 2005).

Table 1.2 Site descriptions of transects used in this study.

Label	Location	Latitude and longitude	Elevation (m)	Description
Pal	Paluma Range	146.13E; 19.00S	900	Simple notophyll vine forest
AU2	Mena Ck	145.42E; 17.47S	200	Simple notophyll vine forest
AU4	Henrietta Ck	145.52E; 17.43S	400	Simple notophyll vine forest
AU6	South Johnston	145.52E; 17.43S	600	Simple notophyll vine forest
AU8	Ravenshoe	145.54E; 17.53E	800	Simple notophyll vine forest
AU10	Charmillin Ck	145.31E; 17.42S	1000	Simple notophyll vine forest
CU6	Mt Lewis	145.26E; 16.51S	600	Simple microphyll vine fern forests
CU8	Mt Lewis	145.26E; 16.51S	800	Simple microphyll vine fern forests
CU10	Mt Lewis	145.26E; 16.51S	1000	Simple microphyll vine fern forests
CU12	Mt Lewis	145.26E; 16.51S	1200	Simple microphyll vine fern forests
BK	Mt Bellenden Ker	145.89E; 17.27S	1550	Simple microphyll vine fern forests

Thesis Outline

My research focused on nine species of frogs in the family Microhylidae in northern Queensland. Host-pathogen dynamics in terrestrial ectotherms are complex, and many factors may interact to determine susceptibility to disease. The findings of this study will aid in determining if terrestrially breeding species are infected with *Bd* in the wild, whether they are inherently vulnerable or invulnerable to infection by the pathogen, and to what extent the innate immune system and microhabitat selection influence the relationship between host and pathogen. They will give insights into the possible causes of differences in susceptibility and may help in future conservation decisions, such as captive breeding, translocation or re-introduction of amphibian species.

Chapter 2 -- The long-term calling dynamics of two microhylid species (*A. robusta* and *C. ornatus*) were analysed to determine stability of population size during a period when other species underwent severe population losses associated with epidemics of chytridiomycosis.

Chapter 3 -- Historic, archived specimens and field sampling across the WT using skin swabbing and qPCR analysis were used to examine the prevalence of *Bd* infection in microhylids in nature.

Chapter 4 -- A series of laboratory experiments were conducted to determine whether the most common microhylid, *Cophixalus ornatus*, could become infected by *Bd*, under standard laboratory conditions that favour the growth of the fungus.

Chapter 5 -- The AMP secretions (and various measures of AMP effectiveness) of microhylids were compared amongst species, and with those of various Queensland frogs as revealed by other studies.

Chapter 6 -- The thermal and hydric microenvironments of microhylids in nature were investigated to determine if they could affect the vulnerability of microhylids to infection by *Bd* in the field.

Chapter 7 -- I synthesised the findings presented in Chapters 2-6 and made suggestions for future research on and management of microhylids.

CHAPTER 2: LONG-TERM STABILITY OF CALLING DYNAMICS AND POPULATION SIZE OF MICROHYLIDS AT PALUMA

Abstract

The breeding activity of some amphibian species is tightly correlated with ambient temperature, and changes in climate have had direct effects on breeding phenology. Responses to climate change have been reported for temperate amphibian species with short breeding seasons, however, little information is available on tropical, prolonged-breeding species. I investigated the population density and breeding phenology of two species of Australian microhylid frogs, *Austrochaperina robusta* and *Cophixalus ornatus*. Both are tropical, terrestrial-breeding species with prolonged breeding seasons. I examined temporal calling patterns using automated recordings of calling activity taken every night during five wet seasons, across an 11-year period. Environmental variables were recorded during each season, allowing examination of correlations between calling and weather conditions. Every season, there was a rapid increase in mean and maximum numbers of calling individuals, followed by a plateau in calling activity (the peak calling period), after which numbers of callers decreased rapidly. These patterns were strongly evident in *C. ornatus*, but weaker in *A. robusta*. Calling activity by both species within the peak period was significantly positively correlated with temperature, humidity and rainfall; these relationships were stronger in *A. robusta*. The onset time, length of the plateau, and level of the plateau varied across seasons, but the date of the end of the calling season was very consistent. There was slight variation among years in the numbers of calls recorded during the peak in *C. ornatus*, but calling activity of *A. robusta* was more variable within years than among them. Overall, there was no evidence for a systematic change in the start date or end date of calling activity in either species over the 11-year period, suggesting that seasonal patterns of calling have not been affected by climate change. Variation among years in levels of calling could be caused by differences in population size, or by differences in weather influencing total amounts of calling. I used a bootstrapping approach to discriminate between these hypotheses for *C. ornatus*. I predicted calling levels in each year based on that year's weather, and a model created using responses of calling activity to weather in all other years. I then compared predicted annual levels of calling activity with observed levels. The results suggested that differences among seasons in overall calling activity were likely not caused by responses to short-term variation in weather, but instead were more likely to be due to small changes in population size. This study suggests that these tropical, prolonged breeding species show strong responses to weather, but have thus far shown no systematic changes in breeding phenology in response to global climate change, and no evidence of large or systematic changes in population size.

Introduction

Populations of animals fluctuate over time, due to biotic and abiotic factors (Leigh 1981). Understanding the causes of population variation is a key issue in amphibian conservation biology (Grafe et al. 2004), which has become especially urgent because amphibians of many species are highly threatened (Wake 1991; Alford and Richards 1999; Houlahan et al. 2000; Stuart et al. 2004).

Amphibian populations range from highly variable to highly stable (Hairston 1987; Hecnar and M'Closkey 1996; Skelly et al. 2003). Regulatory processes may keep populations of some species constant over time, however, environmental variation and demographic factors probably keep the majority of populations far from equilibrium (May 1974; Pechmann et al. 1991; Pechmann and Wilbur 1994; Semlitsch et al. 1996; Alford 1999). Dramatic fluctuations in amphibian population density may be driven by unpredictable aquatic environments acting on larval stages (Alford 1999), and thus populations of species that are independent of these environments may be more stable. There are several examples of terrestrial-breeding species that exhibit population stability, including many Plethodontid salamanders (Green 2003), and direct-developing frogs of the genus *Eleutherodactylus* (Stewart 1995; Woolbright 1996; Canelas and Bertoluci 2007).

Temperature and moisture have pervasive effects on amphibian biology, and therefore amphibians may be more vulnerable than other terrestrial vertebrates to changes in ambient environmental conditions (Donnelly and Crump 1998; Gibbs and Breisch 2001). Long-term population studies suggest that the breeding activity of some amphibian species tracks variation in ambient temperature (Reading 1998; Blaustein et al. 2001). Climate change may be one cause of amphibian declines (Carey 1993; Pounds et al. 2006; Laurance 2008) or of shifts towards earlier breeding times in some anuran and urodele species (Terhivuo 1988; Beebee 1995; Tryjanowski et al. 2003; Scott et al. 2008; Neveu 2009). Relationships between higher temperatures and earlier calling activity (Gibbs and Breisch 2001), and earlier arrival at breeding sites have been noted in some frogs (Reading 1998). These trends are not universal, however, and two long term studies have shown no change in abundance or breeding in response to changes in climate (Meyer et al. 1998; Blaustein et al. 2001).

Although information on long-term population trends is critical to understand population variation, there are few published long-term amphibian population studies (Magnusson et al. 1999; Whitfield et al. 2007). Long-term studies provide detailed information on temporal variation in population size and on the frequency of recruitment failure and other demographic events that could be related to population declines (Blaustein et al. 1994; Skelly et al. 1999). The majority of long-term studies of amphibian ecology and population biology have been conducted on temperate species with short breeding seasons, and there is very little

information on the long-term population biology of tropical, prolonged-breeding species. This lack of long-term data severely limits our understanding of natural population variation of tropical amphibians (Pechmann et al. 1991; Wake 1991), especially as tropical anurans may tolerate less variation in temperature than temperate species (Christian et al. 1988).

It is estimated that 43% of all amphibians are experiencing some form of population decline (Stuart et al. 2004), and of these species, about half have been affected by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*; Skerratt et al. 2007). To date, no declines have been reported for Australian frogs of the family Microhylidae, the majority of which occur in the Wet Tropics (WT) bioregion of northern Queensland (Cogger 1996). Extensive declines associated with epidemic outbreaks of chytridiomycosis have occurred in other species in this region, and the pathogen is now endemic (Berger et al. 2004; McDonald et al. 2005; Woodhams and Alford 2005). Two species of microhylids occur in the Paluma range, where several sympatric hylid frogs suffered from declines caused by outbreaks of chytridiomycosis during the late 1980s- early 1990s, and *Bd* has been documented in the region at least as early as 1989 (IUCN 2010).

This study was carried out to determine whether the temporal calling patterns of two tropical, terrestrial-breeding microhylid species changed over multiple years at one site within the WT, either in response to the appearance of an emerging infectious disease, or in response to changes in ambient environmental conditions. Calls were recorded every night over five summer seasons spanning an 11-year period. I examined how the timing of the onset, peak, and decline of calling, and the duration of peak calling, varied among years to determine whether there was any trend over time that might suggest a response to climate change. I examined measures of mean and maximum calling activity during the peak calling season each year to determine whether numbers of calling individuals varied among years. I tested the hypothesis that observed interannual variation might be due to the short tem-responses of calling activity to weather patterns by modeling calling activity during the peak calling season as a function of weather. Summaries of the predicted activity levels were compared to actual levels to determine whether changes in actual levels were likely to represent actual changes in numbers of males available to call.

Methods

Study Site and Recorder Placement

The temporal patterns of calling of two microhylid species (*A. robusta* and *C. ornatus*) were examined using automated recordings of calling activity each night over five wet seasons, beginning in 1995. Data consisted of recordings made from 3rd October through 24th March

during the 1995-96, 2001-02 (Brooke et al. 2000; Hauselberger and Alford 2005), 2002-03, 2005-06 and 2006-07 wet seasons. Recordings were made along a 550-m transect in simple notophyll vine forest (Tracy 1982) on the "H track" at Paluma, Queensland (19°00'40"S, 146°12'37"E, elevation 905m). Annual mean rainfall at Paluma is 2850mm, with an annual mean temperature of 19-21°C (McDonald 1991). Calling activity was scored from three recorders placed along the transect at 110 m, 410 m and 510 m.

Recorders

Automatic timers (HPM Cat D817/2) were attached to Panasonic cassette tape recorders (model # RQ-2102). Recorders were placed in weatherproof containers (modified Australian army ammunition boxes) for use in the field (Figure 2.1). An extension lead was added to the internal dynamic microphone from each recorder to allow it to be placed exterior to the recorder box (Figure 2.1). I put silica gel inside each box to keep the inside environment as dry as possible. Boxes were located on the ground with the microphone end propped up to improve sound recording quality. Sony normal position (Type I/IEC I), and TEAC normal position (superferro Type I) 90-min cassette tapes were used to record nightly activity. Recorder batteries and tapes were replaced every two weeks. Tapes recorded one-minute samples of vocalisations at 19:30, 22:30, and 01:00.

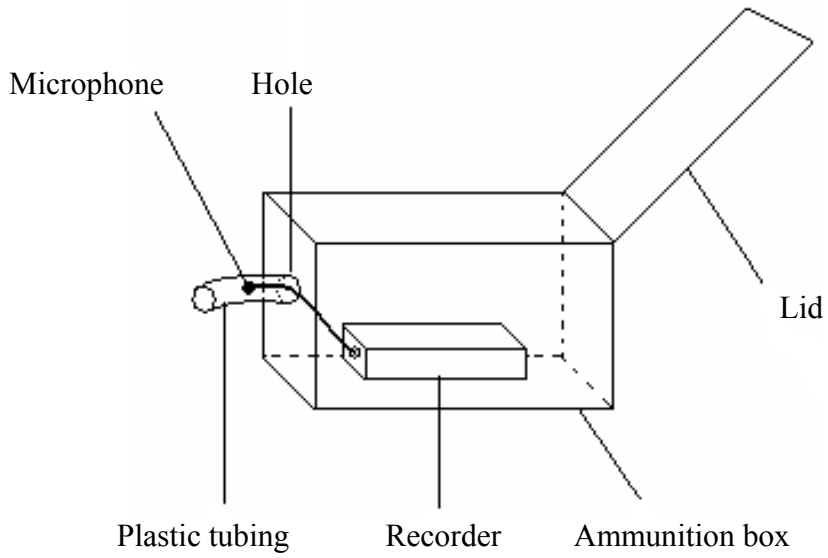

Figure 2.1 Set-up of tape-recorders in ammunition boxes for automatic recording of amphibian vocalisations in the field. A small hole was drilled into one end of the ammunition box, and a piece of plastic tubing was inserted into the hole and sealed with silicone. This tubing allowed an external microphone to be placed outside the box for better sound quality, while still being protected from the elements.

Analysis of recordings

Tapes were analysed by replaying them and tallying the numbers of calls of *A. robusta* and *C. ornatus* that occurred during each one-minute sample. Tapes were replayed on a portable cassette tape player (Panasonic model # RQ-2102), which was set at maximum volume to ensure consistent maximal detection of calls on the tape by the listener. Nights with gaps in recordings caused by equipment failure or by heavy rainfall were omitted from analyses.

Environmental variables

Prior research indicated that levels of *A. robusta* and *C. ornatus* calling are most affected by rainfall, temperature and humidity (Hauselberger and Alford 2005). Measurements of daily minimum temperature and minimum relative humidity (%) at the transect site were obtained for the period 3/10/95-24/3/06 from the Australian Bureau of Meteorology (BOM; Silo Data Drill 2008). These are interpolated daily observations for the location 19º00'S, 146º12'E (Jeffrey et al. 2001). Rainfall measurements consisted of daily recordings made at "Paluma Ivy Cottage", located approximately 200 m northwest of the study site. As rainfall data were recorded for the 24 hours prior to 9:00, I used measures of rainfall reported for 9:00 the morning after each set of frog call recordings was made, so that the rainfall total included rain that occurred on the night being examined. For example, the rainfall data used with recordings made on the night of 12 Dec was rainfall in the 24 hours to 9:00 AM on 13 Dec in the BOM output. I examined correlations of the calling activities of both species across all seasons with these three environmental variables.

Statistical Analysis

Numbers of calls were averaged across the three recording times at all transect locations for each night of the wet season. All calling and rainfall data were $\log_{10}(n + 1)$ transformed prior to analysis to reduce skewness and uncouple means and variances. Visual examination of the relationship between $\log_{10}$(calling activity by *C. ornatus*) and time within each season indicated that calling increased abruptly early in the season, followed by a prolonged plateau period with relatively constant levels of activity, and a final period of abrupt decline to the cessation of calling. Iterative piecewise regression analyses (Ryan and Porth 2007) were used to fit two or three separate linear regressions of $\log_{10}$(calling activity) on time within each season. I first found a preliminary breakpoint between the period of peak calling activity and the period of decline in calling activity late in the season. To do this, a starting point was chosen that fell well within the apparent peak calling period, with the endpoint defined by the end of data collection. I then calculated all possible pairs of regression lines, splitting the time period between points 1 and 2, 2 and 3, … n-1 and n. For each pair of regression lines, I calculated the total sum of the squared deviations of all points from their regression line. I chose the breakpoint that minimised this total sum of squares. I repeated this procedure to find the breakpoint between the period of increasing calling early in the season and the peak period, using data from the date of the first

recording to the time of the breakpoint identified as the end of the peak season in the first analysis. To check that my choice of starting point had not affected the initial analysis, after identifying the breakpoint at the start of the peak calling period, I repeated the initial analysis looking for the second breakpoint; in every case it did not change.

Visual examination of calling data for *A. robusta* showed a pattern similar to that for *C. ornatus*, but with much greater variation in calling levels within the period of peak calling. Calling activity by *A. robusta* responds much more strongly to short-term effects of rainfall than does *C. ornatus* (Hauselberger and Alford 2005). Because of the strong influence of rainfall, although the seasonal pattern of maximum calling activity in *A. robusta* is very similar to the seasonal pattern of mean calling activity in *C. ornatus,* piecewise regression analysis provided poor explanations of the overall pattern, so the data were examined graphically for seasonal pattern but were not analysed statistically. It was clear from these graphs that the peak calling season of *A. robusta* coincides closely with that of *C. ornatus*, so in analyses that examined peak calling periods, the start and end dates estimated for the peak calling season of *C. ornatus* were used for *A. robusta*.

I initially examined responses of calling activity to weather by producing scatterplots of calling activity aggregated across all five peak calling seasons for each species against three weather variables (rainfall in the 24 hours to 09:00 of the day after the recording night, minimum temperature, and minimum relative humidity). These plots suggested that weather set limits on calling activity but did not directly control it in a simple manner. To simplify the response variable and clarify weather effects, I reduced calling activity within the peak period in each season to a binary variable for each species, with a value of 1 for days when calling activity was greater than the overall median value for that species within the peak period across all five seasons, and zero on days when it was less than the overall median. I then used binary logistic regressions for each species to examine how the probability of calling above or below the overall median level was affected by weather.

As a first approach to examining possible trends in calling activity across the period of the study, I examined boxplots for each species that summarised the median, quartiles, and range of calling activity within the peak period for each season. When these suggested that there might be differences among seasons, I performed an additional analysis to determine whether possible differences were consistent with the predicted effects of changes in weather patterns. I modeled the effects of changes in weather patterns by performing a series of logistic regression analyses examining the effects of weather variables on whether the calling level each night was above or below the global median level for the species in question. Each analysis omitted the data for one season. The results of that analysis were then used to predict how many nights in the omitted season should have had calling activity above the global median. Omitting the season for which predictions were made while including all other seasons maximised statistical

power for developing the best possible model of the effects of weather on calling activity, while ensuring that any effects of the season to be modeled that were independent of weather variables were not incorporated in the regression model. If changes across seasons in levels of calling activity were caused by constant responses to weather patterns, the rank order of median levels of calling activity across seasons should be the same as the rank order of predicted number of nights for each season with calling activity above the median. This hypothesis was tested by examining scatterplots and rank correlations of these variables.

Results

Seasonal patters of calling

Calling activity by *C. ornatus* increased to a peak early in the season (November – December) and remained high until late January – early February, followed by a distinct decline in activity (Figure 2.2). In each season, calling ceased in early March. The pattern for *A. robusta* was much more difficult to discern, since activity levels in this species varied greatly from night to night (Figure 2.2). However, the overall pattern seems to coincide with that of *C. ornatus*, with peak calling occurring between November and late January to early February each year.

Recording apparently started after the onset of the peak period in 1995-96 and 2001-02, so for these summers, only peak calling activity and winding down period regressions were estimated. The iterative piecewise analyses for 2002-03, 2005-06, and 2006-07, suggest that the onset of calling always occurred in early October (Figure 2.3; Table 2.1).

The onset, length, and level of calling in the peak calling period varied among seasons (Figure 2.3; Table 2.1). The start date of the peak calling period ranged from 9 October to 12 November (±14 days SD). The end date of the peak calling period ranged from 17th January to 19th February (±14 days SD). The length of the peak calling period ranged from 86-109 ± 9 days. The date of the start of the winding down period (one day later than the end of the peak calling period), was also variable, and the length of the winding down period ranged from 24-55 ± 14 days. The date of the end of the calling period was very consistent across summers and ranged from 11-17 March ± 2 days.

There was no evidence for progressive changes in the starting or ending dates of the peak calling season over time. Overall, the results indicate that calling in *C. ornatus* followed a seasonal pattern in which the onset and peak period of calling varied from year to year. However, the time when calling stopped, and the intensity of calling during the peak period, were not as variable.

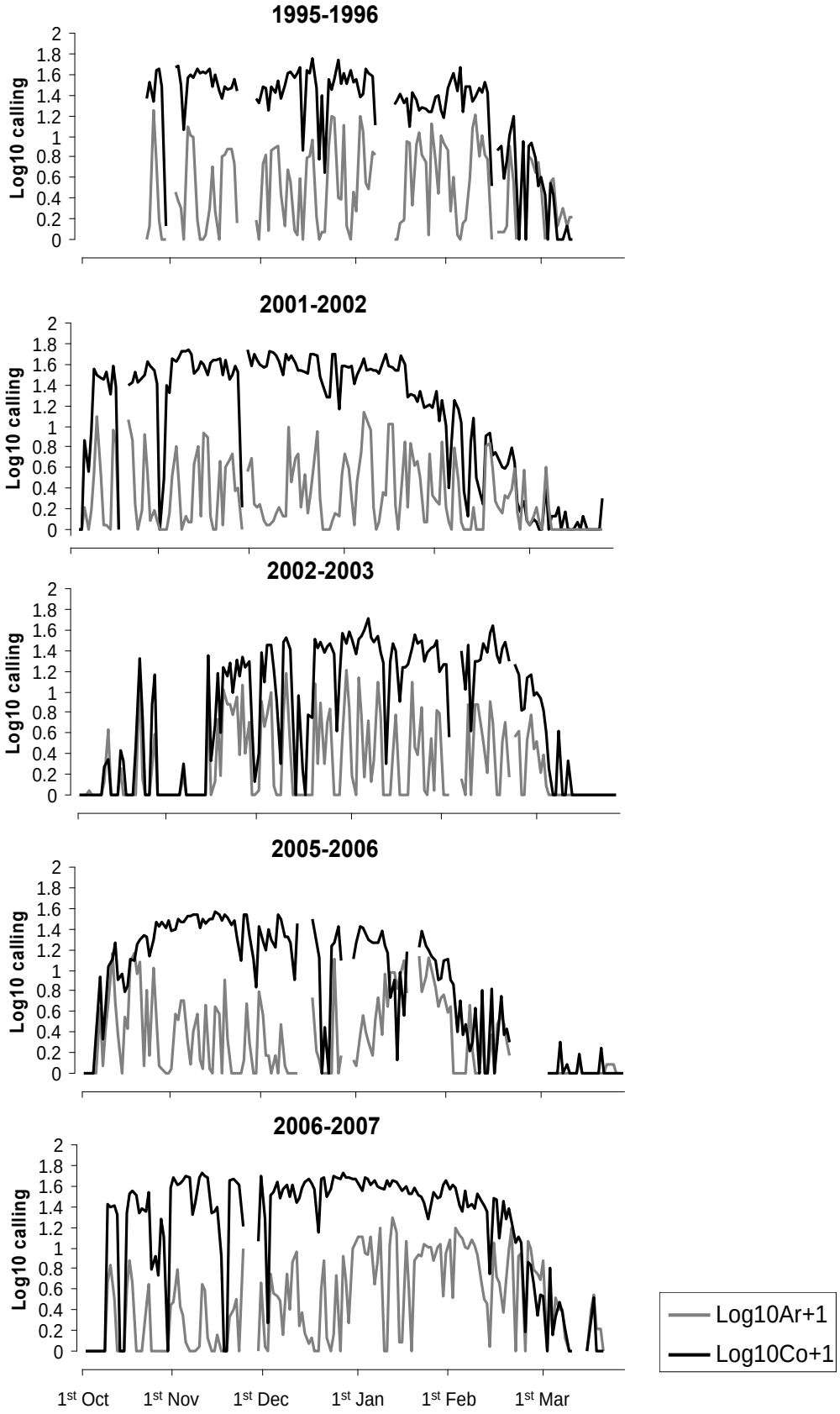

Figure 2.2 $Log_{10}(n+1)$ of the average nightly calling activity of *A. robusta* (grey line) and *C. ornatus* (black line) for each calling season. Gaps in lines indicate dates with no recordings available; zero values are dates with recordings that did not capture any calling activity.

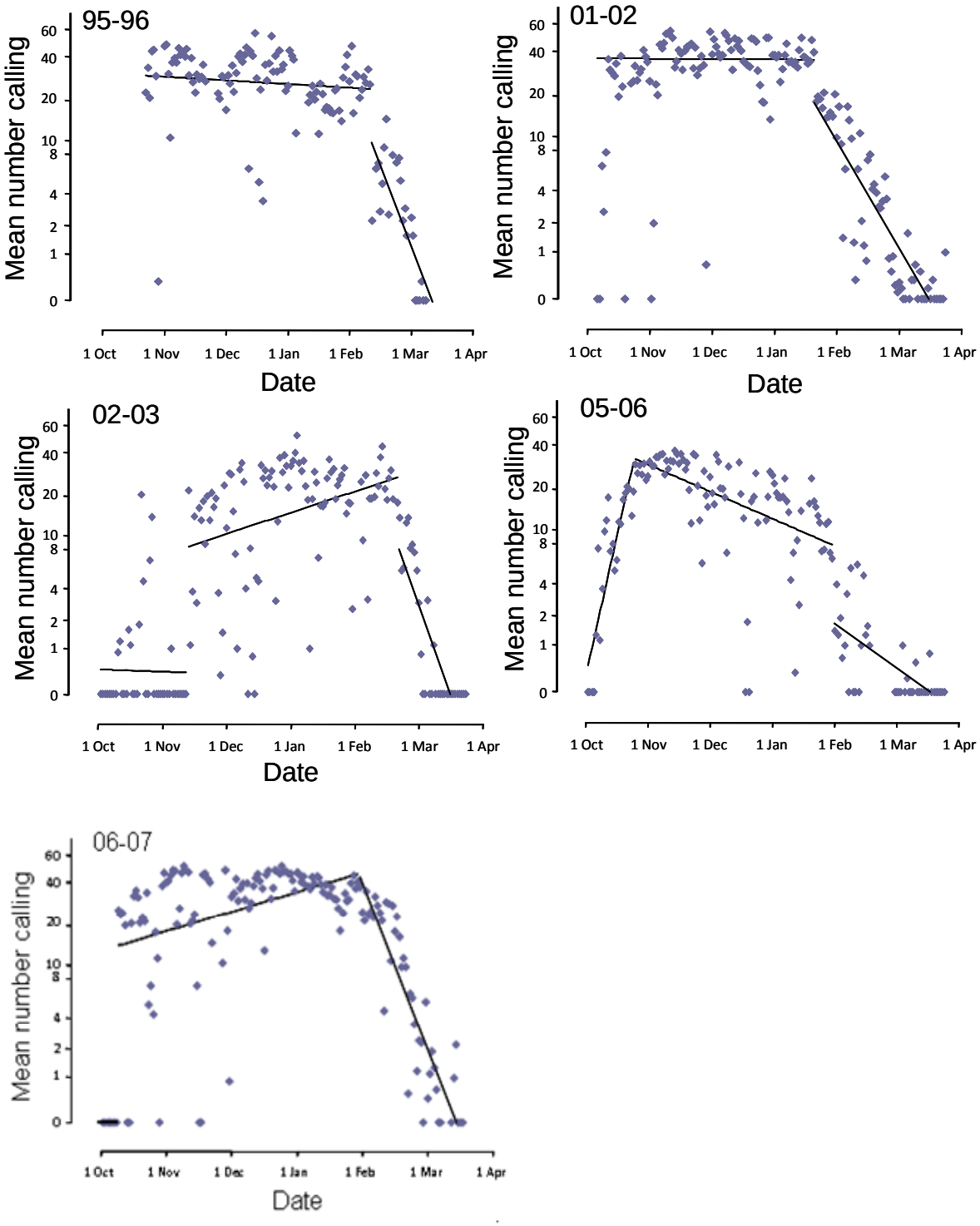

Figure 2.3 Results of iterative piecewise regression analyses of $\log_{10}$(calling activity by *C. ornatus*) across each calling season.

Table 2.1 Iterative piecewise regression analyses on $\log_{10}$(calling activity by *C. ornatus*) across each calling season for peak and winding down calling periods.

Season	Peak calling period					Wind down calling period				
	Start date	Days since October 3 (Day 0)	End date	Days since October 3 (Day 0)	Length of period	Start date	Days since October 3 (Day 0)	End date	Days since October 3 (Day 0)	Length of period
1995-96	23/10/95	20	9/02/96	129	109	10/02/96	130	11/03/96	160	30
2001-02	9/10/01	6	17/01/02	106	100	18/01/02	107	13/03/02	161	54
2002-03	12/11/02	40	19/02/03	139	99	20/02/03	140	16/03/03	164	24
2005-06	26/10/05	23	20/01/06	109	86	21/01/06	110	17/03/06	165	55
2006-07	11/10/06	8	25/01/07	114	106	26/01/07	115	14/03/08	163	48
Mean	22 Oct	19.4	30 Jan	119.4	100	18 Jan	120.4	15 Mar	162.6	42.2
Standard Deviation		13.67		14.08	8.86		14.08		2.07	14.29

Responses of calling to weather variables

Weather variables identified by Hauselberger and Alford (2005) as having the strongest effects on calling activity (rainfall in the 24 hours to 09:00 of the day after the recording night, minimum temperature, and minimum relative humidity) were examined using scatterplots to determine whether they might be related to patterns of calling activity by both species (Figure 2.4). It appears that relationships exist, but they are clearly weak, with all three weather variables acting more to set upper boundaries on potential levels of calling activity than to determine it.

Weather variables accounted for up to 42.8% of the variation in the binary-transformed calling activity in *A. robusta* during the peak period (transformation to a binary variable by rating each night as above or below overall median level during peak periods across all seasons), but only for 7% in *C. ornatus* (logistic regressions, Table 2.2). Calling activity by *A. robusta* was affected very strongly by rainfall. The odds ratios show that as rainfall (mm + 1) increased by factors of 10 (i.e. from 1 to 10, 10 to 100) the odds that calling activity of *A. robusta* was above the median increased almost twentyfold. An increase of one degree in temperature increased the odds of calling above the median by 16.7%, while every percentage point increase in relative humidity increased them by about 5%. The responses of *C. ornatus* were quite different: the effect of rainfall was not significant, and the estimated odds ratio for rainfall was very close to 1, indicating rainfall had little influence on the odds that calling rates would be above the median, while the effect of temperature was similar to that in *A. robusta*, with a 19.6% increase in the odds of above-median calling per degree increase in temperature. The effects of humidity on calling in *C. ornatus* were also similar to those for *A. robusta*; every percentage point increase in humidity increased the odds of above-median calling by about 5%. These results suggest that any differences among seasons in overall calling activity might have been caused by responses to short-term variation in weather, particularly in *A. robusta*.

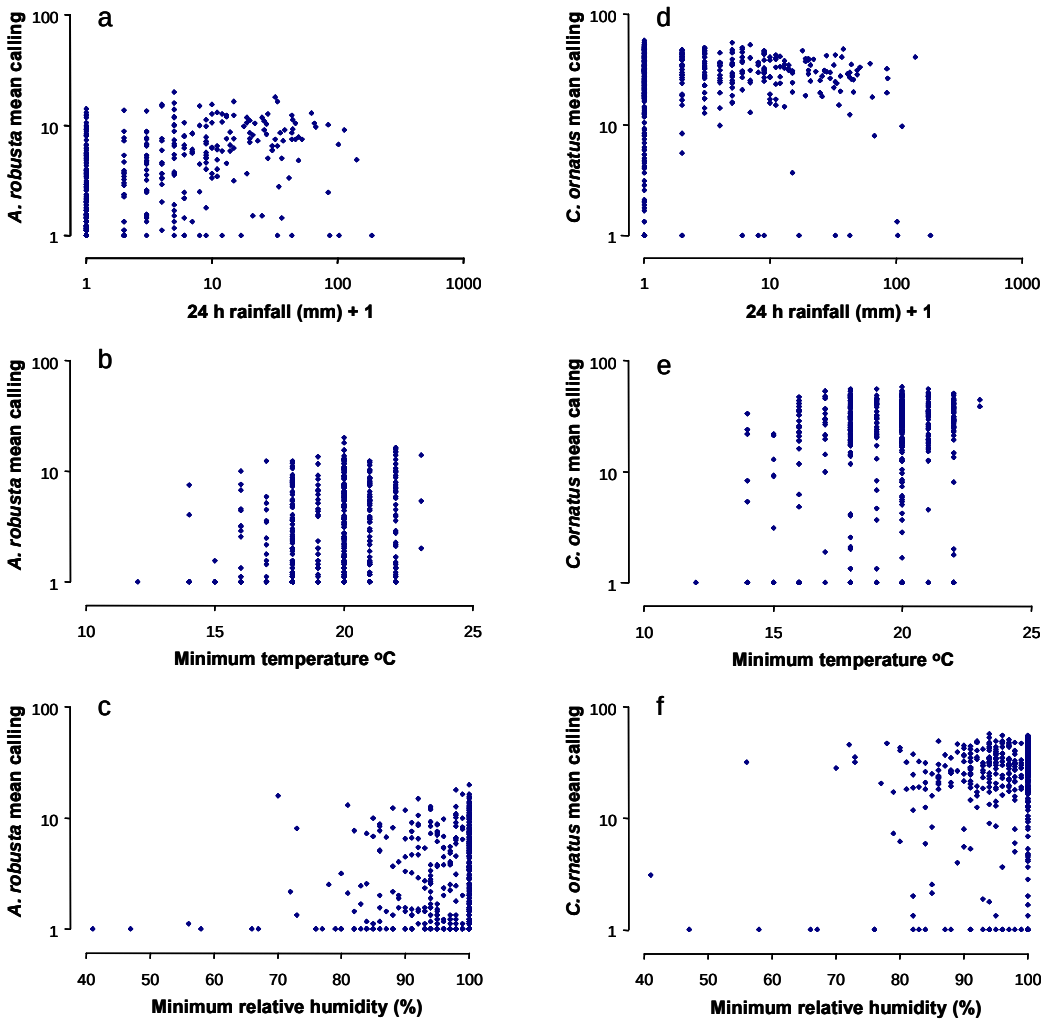

Figure 2.4 (a, b, c) Log_{10} of the mean nightly calling activity during *C. ornatus* peak calling, plotted against weather variables. *C. ornatus* calling activity as a function of log_{10} total 24 h rainfall (mm; r = 0.137, P = 0.003), minimum temperature (°C; r = 0.234, P < 0.001), minimum relative humidity (%; r = 0.282, P < 0.001). **(d, e, f)** *A. robusta* calling activity as a function of log_{10} total 24 h rainfall (mm; r = 0.609, P < 0.001), minimum temperature (°C; r = 0.290, P < 0.001), minimum relative humidity (%; r = 0.177, P < 0.001).

Table 2.2. Logistic regressions on the dichotomised (above or below the global median) calling activity of each species during the peak calling period of *C. ornatus* across all years. The overall models were significant for both species. Nagelkerke R^2 values indicate that the model for *A. robusta* explains 42.8% of the variation in the data, while that for *C. ornatus* explains only 7%.

Factor	*B*	*S.E.(B)*	*Wald* X^2	*d.f.*	*P*	e^B
		Austrochaperina robusta				
Log10(rain (mm) + 1)	2.970	0.320	86.000	1	<0.001	19.500
Minimum temperature (^{o}C)	0.154	0.065	5.608	1	0.018	1.167
Minimum humidity (%)	0.050	0.019	6.948	1	0.008	1.052
Constant	-8.891	2.168	16.814	1	<0.001	0.000
		Cophixalus ornatus				
Log10(rain (mm) + 1)	-0.021	0.181	0.013	1	0.908	0.979
Minimum temperature (^{o}C)	0.179	0.056	10.073	1	0.002	1.196
Minimum humidity (%)	0.050	0.016	10.249	1	0.001	1.051
Constant	-8.097	1.832	19.527	1	<0.001	0.000

Patterns of calling activity across seasons

Levels of calling activity during the period of peak calling were similar across all five seasons in both species (Figure 2.5). Note that no tests of statistical significance were carried out to detect differences among seasons, because statistical tests are used to detect whether samples are likely to be from different populations. For the purpose of examining how patterns of calling activity differed among seasons, I had available the complete populations of data points, so any differences seen are real differences. In *A. robusta*, there was no calling on at least 25% of nights in all seasons. The median level of calling activity across all nights fluctuated between approximately 1.5 and 3 calls per recording per minute, caused by changes in the proportion of nights with no calling activity. The maximum level of calling activity, which should be the best estimate of the actual maximum number of males, was relatively constant across the 11-year period, ranging from a minimum of 13 in 2001-02 and 2005-06 to 19 in 2006-07, a maximum difference of 1.46 times. The pattern of change in *C. ornatus* was different. In all seasons there were relatively few nights with no calling. The median level of calling activity differed among seasons and was lowest in 2002-03, when it was 19.3 calls per recording per minute, and highest in 2006-07, when it was 36 calls per recording per minute. Maximum levels, which (as with *A. robusta*) should be the best reflection of the maximum number of males available to call, and thus of male population size, were similarly variable, ranging between 50.3 and 56.7 except in 2005-06, when the maximum was 35.7, a maximum difference of 1.58 times.

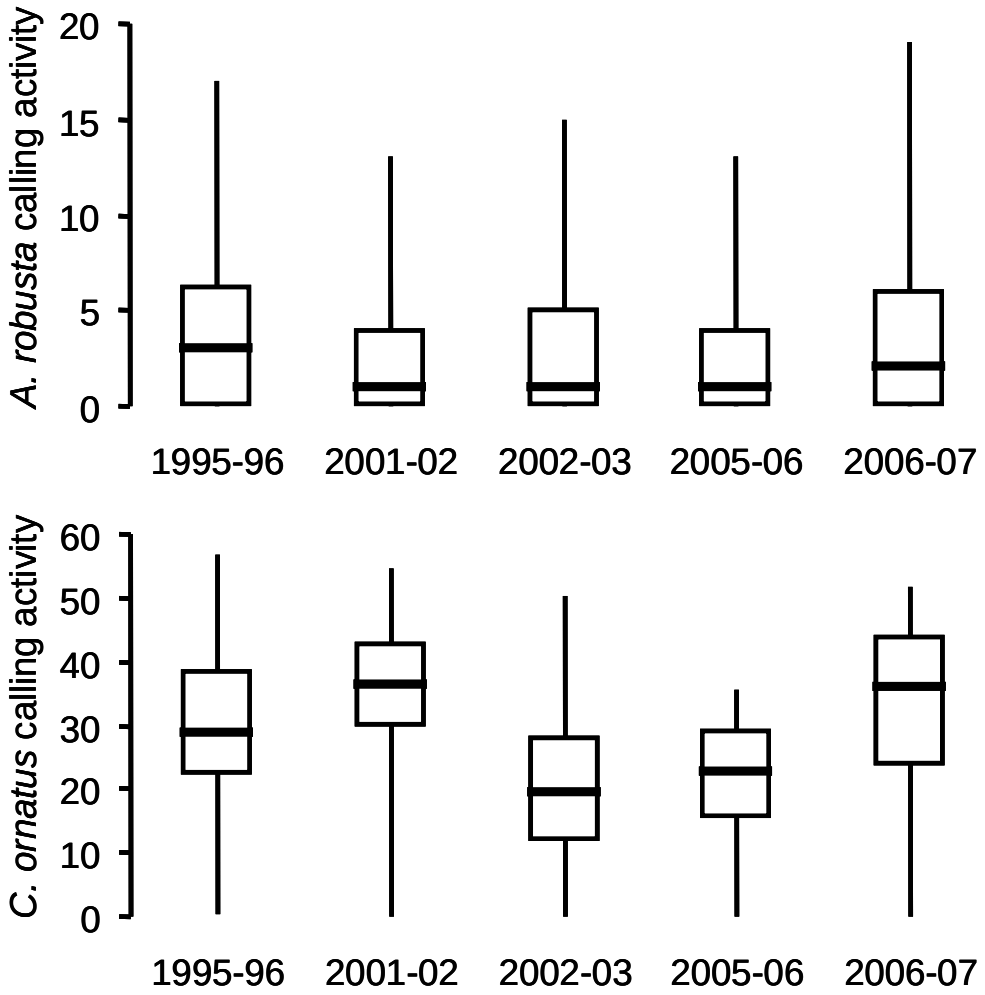

Figure 2.5. Tukey (1977) boxplots illustrating the distributions for each species of mean nightly calling activity during the peak calling period of *C. ornatus* (Figure 2.3) across each season. Heavy lines represent medians, boxes represent upper and lower quartiles, and lines represent ranges.

Role of weather in producing differences among seasons

Although changes in calling activity levels during the peak calling period were relatively small, I carried out a series of analyses to determine if they were likely to be caused by responses of each species to changes in weather patterns among seasons. Logistic regressions for each species with the data for each season omitted, showed that the patterns for each species were consistent (Table 2.3). *Austrochaperina robusta* always responded strongly to rainfall, and less strongly to temperature and humidity, and roughly 40% of the variation in the probability of calling at above-median activity levels was explained by each variable. Rainfall was not included in models for *C. ornatus* because the overall models (Table 2.2) made it clear that it has very little effect on this species. *Cophixalus ornatus* responded strongly to temperature and humidity, the effects of which were consistent across all of the models.

The models presented in Table 2.3 were used to create predictions of relative calling intensity (above or below the global median) for each species on each night within the peak calling period of *C. ornatus* in each season. I used these predictions to evaluate whether the changes I observed among seasons may have been caused largely by changes in weather and the responses of frogs to those changes. Figure 2.6 presents bivariate plots illustrating the relationship for each species between the actual percentage of nights in each calling season with activity levels above the global median, and the percentage predicted to be above the global median using the models presented in Table 2.3. If the responses to weather predicted by those models had caused the observed differences among seasons, there should be a positive linear correlation between the observed and predicted percentages of nights above the median. There was no significant relationship for *A. robusta* ($r = -0.105$, $P = 0.886$), indicating that the observed seasonal differences were not simply responses to weather as modeled by the logistic regressions. There was an apparent negative relationship for *C. ornatus,* however this is not significant ($r = -0.788$, $P = 0.113$). The lack of positive correlations between observed patterns of calling intensity among seasons and patterns predicted using modeled responses to weather variables suggested that the differences I observed in calling intensity (Figure 2.5) were not caused by responses of calling frogs to differences in weather patterns among seasons, but may have been caused by small changes in the number of males available to call, either because of changes in the size of the underlying population or movements of individuals into and out of the study area.

Table 2.3. Logistic regressions on the dichotomised (above or below the global median) calling activity of each species across all seasons, each with one season omitted. These models were used to predict calling activity levels for the omitted season for comparison with actual calling data. All R^2 values are Nagelkerke.

Factor	*B*	*S.E.(B)*	*Wald* X^2	*d.f.*	*P*	e^B
		Austrochaperina robusta				
	1995-96 data omitted, R^2 = 0.491					
Log10(rain (mm) + 1)	3.537	0.399	78.452	1	<0.001	34.366
Minimum temperature (^{o}C)	0.106	0.074	2.037	1	0.153	1.112
Minimum humidity (%)	0.065	0.024	7.218	1	0.007	1.067
Constant	-9.684	2.586	14.020	1	<0.000	0.000
	2001-02 data omitted, R^2 = 0.399					
Log10(rain (mm) + 1)	2.658	0.334	63.334	1	<0.001	14.270
Minimum temperature (^{o}C)	0.159	0.074	4.647	1	0.031	1.172
Minimum humidity (%)	0.053	0.020	6.676	1	0.010	1.054
Constant	-9.018	2.402	14.089	1	<0.001	0.000
	2002-03 data omitted, R^2 = 0.406					
Log10(rain (mm) + 1)	2.804	0.347	65.389	1	<0.001	16.506
Minimum temperature (^{o}C)	0.159	0.071	5.107	1	0.024	1.173
Minimum humidity (%)	0.045	0.020	4.826	1	0.028	1.046
Constant	-8.367	2.307	13.149	1	<0.001	0.000
	2005-06 data omitted, R^2 = 0.469					
Log10(rain (mm) + 1)	3.311	0.381	74.971	1	<0.001	24.717
Minimum temperature (^{o}C)	0.186	0.070	6.996	1	0.008	1.205
Minimum humidity (%)	0.041	0.021	3.712	1	0.054	1.041
Constant	-8.582	2.283	14.136	1	<0.001	0.000
	2006-07 data omitted, R^2 = 0.386					
Log10(rain (mm) + 1)	2.640	0.342	59.407	1	<0.001	14.010
Minimum temperature (^{o}C)	0.176	0.082	4.669	1	0.031	1.193
Minimum humidity (%)	0.056	0.023	6.137	1	0.013	1.058
Constant	-9.832	2.798	12.348	1	<0.001	0.000
		Cophixalus ornatus				
	1995-96 data omitted, R^2 = 0.108					
Minimum temperature (^{o}C)	0.254	0.062	17.049	1	<0.001	1.290
Minimum humidity (%)	0.050	0.017	8.197	1	0.004	1.051
Constant	-9.541	2.008	22.578	1	<0.001	0.000
	2001-02 data omitted, R^2 = 0.038					
Minimum temperature (^{o}C)	0.130	0.062	4.466	1	0.035	1.139
Minimum humidity (%)	0.037	0.017	5.142	1	0.023	1.038
Constant	-6.211	1.962	10.025	1	0.002	0.000
	2002-03 data omitted, R^2 = 0.060					
Minimum temperature (^{o}C)	0.137	0.059	5.329	1	0.021	1.147
Minimum humidity (%)	0.049	0.016	8.833	1	0.002	1.050
Constant	-6.900	1.897	13.320	1	<0.001	0.000
	2005-06 data omitted, R^2 = 0.109					
Minimum temperature (^{o}C)	0.274	0.060	20.761	1	<0.001	1.316
Minimum humidity (%)	0.039	0.017	5.402	1	0.020	1.040
Constant	-8.695	1.876	21.487	1	<0.001	0.000
	2006-07 data omitted, R^2 = 0.074					
Minimum temperature (^{o}C)	0.185	0.071	6.781	1	0.010	1.203
Minimum humidity (%)	0.067	0.020	11.534	1	0.001	1.070
Constant	-10.137	2.465	16.918	1	<0.001	0.000

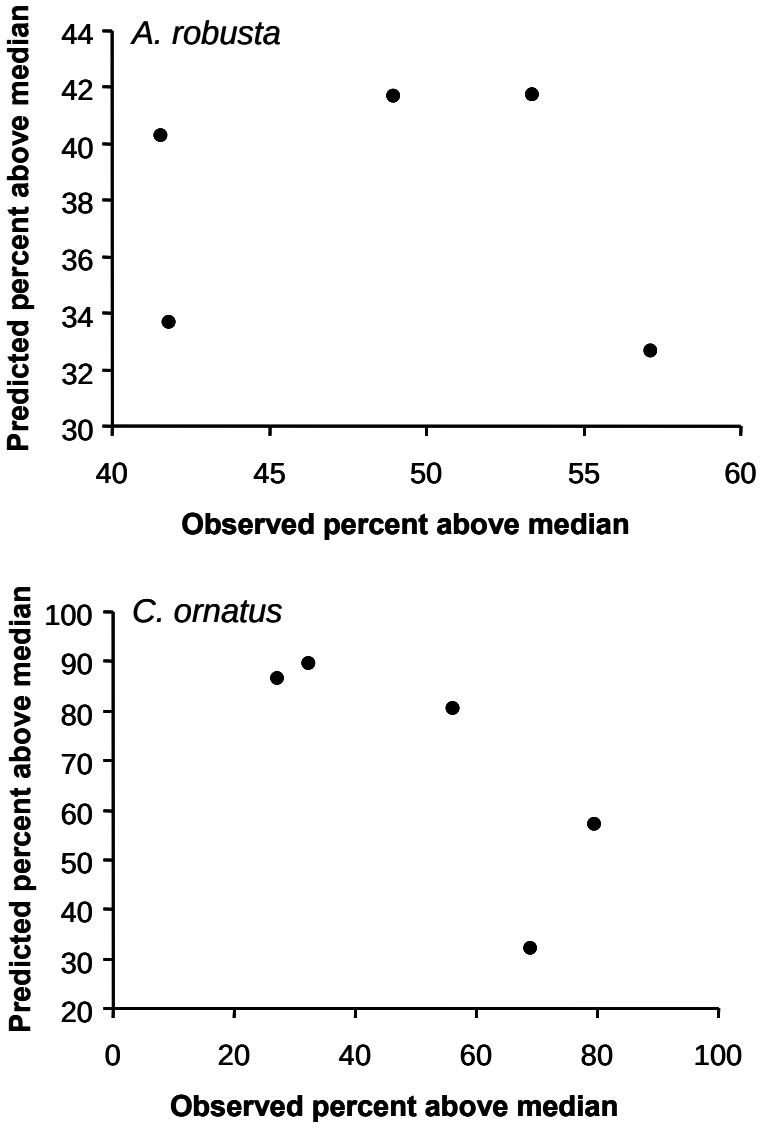

Figure 2.6. Percent of nights in each season predicted to have calling activity above the median for that season, using a logistic regression model that left out data for that season, as a function of the percent of nights that had calling activity above the global median in that season. If differences in calling activity among seasons were caused by responses of frogs to weather data as modeled by logistic regression, there should be a positive linear relationship for each species.

Discussion

Most tropical amphibian species show characteristic patterns of breeding phenology, and are influenced by abiotic factors, including rainfall, temperature, and humidity (Stewart and Pough 1983; Donnelly et al. 1994; Stewart 1995; Magnusson et al. 1999; Gottsberger and Gruber 2004; Hauselberger and Alford 2005). *Cophixalus ornatus* had a clearly defined peak calling period, which extended for roughly three months, in which males tended to call at high levels on most nights, unless temperature or humidity were unseasonally low. For *C. ornatus,*

the timing of onset and end of the peak calling period were variable. On the other hand, the length of the peak calling period and the timing of the end of the calling season were very consistent across all seasons. The high degree of consistency of the end of calling in *C. ornatus* suggested it may be determined by a predictable seasonal cue such as day length, perhaps because of a need to allow time for development and posthatching growth of young before winter. *A. robusta* calling was more variable throughout the season, but the propensity to call appeared to peak around the same time as the peak in *C. ornatus* and end at the same time.

Many studies of other tropical anurans have found seasonal breeding, with frogs typically breeding only in the wet season (Aichinger 1987; Duellman 1995; Gottsberger and Gruber 2004; Canelas and Bertoluci 2007; Fong et al. 2009). Some species can be continuously active (Gascon 1991; Gottsberger and Gruber 2004), whilst others depend on rainfall in the previous 24h for calling to occur (Woolbright 1985; Gottsberger and Gruber 2004). Tropical species that are highly dependent on weather might alter their phenology in response to changes induced by climate change, however, no study to date has shown that such changes have occurred.

There were no consistent patterns of change in any aspect of microhylid reproductive phenology at Paluma through time, indicating that their breeding phenology did not change in any consistent way over the period 1995-2007. Patterns across seasons in the average number of calls each night during the peak period of calling activity were similar for both species. There was little variation in *A. robusta* calling over the sampling period; median values for calling in one minute samples ranged from 1.5-3 calls, and the maximum number of callers ranged from 13-19. For *C. ornatus*, calling activity was slightly more variable, with median values ranging from 19-36 calls, and the maximum number of callers ranging from 50-56, except in the 2005-06 season when the maximum was 35. Variation in calling activity could be caused by changes in the motivation of individuals to call, or by actual changes in the number of individuals in the population. Thus, the maximum number of calls heard in any night in a season gives a more accurate estimate of the actual number of frogs present along the transect, as on 'good' nights it should be tightly correlated with the maximum number of males available to call. There was no downward trend in average or maximum numbers of calls for either species. Although a dip in average calls occurred in one season in *C. ornatus*, the maximum calls for both species were similar at the start and end of the sampling period, and the observed fluctuations were less than two-fold. These results suggest that there was relatively little variation in the total number of frogs along the transect for either species. There was variation in the timing of the onset of calling, the length of the peak calling period, the end of the peak calling period, and to a lesser extent in the end of the calling season, across the study, however there was no consistent pattern of directional change. In summary, although male population sizes and calling phenology of *C.*

ornatus and *A. robusta* at Paluma fluctuated slightly over the study period, neither species showed long term directional changes in either parameter.

The changes that did occur could have been caused by short-term changes in weather patterns. Within the calling season, levels of reproductive effort in terrestrially breeding frogs may track environmental cues such as temperature and rainfall, which could indicate nights of greater or lesser chance of successful mating (Woolbright 1985). The calling activity of both species responded to short-term variation in weather variables in a manner indicating that weather may set limits on calling activity. Logistic regressions of the binary-transformed (above or below the global median) calling activity of both species across all years showed that environmental variables accounted for almost 43% of the variation in calling by *A. robusta*, but only 7% for *C. ornatus*. Rainfall had the strongest effect on calling activity in *A. robusta*, followed by temperature and then humidity. In *C. ornatus*, only temperature and humidity had significant effects on calling activity. The results of this study parallel and reinforce those of my earlier work (Hauselberger and Alford 2005) indicating that the calling activity of *C. ornatus* and *A. robusta* was affected by environmental variables. In that study, calling was strongly related to rainfall in *A. robusta* but not in *C. ornatus*, and both species showed lower levels of calling activity when air temperature or humidity were unusually low. These results may simply reflect the fact that as nocturnal ectotherms, rates of possible energy expenditure decrease as temperature decreases, and as terrestrial frogs, they reduce activity levels under conditions of low humidity to conserve water.

Despite both species showing correlations between calling activity and weather variables, the results of my modeling study suggest that fluctuations in seasonal patterns of calling activity did not reflect responses to changes in short-term weather variables. When the observed patterns of calling intensity within the peak calling period for each season were compared to patterns predicted using logistic regressions, there was no suggestion of a significant positive relationship for either species. This suggests that the differences I observed in calling intensity may have been caused by small changes in the number of males available to call among years, rather than by weather.

Changes in the number of males across seasons could have been caused by an external factor such as the fungal pathogen *Bd*. *Batrachochytrium dendrobatidis* is present at the study site (Berger et al. 1999a; Kenyon 2008), and the earliest season (1995-96) was close in time to the period in which population declines occurred in the Paluma region. Three hylid species experienced population declines in that region during 1989-1992, including two species that disappeared from the area (*Nyctimystes dayi* and *Litoria nannotis*; Richards et al. 1993; Richards and Alford 2005). It is possible that *Bd* could have caused fluctuations in the population size of microhylids at Paluma, however, declines following an initial epidemic of *Bd* are likely to have been missed in this study. Even if the disease took time to reach the study site, initial outbreaks

in these sympatric species would be expected to be synchronous (Lips et al. 2006; Schloegel et al. 2006). The fluctuations that occurred were relatively minor, minimum numbers occurred only in 2001-02 (*A. robusta*) and 2005-06 (*C. ornatus*), between 10 and 15 years after the documented declines in hylid species, and numbers of calling individuals were similar at the beginning and end of the study.

I suggest that the observed fluctuations in numbers of calling male *A. robusta* and *C. ornatus* are more likely to be artifacts of behaviour, or due to natural minor fluctuations in recruitment. Variation in the numbers of male frogs available to call could have been caused by changes in the size of the local population as males moved in or out of the study area. Population variation may also be due to minor fluctuations in recruitment in both species over the years, as amphibians can forgo breeding during years in which the climate is unfavourable (Pechmann et al. 1991).

Although long-term studies dealing with reproductive activity patterns of tropical anurans are rare, those that have been carried out suggest that terrestrial-breeding species tend to exhibit relatively stable populations in comparison to other amphibian species. Relatively high population stability has been documented in frogs of the genus *Eleutherodactylus* and other species of frogs and salamanders with terrestrial, direct development (Hairston and Wiley 1993; Green 2003; Bustamante et al. 2005). In Puerto Rico, for example, population densities of the terrestrial, direct developing frog species *Eleutherodactylus coqui* vary seasonally, but in the long term populations appear to be relatively stable (Stewart 1995; Woolbright 1996). Variation in numbers of adults from year to year were small, numbers were similar at the beginning and end of the survey, and there was only a seven-fold difference between the smallest and largest population density during a survey which included the occurrence of a hurricane (Woolbright 1996). This is a relatively small fluctuation compared to those of temperate aquatic breeding species that are subject to large fluctuations in recruitment from their aquatic larval stages (Alford and Richards 1999), but is large compared to the maximum fluctuation of ca. 1.5 times, that I found over the 11 years of my study.

Tropical anurans may have less physiological plasticity than do temperate species, and may, therefore, be more susceptible to the effects of global warming (Christian et al. 1988). Amphibian responses to climate change are numerous (reviewed by Blaustein et al. 2003; Carey and Alexander 2003; Lips et al. 2008; Bickford 2010), but few studies focus on effects in tropical species with terrestrial breeding, and the evidence for those species is varied. For example, in a comparison of the relative abundance and composition of amphibians across the Andes, the majority of amphibian species that underwent declines had aquatic larvae (Bustamante et al. 2005). Although terrestrial species with direct development (from the genus *Eleutherodactylus*) represented 49% of the species surveyed, they did not show significant changes in species richness. Higher temperatures and an increased number of dry days were

hypothesised to be responsible for the declines. However, in surveys of eight, terrestrially-breeding *Eleutherodactylus* species in Puerto Rico from 1989-2001, species at low and mid-elevations (up to 400m) showed stable populations, but declines and extirpations occurred in high-elevation species (Burrowes et al. 2004). Analysis of weather data indicated significant warming trends and extended periods of drought, which coincided with amphibian declines.

Mountain ecosystems like those in the Australian WT are among the environments most seriously affected by climate change, because they are often refugia for cool-adapted endemic species. Temperature increases are expected to push current climatic envelopes up mountains, decreasing the area of available habitat for specific species, and the bioclimates of some montane species are predicted to disappear entirely with even moderate rises in temperature (Williams et al. 2003). However, the results of the present study indicate that at least two tropical, direct-developing amphibian species with prolonged breeding maintained relatively stable population sizes and reproductive phenologies at one site over an 11-year period from 1995-96 through 2006-07. My study species are, however, two of the most widely distributed Australian microhylids, with relatively wide geographical and elevational ranges. This suggests that they may tolerate a wider envelope of temperatures than narrowly-distributed species. Microhylids with higher degrees of habitat specificity might be more strongly affected by changes in climate. For example, *Cophixalus concinnus* is one of the most restricted amphibian species in Australia, with a total distribution of less than 8km^2 (Hoskin 2004). Bioclimatic modelling suggests that a rise in average global temperatures of 1°C could result in the complete loss of the current range and core habitat of this species (Williams et al. 2003). Long-term studies of this and similar species would be very useful in examining the possible effects of climate change.

Understanding the factors that influence patterns of change in populations is critically important to many areas of basic and applied ecology (Grafe et al. 2004), as the magnitude of population fluctuations may indicate the risk of local extinction (Leigh 1981), may provide information about the processes that drive population dynamics (Semlitsch et al. 1996), and may affect the power of monitoring programs to detect real declines (Gerrodette 1987). Population monitoring should be initiated for species of microhylids with narrowly restricted ranges, as these species are likely to be more sensitive to the effects of future habitat modification and climate change.

CHAPTER 3: PREVALENCE OF *Batrachochytrium dendrobatidis* INFECTION IN DIRECT-DEVELOPING AUSTRALIAN MICROHYLIDS

Abstract

The emerging infectious disease chytridiomycosis has been implicated in the declines and disappearances of amphibian populations around the world. However, the extent of pathological effects appears to vary among hosts, and frogs and salamanders with life-histories that include parental care of direct-developing terrestrial eggs may tend to be less susceptible. I examined a total of 595 samples from nine species of direct-developing Australian microhylids for the presence of infection by *Batrachochytrium dendrobatidis* (*Bd*). Of these, 336 archived samples were collected between 1995 and 2004; 102 were analysed histologically and 234 were examined using quantitative PCR (qPCR). Swab samples from 259 frogs were collected during 2005-08 and were examined using qPCR. None of the 595 samples showed evidence of infection by *Bd*. If these data are regarded as a single sample representative of Australian microhylids, the upper 95% binomial confidence limit for the prevalence of infection in frogs of this family is 0.0062 (less than 1%). Even if only the data from the more powerful diagnostic qPCR tests are used, the upper 95% confidence limit for prevalence is 0.0074 (less than 1%). My data suggest that Australian microhylids have a very low prevalence of *Bd* in nature, and thus are either not susceptible, or are only slightly susceptible, to chytridiomycosis. This may be due to ecological characteristics, or to immune functions such as anti-microbial skin peptides, or antimicrobial symbionts in skin flora.

Introduction

Pathogenic organisms are frequently cited as important drivers of community dynamics (Anderson and May 1986; McCallum and Dobson 1995; Cleaveland et al. 2001; Hudson et al. 2001), and disease-causing organisms have been implicated in the decline and extinction of a wide variety of wildlife species (Warner 1968; Van Riper III et al. 1986; Berger et al. 1998; Cunningham and Daszak 1998; Daszak et al. 1999). Pathogens can cause mass mortality of species, converting species rich systems into depauperate communities that are dominated by a few resistant species (Burdon 1991). This phenomenon has been observed in island birds (Warner 1968; Van Riper III et al. 1986), forest trees (Burdon 1991), and rainforest frogs (Lips et al. 2006). In these scenarios, some species experience severe declines, others decline less severely, and others suffer no losses. Host susceptibility can differ greatly among

and even within species (Wakelin 1978), and disease dynamics are affected by factors relating to the host, the pathogen, and the surrounding environment (Warner 1968; Van Riper III et al. 1986; Lips et al. 2006).

Chytridiomycosis is an emerging infectious disease caused by *Batrachochytrium dendrobatidis* (*Bd*), a chytrid fungus that parasitises the mouthparts of larvae and the keratinised epidermis of post-metamorphic amphibians (Berger et al. 1998; Longcore et al. 1999). This pathogen has been associated with population declines of amphibian species in many regions (Berger et al. 1998; Daszak et al. 2003; Lips et al. 2006; Skerratt et al. 2007), including at least 14 threatened Australian species (Speare and Berger 2004b). *Batrachochytrium dendrobatidis* causes mortality in a large range of amphibians, however, susceptibility to infection and the extent of pathological effects vary greatly among host species (Davidson et al. 2003; Daszak et al. 2004). Where outbreaks of chytridiomycosis have occurred, it is common for some species to suffer local extinction, while others decline and some suffer no population-level effects (Lips and Donnelly 2002; Retallick et al. 2004; Lips et al. 2006; Schloegel et al. 2006). Experimental data have demonstrated that significant inherent differences exist within (Davidson et al. 2003), and among species infected with *Bd* in a laboratory setting (Woodhams et al. 2007a). There have been four patterns of effects of *Bd* on frog species:

1. It is presumed to have caused global extinctions of species (Retallick et al. 2004; Schloegel et al. 2006; Gewin 2008).
2. Species have suffered extinctions of local populations, but have escaped global extinction (Ingram and McDonald 1993; Gillespie and Marantelli 2000).
3. Populations have declined during initial outbreaks but have subsequently recovered and show apparently stable associations with *Bd* (Retallick et al. 2004; McDonald et al. 2005).
4. Many species have appeared to be unaffected at the population level (McDonald and Alford 1999).

The association of *Bd* with frog species thus provides an excellent model system in which to study variation in disease susceptibility.

Variation in susceptibility to *Bd* can be associated with ecological and life-history traits of host species (Williams and Hero 1998; Lips et al. 2003; Retallick et al. 2004; Woodhams and Alford 2005). Because the fungus requires a moist environment to multiply and release infective zoospores (Powell 1993; Pessier et al. 1999), the habitat or microhabitat used by species may affect the extent or likelihood of initial contact with *Bd* (Rowley and Alford 2007a). Habitat and microhabitat use reflect behaviour, and thus pathogen transmission, by affecting the frequency of contact with infected individuals, infected water bodies, or contaminated environmental substrates (Lips et al. 2006; Rowley and Alford 2007a). For

example, frog species that have not declined due to *Bd* may simply not come into contact with *Bd* zoospores. After individuals become infected, the thermal and hydric environments experienced by the host species can have a large influence on disease development (Woodhams 2003; Woodhams et al. 2003), and species that are not affected by chytridiomycosis at the population level may exist in environments that limit the growth of the fungus.

Several studies have found correlations between species that have exhibited mass mortalities and their behavioural, ecological or life-history characteristics. Declining amphibian species tend to have low fecundity (Williams and Hero 1998); but note that this study excluded microhylids to arrive at this conclusion), specialised habitat requirements (Murray and Hose 2005), montane distributions (Blaustein and Wake 1990; Wake 1991; Richards et al. 1993; Lips 1998; McDonald and Alford 1999), and reproduction associated with streams (Lips 1998; McDonald and Alford 1999; Lips et al. 2003; Woodhams and Alford 2005).

In Australia, frog species with life-histories incorporating direct development, which therefore have no aquatic, tadpole stage, have not frequently been found infected by *Bd*, and are not known to have suffered from population declines associated with outbreaks of chytridiomycosis. In a survey of frog species in south-eastern Queensland, only a single terrestrial-breeding frog (*Assa darlingtioni*) was found infected with *Bd* out of 23 individuals sampled (prevalence of 4.3%). In contrast, 156 permanent and ephemeral stream and pond breeders out a total of 496 individuals were found infected with *Bd* (prevalence of 31.5%; Kriger and Hero 2007b). Of 60 amphibian species from upland (<300m) areas in eastern Australia, only one species (*Philoria frosti*) out of 20 with direct development has undergone declines, whilst 20 of the 40 species with an aquatic stage in their life-history have experienced population declines (Hero et al. 2005).

Declining species are also characterised by having aquatic larvae in Brazil (Eterovick et al. 2005), Ecuador (Ron et al. 2003; Bustamante et al. 2005), Venezuela (La Marca 1995), Costa Rica (Crump et al. 1992; Pounds et al. 1997; Lips 1998), Panama (Lips et al. 2006; Brem and Lips 2008), Spain (Bosch et al. 2001), and the USA (Muths et al. 2003). World-wide, relatively few species of terrestrial, direct-developing frogs that bypass a free-swimming tadpole stage have been found infected by *Bd* (Appendix 1). Few examples of population crashes of species with terrestrial oviposition and direct development have been recorded in the literature and even fewer can cite the presence of *Bd* as a possible cause of the declines. Examples come from Central America (Lips et al. 2006; Brem and Lips 2008), Puerto Rico (Burrowes et al. 2004), and New Zealand (Bell et al. 2004).

The apparently lower vulnerability of direct-developing species may be due to the absence of tadpoles, which may often be involved in transmission of *Bd*, because they are likely to be exposed to the fungus' aquatic zoospores (Daszak et al. 1999). In addition to the absence of a tadpole stage, direct-developing species commonly exhibit some form of parental care of

the egg clutch, which may prevent fungal infestation of the developing embryos (Forester 1979; Simon 1993). Amphibians that brood their eggs can possess antimicrobial skin secretions or symbiotic bacterial flora that inhibit the growth of a range of bacteria and fungi, including *Bd* (Austin 2000; Harris et al. 2006). Thus, adaptations that normally serve to protect developing egg clutches may predispose terrestrial breeders to a reduced susceptibility to *Bd* infection.

The extensive research undertaken on the distribution of occurrence of *Bd* in Australia and its effects on frogs has focused on taxa recognised as being threatened by declines. *Batrachochytrium dendrobatidis* has been found on 57 Australian species from four families (Hylidae, Microhylidae, Myobatrachidae and Bufonidae; Speare and Berger 2004a; Kenyon 2008). The families Microhylidae and Ranidae are the only native families of Australian frogs for which no declines related to chytridiomycosis have been reported.

Most microhylid species occur in the Wet Tropics (WT) region of Queensland (Cogger 1996), where extensive declines have occurred in other species and the pathogen is now endemic (Berger et al. 2004; McDonald et al. 2005; Woodhams and Alford 2005). Although they do not breed aquatically, microhylids inhabit cool, moist habitats; environments that should be favourable for *Bd*. They also possess ecological traits that characterise declining species, such as occurring at high altitudes and having low reproductive output and restricted distributions (Laurance et al. 1996; Lips 1998; McDonald and Alford 1999; Murray and Hose 2005). Although no long-term monitoring studies have been published, it appears unlikely that species in this family have undergone declines, as population densities appear to be high (Hauselberger 2001; Hauselberger and Alford 2005; Williams 2007).

Only two species of Australian microhylids (*Austrochaperina robusta* and *Cophixalus ornatus*) from two separate populations within the WT (Paluma and Babinda) have been surveyed for infection by *Bd*. Histological surveys of *A. robusta* individuals at Pauma failed to detect any infected animals (D. Mendez pers. comm.; Hauselberger 2001), whilst a single *C. ornatus* collected in September 2005 tested positive for *Bd* DNA using a diagnostic quantitative PCR assay (Kriger and Hero 2006a). This single sample was tested in triplicate (K. Kriger, pers comm.), and establishes that it is possible for microhylids to be infected. However, the results of a single sample provide no information about the prevalence of *Bd* on microhylids or its distribution among species or populations.

This study aims to determine the extent to which Australian microhylids are infected with *Bd* in nature. This information is a necessary preliminary to understanding the extent to which they may be threatened by chytridiomycosis. If *Bd* infections were common in Australian microhylids, the future of several species would be in doubt due to their extremely small geographical ranges. Small changes in environmental conditions can greatly affect the prevalence and virulence of the pathogen in other species (Woodhams et al. 2003; Pounds et al. 2006; Richards-Zawacki 2009), so alterations in environmental factors could affect entire

species over a very short period of time. Given the recent, dire predictions regarding possible responses of narrowly-distributed species to climate change in the WT (Williams et al. 2003), the added pressure if tolerance to endemic chytridiomycosis decreased, could result in the extinction of microhylid species. If *Bd* occurs at very low prevalences or is absent from many populations, it is probably due to factors innate to microhylids, such as their ecological or life history traits, and chytridiomycosis may not constitute a great threat. If innate mechanisms are responsible for a high degree of resistance to infection, understanding these mechanisms may aid in captive management and treatment of frogs that are more vulnerable to the pathogen.

Materials and methods

Analysis of *Bd* infection

One hundred and two archived samples were analysed for *Bd* infection using histological techniques. 493 samples were analysed using the qPCR assay methods developed by Boyle et al. (2004). Diagnostic qPCR is a much more sensitive assay and is highly specific (Boyle et al. 2004; Kriger and Hero 2006b; Hyatt et al. 2007). Of the samples analysed using qPCR, 451 samples were analysed at James Cook University (JCU), Townsville by R. Campbell, S. Garland and A. Phillot, and 42 samples were analysed at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Australian Animal Health Laboratory (AAHL), Geelong, under the supervision of A. Hyatt. Standard protocol in both the JCU and AAHL CSIRO PCR laboratories includes running each sample in triplicate and including a positive control in each PCR run that should reveal the presence of inhibition in samples.

Archived samples

Three hundred and thirty six archived samples of seven microhylid species (*Cophixalus aenigma*, *C. bombiens*, *C. hosmeri*, *C. infacetus*, *C. neglectus*, *C. ornatus* and *Austrochaperina robusta*) were available from previous research. The samples consisted of toe-clips and tissue sections preserved in 70% ethanol, or prepared slide sections of toe clips, stored at JCU and the University of Queensland (UQ). Samples dated back to 1995, and came from five separate research studies (Brooke et al. 2000; Hauselberger and Alford 2005; Felton et al. 2006; Williams 2007; C. Hoskin pers. comm.; Table 3.1).

Table 3.1 Location of archived sample collection sites within the WT region of northern Queensland.

Label	Location	Latitude S	Longitude E	Elevation (m)	Collector
AU2	Meena Ck	17°40.01'	145°52.60'	200	Williams
AU4	Henrietta Ck	17°36.92'	145°45.32'	400	Williams
AU6	South Johnston	17°40.09'	145°43.25'	600	Williams
BF1	Mt Bartle Frere	17°26.11'	145°51.29'	100	Hoskin
BF7	Mt Bartle Frere	17°24.98'	145°50.12'	700	Hoskin
BF13	Mt Bartle Frere	17°24.36'	145°49.31'	1350	Hoskin
BK	Mt Bellenden Ker	17°15.84'	145°51.31'	1550	Williams
Hai	Mt Haig	17°05.60'	146°36.09'	900	Hoskin
Mau	Mausman	17°20.79'	145°41.67'	740	Hoskin
Pal	Paluma	19°00.42'	146°12.43'	900	Brooke Felton Hauselberger

All ethanol-preserved tissue samples were processed for the detection of *Bd* DNA using qPCR. Each sample was removed from its storage vial using forceps and transferred to an individually labeled Eppendorf tube containing 50% ethanol. Forceps were sterilised, and any DNA that might have adhered to them was destroyed, by submerging in 100% ethanol and flaming between each use. Brooke et al. (2000) collected several hundred toe-clips from *C. ornatus* at Paluma during August 1995 - March 1996; these had been stored at JCU. 201 samples were used in qPCR analysis for detection of *Bd* DNA. Williams et al. (2006) collected toe-clips from various microhylid species throughout the WT during September 2003 – March 2004; I used 18 samples from 5 species for diagnostic qPCR. I also examined samples from *C. ornatus* and *C. neglectus* that were originally collected by J. Milton and C. Hoskin, and were stored at UQ. Several frogs collected from Mt. Bartle Frere in January 2001 were brought back to UQ in Brisbane and died in captivity shortly thereafter. These individuals were thought to have died from chytridiomycosis, and were stored in 70% ethanol. I obtained 15 tissue samples from these individuals, each consisting of a strip of skin cut from the ventral thigh or abdominal region. They were treated in the same manner as toe clips.

Samples that existed as prepared histological slides (originally used for skeletochronological determination of age) were examined using a light microscope to detect *Bd* infection by searching for fungal structures in the *stratum corneum* (Berger et al. 2000a). These samples had been prepared histologically as 7um sections, stained in heamotoxylin and eosin, and mounted on microscope slides. Felton et al. (2006) collected toe-clips from 47 *C. ornatus* at Paluma from 1998-1999, and Hauselberger and Alford (2005) collected toe-clips from 55 *A. robusta* in 2000-01 at Paluma.

Swab samples

Seven species (*Austrochaperina fryi*, *A. pluvialis*, *A. robusta*, *Cophixalus aenigma*, *C. hosmeri*, *C. neglectus*, *C. ornatus*) of microhylid frogs were sampled for the presence of *Bd* infection using skin swabs. Nine, 500m transects were set up within the WT region (Table 3.2), and each transect was sampled at least once each wet season (September-March) from 2004-08. Frogs were located by their vocalisations during night surveys, or during the day in log turning surveys. Night surveys began after sunset (19:00h), and continued until the full transect was sampled or until males ceased calling. Male frogs were found by walking along transects and listening for advertisement calls, whereas females, immatures and non-calling males were located opportunistically. Day surveys consisted of lifting large pieces of wood or logs that were encountered along transects and examining the habitat underneath for frogs. Samples were collected up to approximately 50m on each side of the transect line. To determine the body temperature of individual frogs before capture, the surface temperature of each frog was measured using an infared (IR) thermometer (Raytek ST80 Pro-Plus Non-contact laser thermometer). The IR thermometer was placed approximately 20cm away from the frog aiming at the lower dorsal area. Emissivity on the thermometer was set at 0.95. Temperatures measured in this way very closely match both surface temperatures of frogs measured using contact thermocouples (Rowley and Alford 2007b).

Frogs were captured in clean, unused, 20x20cm plastic bags, and each frog was handled with a new pair of latex gloves. Strict hygiene protocols for the handling of individual frogs and for disinfection of equipment between field sites were followed (Speare et al. 2004). A sterile medical cotton swab (Medical Wire & Equipment Co. Bath Ltd., Wiltshire, UK) was run over the dorsal and ventral surfaces of each frog, including the forelegs, and hindlegs, according to methods described by Skerratt et al. (2008). This procedure generates the highest sensitivity using the qPCR assay for detection of *Bd* DNA (Hyatt et al. 2007). It allows sampling of all dorsal and ventral surfaces, is non-invasive and not harmful to the individual. To prevent contamination and reduction in the number of zoospores detected by the qPCR assay, each swab was placed directly into its individual container after the swabbing procedure, and stored in a refrigerated environment as soon as possible after collection (<2hr; Van Sluys et al. 2008). When a refrigerator was not available, samples were placed on ice in a portable, insulated container.

Table 3.2 Location of collection sites within the WT region of northern Queensland.

Label	Location	Latitude S	Longitude E	Elevation (m)
AU2	Meena Ck	17°40.01'	145°52.60'	200
AU4	Henrietta Ck	17°36.92'	145°45.32'	400
AU6	South Johnston	17°40.09'	145°43.25'	600
AU10	Charmillin Ck	17°40.86'	145°31.19'	1000
BK	Mt Bellenden Ker	17°15.84'	145°51.31'	1550
CU8	Mt Lewis	16°35.38'	145°17.35'	800
CU10	Mt Lewis	16°35.21'	145°16.36'	1000
CU12	Mt Lewis	16°30.56'	145°16.35'	1200
Pal	Paluma	19°00.42'	146°12.43'	900

Batch samples

Due to the cost of qPCR analysis, some samples were analysed in batches. One hundred archived samples from Brooke et al. (2000) were analysed in batches of ten, where ten toe clips were placed into a single container for qPCR analysis. This was only carried out on samples that contained more than one toe clip, so that if a positive result occurred, individual toes could be retested. Eighty field samples collected from 2004-08 were run in batches of four, where four swabs were prepared together for qPCR analysis. All field samples collected consisted of two swabs per individual, so that if a positive result was found in the qPCR analysis, the second set of swabs could be individually retested.

Statistical Analysis

I calculated two-tailed Clopper-Pearson 95% binomial confidence limits for the prevalence of *Bd* in a variety of populations, using the program StatXact 4.0 (Cytel Software Corp. 1998). Sample sizes and two-tailed Clopper-Pearson 95% binomial confidence limits were calculated for all areas surveyed both in field and archived samples. For field sampling, results for the three years of surveys (2004-08) were collated, and confidence limits for infection prevalence in populations at each of the sites surveyed during this time period were estimated. For the archived data, samples were not collated, and confidence limits were calculated independently for each sample at each location.

Results

Archived samples

Three hundred and thirty six archived samples, taken from seven species, were analysed for the presence of *Bd* using histological and qPCR techniques. All samples were negative (Table 3.3). This included a group of *C. neglectus* and *C. ornatus* individuals that were collected from Mt Bellenden Ker in early 2001 and died in the laboratory shortly after. It had been assumed these individuals succumbed to chytridiomycosis; however analysis of samples

indicated no fungal DNA. The small sizes of many samples have led to high upper 95% confidence limits for prevalence, but the three larger samples had upper 95% confidence limits below 10%, and the sample for Paluma in 1996, which was analysed using the sensitive qPCR assay, had an upper 95% confidence limit of 1.8%. Aggregated across all archived samples, the upper 95% confidence limit for prevalence was 1.1%.

Table 3.3 Archived samples analysed for the presence of *Bd* using qPCR and histological techniques. All samples returned negative results.

Species	Date	Location	Analysis	n	Upper 95% CL
A. robusta	Oct 2000 – Mar 2001	Pal	Histology	55	0.065
C. aenigma	Sep 2003 – Mar 2004	AU6	qPCR	4	0.602
C. bombiens	Sep 2003 – Mar 2004	AU4	qPCR	1	0.975
C. hosmeri	Sep 2003 – Mar 2004	AU2	qPCR	3	0.708
C. infacetus	Sep 2003 – Mar 2004	AU4	qPCR	6	0.459
C. neglectus	Jan 2001	BF13	qPCR	1	0.975
	Sep 2003 – Mar 2004	BK	qPCR	4	0.602
C. ornatus	Sep 1995 – Mar 1996	Pal	qPCR	201	0.018
	Oct 1998 – Mar 1999	Pal	Histology	47	0.075
	Jan 2001	BF1	qPCR	4	0.602
	Jan 2001	BF7	qPCR	4	0.602
	Jan 2001	BF13	qPCR	4	0.602
	Jan 2001	Hai	qPCR	1	0.975
	Jan 2001	Mau	qPCR	1	0.975
Total				336	0.011

Field data

None of the 259 swab samples taken from seven species during surveys in 2004-08 tested positive for the presence of *Bd* using diagnostic qPCR (Table 3.4). The small sample sizes at many sites mean that upper 95% confidence limits for prevalence were high, but at the three sites with larger samples they were below 10%. Aggregating all the field samples taken during 2004-2008 to estimate an upper 95% confidence limit for microhylid frogs in the Australian WT produced an upper 95% binomial confidence limit for prevalence of 1.4%

The surface temperatures of 237 of the frogs from which samples were collected were recorded. Temperatures ranged from 10.8-24.3°C (Table 3.5); the mean surface temperature was 18.1°C.

Table 3.4 Field samples (2004-2008) analysed for the presence of *Bd* using qPCR techniques, and upper Clopper-Pearson 95% binomial confidence limits for population prevalence of *Bd* across field sites. All samples were negative for the presence of *Bd*.

	AU2	AU4	AU6	AU10	CU8	CU10	CU12	Pal	BK	Total
A. fryi					5	2				7
A. pluvialis	1				3					4
A. robusta		2	1	1				22		26
C. aenigma					1	2				3
C. hosmeri	2									2
C. neglectus									132	132
C. ornatus				57	2		1	23	2	85
Total	3	2	1	58	11	4	1	45	134	259
Upper 95% conf. limit	0.71	0.84	0.98	0.06	0.28	0.6	0.98	0.08	0.03	0.014

Table 3.5 Maximum, minimum and mean surface temperatures of frogs collected during the 2004-2008 field season.

Temperature (ºC)	AU2	AU4	AU6	AU10	CU8	CU10	CU12	Pal	BK	Total
Maximum	24.3	22.1	20.4	23.8	21.9	19.6	18.4	24	20.4	
Minimum	21.2	21.9	20.4	17.1	20.6	19.2	18.4	19.2	10.8	
Mean	22.8	22	20.4	19.8	21.2	19.5	18.4	21.2	15.7	18.1
N	3	2	1	56	11	4	1	41	118	237

If all of the data for diagnostic qPCR assays of archived and survey samples (0 positives from 493 individuals) are combined as a sample of WT microhylids in general, the upper 95% binomial confidence limit for prevalence in this sample is 0.75%. The upper 95% confidence limit for the 102 samples examined histologically (all found to be negative) is 3.55%. Considering the total of 595 negative samples together gives an upper 95% binomial confidence limit of 0.62% for prevalence.

Discussion

None of the samples analysed in this study tested positive for the presence of *Bd* DNA. Considering only the results of the most sensitive, widely accepted procedure, diagnostic qPCR of swab samples, at two sites (Bellenden Ker and Paluma) sample sizes were large enough to indicate that the prevalence of *Bd* infection is below 5% and 10%, respectively. If *Bd* infections occur in these populations, they occur at prevalences well below those documented for Australian, stream-associated frogs (Berger et al. 2004; Kriger and Hero 2007a; Kriger et al. 2007; Kenyon 2008; Murray et al. 2009). Although aggregating samples taken across multiple

species and sites is potentially problematic, it has commonly been done in the literature on chytridiomycosis (Weldon et al. 2004; Sanchez et al. 2008). The results for my swab samples indicate that *Bd* infection occurs in the sample aggregated across species and populations at a mean prevalence less than 1.4%. Including the results of the potentially less sensitive tests on alcohol-reserved samples raises the total to 493 individuals that returned negative diagnostic qPCR results, and reduces the upper 95% confidence level for prevalence in the sampled species to less than 1%. Including the demonstrably less powerful histological results for the total of 595 negative individuals reduces it further. These data indicate that the prevalence of *Bd* in WT microhylids is very low in nature, and suggests that Australian microhylids have low susceptibility to infection by the pathogen. Results far less powerful than this have been used in the literature on chytridiomycosis to suggest that the pathogen is absent from entire faunas (Weldon et al. 2004).

It is possible that my results may include false negatives. The use of histological analysis instead of diagnostic PCR, low infection intensity, degradation of fungal DNA due to inhibitors or temperature, and batching samples can all lead to reduced sensitivity of diagnostic assays. Hyatt et al. (2007) demonstrated that diagnosis of *Bd* infection via histology is less sensitive than qPCR in all but the very early stages of infection, but that qPCR assays of toe-clips have a sensitivity similar to swab samples. All archived samples analysed using qPCR were fixed and stored only in ethanol, which is not known to degrade the sensitivity of qPCR tests (Soto-Azat et al. 2009), and is used as standard procedure by some laboratories (Hyatt et al. 2007). It is thus unlikely that the use of archived toe-clip samples in this study produced false negatives.

Inhibitors may be present in a swab sample if foreign material such as dirt or detritus is picked up on the swab, or may be present in skin secretions of some frog species or life stages (Hyatt et al. 2007). However, standard protocol in both the JCU and AAHL/CSIRO laboratories includes a positive control in each PCR run that should reveal the presence of inhibition, and no evidence of inhibition was ever detected in our samples. As it was not always possible to refrigerate samples collected in remote areas, increased temperatures may have led to a reduction of the amount of *Bd* DNA detected on swabs using the qPCR assay (Van Sluys et al. 2008). This effect was minimised, as air temperatures at sampling sites were typically below 30^{o}C, and swabs were always refrigerated or stored on ice < 2hr after the samples were taken. Hyatt et al. (2007) demonstrated that storing swabs at 23°C in the laboratory for 18 months did not reduce detection sensitivity.

The main factor in the methodology that could compromise the sensitivity of the qPCR assay to unacceptable levels, is batching of samples into groups of ten. Hyatt et al. (2007) showed that the maximum number of swabs that can be pooled without lowering the sensitivity of the qPCR assay is five. Batching groups of greater than five lowers the sensitivity of the test

in laboratory trials, especially for samples that contain a low number of *Bd* zoospores. Batching samples in groups of 10 produced false negative results in 30% and 50% of samples that were spiked with one and ten zoospores respectively. No negative results occurred in samples that contained 100 zoospores (Hyatt et al. 2007). In this study, batch samples that were run in groups of 10 were whole toe clip samples rather than skin swabs. As heavier infections of *Bd* are concentrated on the legs and feet in some amphibian species (North and Alford 2008), and because whole tissue samples are expected to hold more *Bd* DNA than swabs, It is unlikely that the reduced sensitivity of the test produced more than a small number of false negatives, if it produced any. The pooling procedure was only used for half of the Paluma collection of archived samples. The other half of the samples were analysed individually and also returned uniformly negative results.

Although it is not possible to rule out with 100% certainty the possibility that false negatives occurred in some of my 493 diagnostic qPCR results, they are unlikely to have affected a high proportion of the samples. As diagnosis of *Bd* infection via histology is less sensitive than qPCR (Hyatt et al. 2007), results for the 102 histological samples diagnosed may have suffered from a higher rate of false negatives than my qPCR samples. However, they form a relatively minor part of the data, and even if false negative rates for these samples are assumed to be high, the conclusion that prevalence in WT microhylids must be very low is not affected.

For three specific sites (Paluma, site AU10 at Charmillin Creek on the Atherton Tableland, and Mt. Bellenden Ker) sample sizes from the swab surveys are sufficient in themselves to demonstrate that the prevalence of *Bd* in microhylids is very low, if it is present at all. The fungus has been present in stream-associated frogs in all three areas for a substantial period of time. At least two frog species (*Litoria nannotis* and *L. genimaculata*) have been found infected with *Bd* at Paluma since 1989 (Berger et al. 1999a; Kenyon 2008; IUCN 2010). In a study that documented *Bd´*s spread upon arrival at a site (El Cope in Panama; Lips et al. 2006), it spread throughout the amphibian fauna at the site, including species not closely associated with streams, within months of its first appearance. There were numerous declines of frog populations on the Atherton Tablelands in the region of site AU10 (Richards et al. 1993) which have subsequently been attributed to chytridiomycosis; histological examination confirmed the presence of the fungus at multiple sites on the Atherton Tableland in the late 1990s and early 2000s (IUCN 2010). Frogs declined in the rainforest block containing Mt. Bellenden Ker in the early 1990s (Richards et al. 1993), and *Bd* has been present there since at least 1999 (IUCN 2010). The positive record for *Cophixalus ornatus* at Babinda Creek (Kriger and Hero 2006a) is from a stream that drains from this rainforest block. These results make it clear that the absence, or extremely low prevalence, of infections by *Bd* in the frogs at these widely-separated sites must reflect the biology of the microhylid frogs.

One possible explanation for the absence or low prevalence of *Bd* infection in microhylids could be due to a very high sensitivity to chytridiomycosis. Individuals may be killed shortly after infection in the wild, which would drastically reduce the probability of infection – even with large sample sizes. However, experimental infection in one species of microhylid did not yield a fatal response, even under environmental conditions ideal for the growth of *Bd* (Chapter 4).

Another possible explanation for the absence or low prevalence of *Bd* infection in Australian microhylids could be the terrestrial habits of these frogs; they might simply not come into contact with fungal zoospores. However, terrestrial habits did not protect frogs at El Cope, Panama from becoming infected shortly after the pathogen first appeared at the site (Lips et al. 2006). Lips et al. (2006) suggested that within sites, chytridiomycosis spreads by a combination of frog-frog and environment-frog transmission. These modes of transmission have been demonstrated in the laboratory (Davidson et al. 2003), and in field mesocosms (Parris and Cornelius 2004). Riparian species may be more susceptible to transmission of the disease because zoospores survive in water, however, exclusively terrestrial species can also be infected (Burrowes et al. 2004; Lips et al. 2006; Puschendorf et al. 2006a). *Batrachochytrium dendrobatidis* has been found on moist rocks, sticks and leaves during an epidemic, which supports the hypothesis that it can be transmitted by contaminated environmental substrates (Lips et al. 2008; Richards-Zawacki 2009). This suggests that Australian microhylids should not completely escape infection through lack of opportunity for transmission. Although they are not known to enter water, they occur commonly within a few meters of streams (pers obs.), and the substrates they come into contact with are frequently transited by more aquatic species as they move into and out of the forest (Rowley and Alford 2007c).

Kriger and Hero (2006a) detected 31 zoospore equivalents in a *C. ornatus* individual within the WT using qPCR techniques. A single individual was sampled at Babinda in September 2005, and was located approximately 2m from Babinda creek. Kriger et al. (2007) also sampled 34 *Litoria leseurii* individuals along the same stream, and found a chytrid prevalence of 44% in these samples. The fact that I did not detect *Bd* in any of the 595 microhylids surveyed for the present study suggests that although infected microhylids can be found in nature, they are rare.

The intensity of infection by *Bd* in Queensland's frogs undergoes seasonal changes, with *Bd* infection levels decreasing substantially in the warmer months (Berger et al. 2004; Retallick et al. 2004; McDonald et al. 2005; Woodhams and Alford 2005; Kriger and Hero 2007a). It is likely that the higher temperatures that exist in the tropical lowlands of northern Queensland in the summer months decrease the incidence of chytridiomycosis in some amphibian populations, and may entirely eliminate it from others. *Batrachochytrium dendrobatidis'* growth may be limited by high summer temperatures, which in many subtropical

and tropical regions should reach levels lethal to *Bd*. This would explain the pattern of amphibian declines that occur during the cooler months and at high elevations, and the absence of population declines in lowland, equatorial amphibians (Berger et al. 1998; Bradley et al. 2002).

In this study, field sampling occurred from October - March each wet season, when frogs were calling. It is possible that because sampling was carried out in summer, prevalence of *Bd* infection was reduced, and that this survey is an under-estimate of true *Bd* prevalence. However, in montane regions, summer maximum temperatures may never attain levels lethal to *Bd*, and optimal chytrid growth temperatures may actually occur in summer. Kriger and Hero (2008) found a significant interaction between altitude and season in southeast Queensland, with infection levels remaining high in upland frog populations well into summer, but dropping off in the lowlands as temperatures warmed. Multiple studies of the prevalence of infection by *Bd* in stream-associated frogs in the WT (Berger et al. 2004; Woodhams and Alford 2005; Kenyon 2008) have shown that although prevalence at montane sites declines in summer, it remains well above the upper 95% confidence limits I found for microhylids, both as a group and at the three specific sites with large samples sizes. Of the 595 samples used in this study, 563 (95%) were collected at locations above 900m altitude. Studies have also found that *Bd* infection prevalence increases in northern Queensland at altitudes above 300m (McDonald et al. 2005)

Studies of the biology of *Bd in vitro* indicate that *Bd* is able to survive and grow at 4°C, but that the best conditions for growth occur at 17-25°C, with optimal growth at 23°C, reversible cessation of growth at 28°C and death at 30°C for extended periods of several days (Longcore et al. 1999; Piotrowski et al. 2004). The surface temperatures of frogs in this study ranged from 10.8-24.3°C, which are within the envelope that supports *Bd* growth. The average temperatures of frogs at each of the nine field sites are within the preferred conditions of growth for *Bd*. It is thus unlikely that the surface temperatures of microhylids, even in the summer months, are sufficiently high to inhibit *Bd* growth.

Although reduced opportunities for transmission may contribute to the extremely low prevalence of *Bd* infection from Australian microhylids, it seems likely they also have an unusually high degree of resistance to infection by the pathogen. Resistance to infection is determined by many factors, including environmental conditions, host behaviour, and pathogen and host biology, and innate or acquired immune responses may provide microhylids with inherent protection from infection by *Bd*. Innate immune defenses in amphibian skin may be important in conferring resistance to chytridiomycosis (Woodhams et al. 2007b). Amphibian species that lay eggs in terrestrial nests that are brooded or tended by parents may be preadapted to being resistant to *Bd* because they have particularly effective innate immune defenses such as antifungal skin secretions or microbial symbionts as a means to reduce fungal infestations that would otherwise overwhelm brooded egg clutches (Austin 2000; Lauer et al. 2007). Studies

have shown that terrestrial egg clutches suffer increased mortality when brooding adults are removed from nests (Forester 1979; Townsend et al. 1984; Simon 1993), and the adaptations that normally serve to protect developing egg clutches may predispose terrestrial breeders to a reduction in susceptibility of *Bd* infection. Innate immune defenses such as the production of antimicrobial peptides have been studied in some detail, and secreted peptide mixtures from the skin of a range of amphibian species can inhibit *Bd* growth (Rollins-Smith et al. 2002b; Woodhams et al. 2005). Antimicrobial skin flora are also likely to have a major role in determining the susceptibility of frogs to chytridiomycosis, and several brooding species of terrestrial salamanders possess diverse bacterial flora that inhibit the growth of a range of microbes and fungi, including *Bd* (Austin 2000; Harris et al. 2006; Lauer et al. 2007; Banning et al. 2008).

Amphibian declines caused by chytridiomycosis probably result from a complex web of factors including amphibian behaviour, environmental conditions, host immune function, and microbial symbionts. Further research into the innate immune defenses of terrestrial breeding amphibians is required to determine if the low prevalence of *Bd* infection in these species is due to aspects of their terrestrial mode of reproduction, and to determine whether this may be applicable to more susceptible taxa.

CHAPTER 4: EXPERIMENTAL INFECTION OF *Cophixalus ornatus*, AN AUSTRALIAN DIRECT-DEVELOPING MICROHYLID, WITH *Batrachochytrium dendrobatidis*

Abstract

The frog family Microhylidae reaches its highest diversity in Australia in the Wet Tropics bioregion of northern Queensland, where there are 19 species, including 14 restricted to the region. Frogs of other families suffered severe declines and extinctions in the late 1980s to the mid 1990s in association with outbreaks of chytridiomycosis, caused by *Batrachochytrium dendrobatidis* (*Bd*). There is no evidence that microhylids experienced similar declines, although they are extensively sympatric with species that suffered population losses. There are two possible reasons why microhylids may have avoided declines. Either they are not susceptible to the disease, or they might be susceptible, but the disease does not cause declines in the wild (as has been shown for various other frog groups). As the susceptibility of microhylids to infection by *Bd* and to the pathogenic effects of chytridiomycosis is unknown, I tested the most common species of terrestrial microhylid (*Cophixalus ornatus*) to infection with *Bd*. Seven *C. ornatus* and five *Litoria wilcoxii* (susceptible controls) were sequentially exposed to increasing numbers of *Bd* zoospores and tested for infection using quantitative PCR assays. All *C. ornatus*, and four out of five *L. wilcoxii*, became infected by *Bd* at some point during the experiment. Neither species showed any clinical symptoms of chytridiomycosis, but the mean number of zoospore equivalents in positively infected individuals was significantly higher in *L. wilcoxii* than in *C. ornatus*. All *C. ornatus* individuals eliminated their infections by day 122 of the experimental trials, whereas *L. wilcoxii* individuals retained relatively intense infections. A combination of life-history traits coupled with an effective immune response, which may include anti-fungal microflora, could be accountable for absence of clinical infection in *C. ornatus,* and the ability of this species to eliminate infection over time. The lower level of susceptibility to the clinical effects of chytridiomycosis by *C. ornatus* suggests that the threat of disease to wild populations may not be severe. Future studies should determine if other microhylid species also resist disease progression.

Introduction

Amphibian declines currently rank among the most critical issues in conservation biology; factors thought to be responsible include habitat loss, climate change, overexploitation, chemical contaminants and emerging infectious disease (Stuart et al. 2004; Mendleson et al.

2005). Current estimates suggest that 43% of all known amphibian species are experiencing some form of population loss (Stuart et al. 2004). One major cause of declines is chytridiomycosis, an invasive skin disease of amphibians caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*; Berger et al. 1998; Longcore et al. 1999). Chytridiomycosis has been associated with mass mortality, population declines, and extinctions of amphibian species in many regions, and has been found in the Americas (Ron and Merino 2000; Lips et al. 2003; Muths et al. 2003; Barrionuevo and Mangione 2006; Carnaval et al. 2006; Lampo et al. 2006; Felger et al. 2007); Puerto Rico (Burrowes et al. 2004), Cuba (Díaz et al. 2007), the West Indies (Alemu et al. 2008), Europe (Bosch et al. 2001; Cunningham et al. 2005; Garner et al. 2005); Africa (Hopkins and Channing 2003), Indonesia (Kusrini et al. 2008); Japan (Goka et al. 2009); South Korea (Yang et al. 2009); Australia (Berger et al. 1998), and New Zealand (Waldman et al. 2001).

In many cases, population declines attributed to *Bd* have been dramatic, causing rapid extinction of up to 50% of amphibian species at a particular site (Lips et al. 2006). However, some species appear unaffected at the population level, indicating that the prevalence and intensity of infections and the extent of pathological effects differ strongly among host species (Retallick et al. 2004; Lips et al. 2006). High-elevation populations often suffer more severe declines (Wake 1991; Laurance et al. 1996; Lips 1998; McDonald and Alford 1999), and affected species often have aquatic larvae associated with streams and spend a large proportion of their time in or near streams (Lips 1998; McDonald and Alford 1999; Lips et al. 2003; Woodhams and Alford 2005). Tadpoles may be an important life-stage with respect to disease transmission, as they stand a high chance of being exposed to the fungus' aquatic zoospores and they may serve as carriers to maintain the pathogen in the environment (Waldman et al. 2001; Lamirande and Nichols 2002; Rachowicz and Vredenburg 2004).

While most anurans mate and deposit eggs in water, numerous species lay their eggs on land and undergo direct development, which is the direct, embryonic formation of adult features with no free-living, aquatic larval stage (Jennings and Hanken 1998). Direct development has evolved independently within each of the three extant orders of amphibians (frogs, salamanders, and caecilians). It characterises hundreds of living species in over a dozen families, including the Australian microhylids, and is the predominant reproductive mode in some clades, e.g., plethodontid salamanders (Wake and Hanken 1996).

Evidence of declines of amphibians with terrestrial direct development is disproportionately scarce in comparison to species that have a tadpole stage in their development. For example, in a study of 60 frog species from eastern, upland (<300m) areas of Australia, only one species (*Philoria frosti*) out of 20 (5%) with direct development suffered from declines, whilst 20 out of 40 (50%) species with an aquatic stage in their life-history experienced population losses (Hero et al. 2005). In a study of 30 Costa Rican species, two out

of 15 (13%) species with direct development were in decline, whilst nine out of 15 (60%) species with an aquatic stage in their development, were declining (Puschendorf et al. 2006a). There are very few examples of population crashes of species with direct development in the literature, and even fewer can cite the presence of *Bd* as a possible cause of the declines (Pounds et al. 1999; Bell et al. 2004; Burrowes et al. 2004; Lips et al. 2006, Brem and Lips 2008). Two exceptions come from Panama where direct-developing frogs and salamanders have declined and disappeared from sites during epidemic outbreaks of chytridiomycosis (Lips et al. 2006; Puschendorf et al. 2006a).

It is possible to experimentally infect direct developing species with *Bd*, as several terrestrial, direct-developing, plethodontid salamanders have been infected in the laboratory. One hundred percent of infected individuals of the species *Batrachoseps attenuatus* died when maintained in the laboratory, however, wild populations showed stable infections with *Bd* (Weinstein 2009). Similarly, at least two species in the genus *Plethodon* (*P. metcalfi* and *P. glutinosus*) exhibited mortality when infected with *Bd* zoospores (Chinnadurai et al. 2009; Vazquez et al. 2009). *Plethodon cinereus* has been infected with *Bd* in the laboratory, but did not die, and only lost weight in response to infection, compared to controls (Harris et al. 2009). The lack of mortality suggests that this species is not vulnerable to the effects of chytridiomycosis. Other direct-developing species may show some resistance to chytridiomycosis, as three species in the genera *Eleutherodactyus* and *Craugastor* appear to coexist in stable associations with *Bd* (Puschendorf et al. 2006a). At least one species (*E. coqui*) did not show significant mortality following experimental exposure to *Bd* under laboratory conditions (C. Carey pers. comm.).

In Australia, the family Microhylidae includes 19 direct developing species, of which 14 are endemic to the Wet Tropics (WT) region of northern Queensland (Zweifel 2000; Hoskin 2004). Microhylids commonly occur in moist, tropical habitats at high elevations, which are environments that are favourable to the growth of *Bd*. In many regions where microhylids occur, sympatric species have suffered from *Bd*-associated population declines (McDonald et al. 2005; Woodhams and Alford 2005). Despite the lack of extensive population data, it does not appear that microhylids have undergone population losses, associated with *Bd*, as anecdotal observations suggest that numbers of individuals of most species appear to be high and unchanging (Hauselberger and Alford 2005; Williams 2007). The number of calling individuals along a transect in the WT showed that populations of two microhylid species were relatively stable from 1995-2007 (Chapter 2).

The ability of *Bd* to infect direct-developing microhylids under standard laboratory conditions is unknown. Until recently, only two Australian species of microhylids (*Austrochaperina robusta* and *Cophixalus ornatus*) had been extensively surveyed histologically for chytrid infection, with negative results (Hauselberger 2001; D. Mendez pers.

comm.). Kriger and Hero (2006a) reported a positive diagnostic PCR assay on a sample from *C. ornatus*, however, only a single animal was sampled. The combination of their result with the results of the histological surveys suggests that the prevalence of *Bd* on Australian microhylids is very low. This was confirmed by extensive surveys of 595 samples of Australian microhylids across nine species, which indicated that infection by *Bd* was extremely rare or absent in nature (Chapter 3).

The near absence of *Bd* infections from Australian microhylids could have several causes. First, the terrestrial environment may not be an appropriate reservoir for *Bd*, because it is drier and warmer than riparian habitats (Brem and Lips 2008). Thus, as microhylids do not reproduce aquatically, and do not enter stream water, they may not be exposed to infective zoospores (e.g., Rowley and Alford 2007a). Second, microhylids as a group may be highly resistant to infection by *Bd* because they have particularly effective innate immune defenses. Australopapuan microhylids typically brood their egg clutches, and if a brooding parent is removed from a nest, egg clutches are often overgrown by fungus (Townsend et al. 1984; Simon 1993). These species may secrete antimicrobial peptides (AMPs) and alkaloids in order to inhibit the growth of pathogenic fungi (Simmaco et al. 1998; Rollins-Smith et al. 2002b). They may also possess symbiotic cutaneous microbiota that produce antibiotic substances that inhibit the growth of fungal pathogens of embryos (Rollins-Smith et al. 2002b; Harris et al. 2006; Lauer et al. 2007; 2008; Woodhams et al. 2007b; Banning et al. 2008).

If the apparent immunity of microhylids to chytridiomycosis is due to AMPs or symbiotic microbiota, there may be potential to use these agents to control *Bd* in other amphibian species in the laboratory, or even in the field (e.g. Harris et al. 2009). A first step towards discovering what is responsible for the extremely low prevalence or absence of infection in Australian microhylids in nature, is to determine whether they are constitutively immune to infection by *Bd*. I therefore carried out a series of laboratory experiments to determine whether the most common species, *Cophixalus ornatus*, can become infected by *Bd*, and if so, under what conditions this may occur.

Methods

Experimental design

Fifteen frogs were used in an infection experiment. Seven *C. ornatus* and five *L. wilcoxii* (as positive controls, since *L. wilcoxii* is vulnerable to infection by *Bd*) were sequentially exposed to increasing numbers of *Bd* zoospores (100 on day 0), while three *C. ornatus* were used as negative controls. The relatively low level of replication was due to permit restrictions, but sufficed to demonstrate effects. Frogs were housed individually at all times. A

single isolate of *Bd* (Tully-L. rheocola 06-LB-1) was used (isolated in 2006 from a L. rheocola individual located at Tully), and each exposure consisted of administering 20uL of dilute salts (DS) solution (Boyle et al. 2003), that was either sterile (control animals), or contained *Bd* zoospores (experimental animals) directly onto the posterior dorsal surface of the frog using a micro-pipetter. Animals were sequentially exposed to 100, 1000, 10,000 and 10,000 living *Bd* zoospores on experiment days 0, 17, 46, and 66 respectively.

Diagnostic samples were collected using a non-invasive, standardised technique. A sterile medical cotton swab (Medical Wire & Equipment Co. Bath Ltd., Wiltshire, UK) was run over the dorsal and ventral surfaces of each frog, including the forelegs and hindlegs, as described by Skerratt et al. (2008). To prevent contamination and reduction in the number of zoospores detected by the real-time TaqMan polymerase chain reaction assay (qPCR; Boyle et al. 2004), each swab was replaced directly into its individual container after the swabbing procedure, and was refrigerated as soon as possible after collection. Samples were analysed at James Cook University (JCU), Townsville using the qPCR assay methods developed by Boyle et al. (2004). qPCR assays were performed in triplicate, with the addition of a positive control in each run, designed to reveal the presence of inhibition in samples. Previous research has shown that qPCR can detect *Bd* infection in frogs as early as 7 days post infection (Boyle et al. 2004).

Collection and maintenance of experimental animals

10 calling adult male *Cophixalus ornatus* were collected during nocturnal searches along a 500m transect located on the "H track" at Paluma, Queensland, Australia (19°00.42'S, 146°12.43'E) during the 8-10th February 2008. All weighed between 0.76-1.03g, with snout-vent lengths (SVLs) of 18.4-22.03mm. Individuals were located by their mating calls, captured in an inverted plastic, self-sealing sandwich bag with a handful of leaf litter, swabbed to detect *Bd* DNA, and transferred to a larger sealed bag. Bags containing frogs were placed in a small insulated cooler to preclude rapid changes in ambient environmental temperature during transport to the laboratory. Collection sites were marked with fluorescent flagging tape and labeled with the frog's individual number, so that they could be released at the point of capture after the conclusion of the experiment, if they were uninfected.

Animals were housed in a controlled temperature laboratory at JCU, maintained at approximately 23°C. Hourly measurements, using i-button dataloggers (Thermochron iButtons by Dallas semiconductor, Dallas, Texas, USA; diameter 15mm, height 6mm) revealed temperatures ranging from 21-24.5°C and relative humidity from 60-75%. The laboratory was illuminated by a large window, and animals experienced a natural diurnal light cycle. Frogs were acclimatised to laboratory conditions for 14 days before the infection experiment began.

All *C. ornatus* were housed individually in 1.6L transparent plastic enclosures (18x11x12cm) with ventilated lids (Critter Keeper^{TM}, Rolf Hagen & Co. Inc: Mansfield, USA). Containers were filled with 2-3cm of autoclaved peat soil which was moistened weekly.

Containers also contained autoclaved leaf litter and small pieces of wood from the natural environment, to provide shelter for frogs. Swabs taken from all animals at the time of collection tested negative for *Bd* DNA, indicating that all animals were uninfected at the start of the experiment.

Five *L. wilcoxii* individuals were used in infection experiments as positive controls. These frogs were raised from tadpoles collected at Crystal Creek, Paluma Range National Park, Queensland, Australia (S18°58′54″ E146°12′′01″) by N. Kenyon (Kenyon 2008). At the start of the experiment, *L. wilcoxii* weighed between 4.97-11.65g with SVLs ranging from 31.93-47.60mm. They were housed in 32x21x17cm plastic containers with removable, perforated lids, containing a small amount of autoclaved leaf litter. They were provided with a 12x6cm plastic tub of rainwater (collected and stored in a water tank) that was changed weekly. All individuals were swabbed for detection of *Bd* prior to the experiment and were uninfected.

All experimental frogs were fed cultured crickets (*Acheta domesticus)* weekly *ad libitum*. *Cophixalus ornatus* were fed early instar "pin-head" crickets and *L. wilcoxii* individuals were fed larger crickets. Once a month, 0.5 mL of a liquid nutritional supplement (2mL Calcivet/100mL rainwater) was applied dorsally to each *L. wilcoxii*.

Culture and maintenance of *Bd*

All manipulations of *Bd* culture were conducted at the School of Veterinary and Biomedical Sciences at JCU in the laboratory of R. Speare. Transport of prepared zoospore solutions from the Speare laboratory to the experimental frog facility was conducted in an insulated, cooled Styrofoam container. Experiments were carried out with appropriate sterilisation of clothing, equipment, and effluent, using established protocols (e.g., Speare et al. 2004) to prevent the escape of the pathogen to the environment, or any cross-contamination of animals. To prevent cross-infection, disposable latex gloves were worn at all times and changed between frogs and animal enclosures. A single isolate of *Bd* was used in this study (Tully-L.rheocola 06-LB-1). Since its isolation in 2006 from a *Litoria rheocola*, Tully-L.rheocola 06-LB-1 had been serially passed *in vitro* 24 times. The fungus was maintained at 23°C in 50% nutrient tryptone-gelatine hydrolysate-lactose (TGhL) broth (8 g tryptone, 2 g gelatin hydrolysate, 1 g lactose, 1000ml distilled water) in 25cm^2 tissue culture flasks (TPP9025, JRH Biosciences). Stock culture was passaged at intervals of 4-7d to assure that cells were in an active phase of growth at time of collection. Culture flasks were scraped using a TPP cell scraper (CSL Biosciences), and 1mL of this stock solution was transferred into a new flask containing 9ml TGhL broth and incubated at 23°C.

Harvesting zoospores

Zoospores were harvested using the methods described by Rollins-Smith et al. (2002a). Stock culture at maximum growth was transferred to 50% nutrient TGhL agar (8 g tryptone, 2 g gelatin hydrolysate, 1 g lactose, 10 g agar, 1000ml distilled water) in 9 cm Petri dishes (Sarstedt,

Australia, Inglefarm, SA). Culture flasks were scraped (as described above), and 0.75mL of stock solution was transferred to agar plates with a sterile pipette. Inoculated plates were left open for approximately 5-10 minutes in a class II biosafety cabinet until the added broth had reduced to a wet smear. Petri dishes were then labeled, sealed with Parafilm®, and incubated at 23°C for approximately three days until maximum zoospore release was observed. Agar plates were then flooded with 3ml of sterile DS solution and were left to sit for 20min while zoospores accumulated in the liquid. The supernatant was then collected using a sterile pipette and transferred into a labeled centrifuge tube. A sterile TGhL agar plate (containing no *Bd*) was also flushed with DS solution and the supernatant collected in the same way, to be used as a negative control in the experiment. Zoospores may stay motile (and thus infective) for up to 24 hours, however, most encyst before this time (Longcore 2008); in this study, all zoospores were used in experimental trials within 2hr of collection.

The number of zoospores in the DS solution was estimated by counting live and moving zoospores using a haemocytometer as described by Boyle et al. (2004). A count was completed on each of 5 0.005mm^2 squares and this was replicated using the second counting chamber. The two results were averaged, to create the final count of zoospores, and a zoospore count per mL of culture calculated. Using this result, the zoospore solution was diluted with DS solution to the level needed in each experimental trial; the negative control solution was also diluted to the same extent.

Experimental trials

The infection experiment consisted of four trials in which frogs were exposed to increasing numbers of zoospores (100, 1000, 10,000 and a repeat of 10,000). These trials were continued until the majority of exposed frogs became infected. Frogs underwent the first infection trial on day 0, (25/2/2008) and were each exposed to approximately 100 *Bd* zoospores. 20μL of zoospore solution (5000 zoospores/mL) were administered to each frog. The three negative control *C. ornatus* were administered 20μL of sterile DS control solution. Because the *C. ornatus* and *L. wilcoxii* individuals were usually quite moist, frogs were first blotted dry using paper towel, to ensure that the solution would adhere to their skin. In this and subsequent trials, all frogs were checked daily, fed weekly, and swabbed 7 and 14 days after exposure to determine *Bd*-infection status.

The second trial of the experiment occurred on day 17 (13/3/2008). A dose of approximately 1000 zoospores per frog (20 μL of 50,000 zoospores/mL) was prepared and administered to experimental frogs as outlined for trial 1. Control frogs were re-exposed to control inoculum. The third trial was carried out on day 46 (11/4/2008). A dose of approximately 10000 zoospores per frog (20 μL of 500,000 zoospores/mL) was prepared and administered to experimental frogs as before. Control frogs were re-exposed to control inoculum. The fourth trial began on day 66 (1/5/2008). Exposures and controls were treated as

in the third trial, with a second dose of 10000 zoospores administered to each experimental frog. Following the fourth trial exposure, all animals were swabbed 7, 14, 21, 28, 35, 50 and 56 days post-exposure to follow the progress of infections. Two experimental *C. ornatus* died between days 122 and 132 of the experiment. Autopsies indicated that these deaths were not related to chytridiomycosis, and both individuals were clear of infection on day 122.

Disposal of frogs

The majority of animals used in infection experiments were clear of infection by the end of the experimental phase. Individuals that remained infected were treated with heat after the experimental study was completed, to clear their infections. This consisted of placing enclosures in a position in the laboratory that received direct sunlight for 1h each day for five consecutive days. Animals were swabbed intermittently, and when 2 consecutive samples showed a negative result for the presence of *Bd* DNA, *C. ornatus* were released at the point of capture. Two *C. ornatus* individuals died after the experimental trial. They were preserved in ethanol and stored at JCU. All *L. wilcoxii* individuals were maintained in the laboratory for future use.

Statistical analysis

Quantitative PCR results were considered positive only if all three replicate wells for each sample returned positive results (Hyatt et al. 2007). Weaker positives could reflect residual DNA from initial exposures or DNA remaining on animals that have lost their infections, rather than actual infections. For positive samples, the mean number of zoospore equivalents for the three replicate wells was calculated and is reported. Mean intensity of infection was calculated for each individual that became infected, using only the data for results with an intensity greater than zero. These individual means were compared between species using a Mann-Whitney-U test. Omitting data for samples that did not test positive in all three of three replicate wells during qPCR may bias estimates of intensity of infection slightly towards higher intensities.

Results

All frogs tested negative for *Bd* infection at the onset of the study. No animals became infected in trials 1 and 2, at doses of 100 or 1000 zoospores (Figure 4.1; Table 4.1). Trial 3, the first trial in which animals were exposed to 10000 zoospores, produced relatively light infections in two *C. ornatus* and four *L. wilcoxii* (Figure 4.1; Table 4.1). The apparent infection in *C. ornatus* individual 10 at seven days after exposure in this trial was positive in less than three of the replicate wells for the qPCR, and might have been caused by the continuing presence of DNA from the initial infective dose of zoospores, instead of indicating an actual infection. In Trial 4, the second inoculation with 10000 zoospores, all 7 *C. ornatus* individuals

were infected at 14 days following exposure (Figure 1; Table 4.1), and 2 of the *L. wilcoxii* individuals were infected. By day 101, 35 days after exposure in trial 4, only one *C. ornatus* showed a suspicious positive result (Table 4.1), while two of the positive controls retained relatively intense infections (Figure 4.1). By day 122, 56 days after the exposure in Trial 4, no *C. ornatus* returned positive results, while two *L. wilcoxii* retained relatively intense infections. Because frogs were maintained at high humidities and temperatures close to 23^{o}C, environmental temperature and humidity could not be responsible for the observed losses of infections.

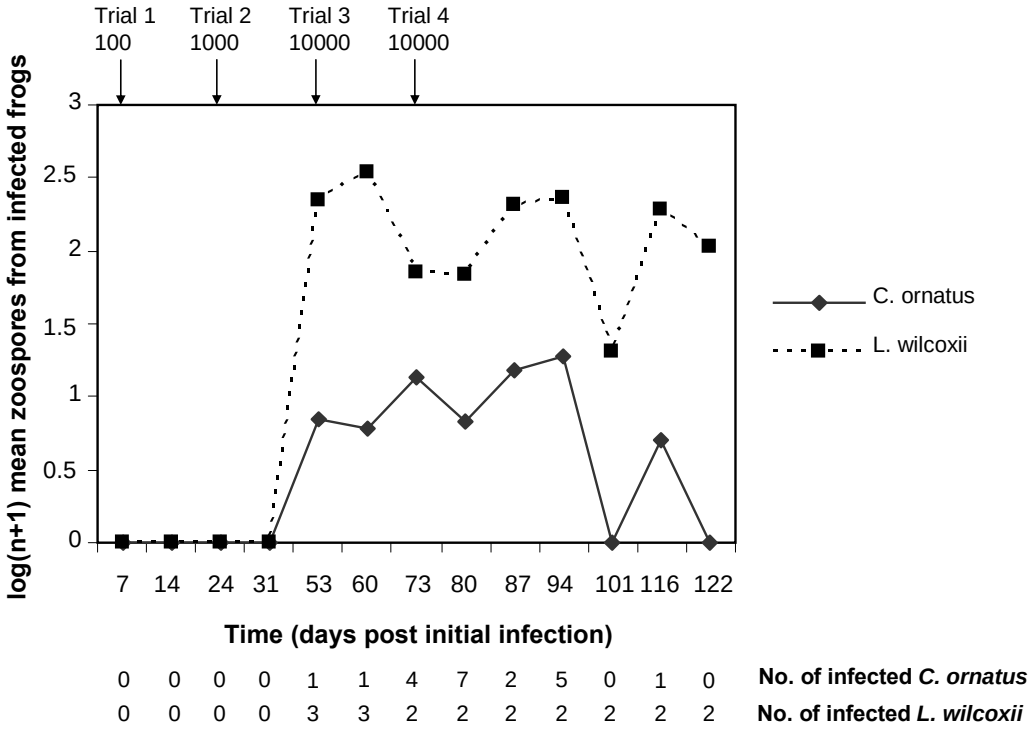

Figure 4.1 Mean zoospore equivalents from skin swabs of infected *C. ornatus* and *L. wilcoxii* individuals as determined by qPCR analysis.

Table 4.1 Results of the infection experiment. Zeros indicate completely negative diagnostic qPCR results, S indicates suspicious positives (less than three replicate wells returned positives, all with very low numbers of zoospore equivalents), numbers greater than zero are mean number of zoospore equivalents, rounded to the nearest whole number.

Experimental trial			1		2		3		4									
Day since initial trial			7	14	24	31	53	60	73	80	87	94	101	116	122	132	155	
Days since most recent exposure			7	14	7	14	7	14	7	14	21	28	35	50	56	66	89	
Species	Individual	Control or Exposed	qPCR results (mean number of zoospore equivalents if three replicate wells were positive)															Mean of positive samples
C. ornatus	1	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	--	0
	2	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	--	0
	3	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	--	0
	4	E	0	0	0	0	6	0	4	4	0	7	S	0	0	0	--	5.3
	5	E	0	0	0	0	0	0	34	67	0	6	0	0	S	0	0	35.7
	6	E	0	0	0	0	0	5	11	11	46	0	0	S	0	0	0	18.3
	7	E	0	0	0	0	0	0	0	3	0	77	0	0	0	0	--	40.0
	8	E	0	0	0	0	0	0	S	3	0	20	0	0	0	--	--	11.5
	9	E	0	0	0	0	0	0	S	2	4	S	0	4	0	0	--	3.3
	10	E	0	0	0	0	S	0	15	2	0	24	0	0	0	--	--	13.7
L wilcoxii	1	E	0	0	0	0	785	1064	22	34	312	176	106	264	106	--	--	318.8
	2	E	0	0	0	0	24	12	213	135	135	308	3	135	102	--	--	118.6
	3	E	0	0	0	0	554	S	0	0	0	0	0	0	0	--	--	554.0
	4	E	0	0	0	0	0	2919	S	S	0	0	0	0	0	--	--	2919.0
	5	E	0	0	0	0	0	0	0	0	0	0	0	0	0	--	--	0

The mean intensity of infection (mean number of zoospore equivalents in samples with confirmed positives) for infected *C. ornatus* individuals was 18.2 (S.D. = 14.3, N = 7). The maximum for any individual sample was 77. In contrast, the mean intensity for infected *L. wilcoxii* was 977.6 (S.D. = 1306.5, N = 4), with a maximum for any sample of 2919 (Table 4.1). The mean number of zoospore equivalents in infected individuals was significantly higher in *L. wilcoxii* (Mann-Whitney U = 28 with 7 and 4 d.f., P = 0.006, Figure 4.2).

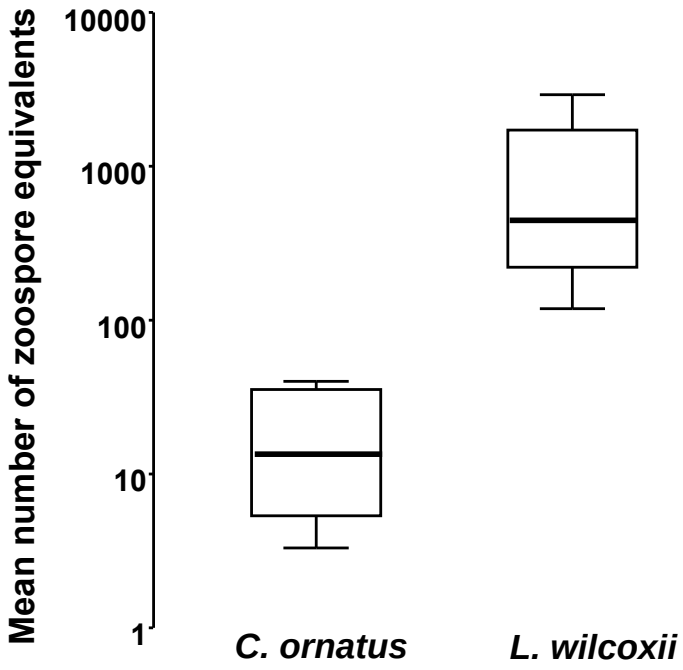

Figure 4.2 Mean number of zoospore equivalents recovered from positive swab samples in *C. ornatus* and *L. wilcoxii* individuals.

Discussion

This is the first demonstration that an Australian microhylid species can become infected with *Bd* under standard laboratory conditions. All seven experimental *Cophixalus ornatus* became infected during trial 4 of the experiment. Four of the five *Litoria wilcoxii* positive controls tested positive for relatively high numbers of zoospore equivalents at some point during trials 3 and 4. None of the negative control *C. ornatus* ever tested positive for *Bd*.

Positive qPCR results were not recorded after doses of 100 or 1000 zoospores in either *C. ornatus* or the positive control species, *L. wilcoxii*. The first dose of 10000 zoospores produced light positive infections in two *C. ornatus*, and a possible positive in a third, while it produced intense infections in four of the five positive control animals. This suggests that *C. ornatus* may have greater innate resistance to acquiring *Bd* infections than *L. wilcoxii*. After a second exposure to 10000 zoospores, all *C. ornatus* had positive qPCR results. Levels in all

individuals increased at some time after day seven following exposure, making it very unlikely that positives were caused by residual DNA remaining from the zoospores used to expose the frogs.

These results indicate that *C. ornatus* can become infected by *Bd* when exposed to relatively high numbers of zoospores in the laboratory. However, they also suggest that once acquired, infections are cleared relatively rapidly, and are unlikely to persist or develop into chytridiomycosis. The number of zoospore equivalents detected on swabs taken from *C. ornatus* was always relatively low, between six and 77 zoospore equivalents. All *C. ornatus* had cleared their infections by day 132, 66 days after their last exposure to an infective dose of zoospores, and it is likely that all were uninfected by day 122, since the one individual (number 5) that returned a suspicious positive on that date had returned negative results on the two preceding dates. In contrast, two of the four *L. wilcoxii* that became infected in trial 3 retained relatively intense infections on the last date they were sampled, day 122, at least 56 days after the trial 4 exposure and 69 days after the trial 3 exposure. The *L. wilcoxii* that became infected also developed significantly more intense infections, with mean numbers of zoospore equivalents 51 times as high as in *C. ornatus* (Figure 4.1). This difference was not due simply to the body size of the two species. The mass of experimental *L. wilcoxii* was from 5-15 times greater than that of experimental *C. ornatus*, indicating a 4.6 fold difference in surface area. Differences in infection intensity were up to 40 fold, and thus up to an order of magnitude of the higher mean intensity of zoospores in *L. wilcoxii* remains unexplained by differences in surface area.

It is possible that there were problems in detecting low-level infections in the individuals in this study, as some frogs lost and regained infections. It is also possible that microhylids may fight an initial infection by *Bd*, but become infected by zoospores surviving in the substrate or water of their enclosure.

The results of the present study support the hypothesis that Australian microhylids have a low level of susceptibility to the clinical effects of chytridiomycosis. Experimental environmental conditions were within the optimal range for the growth of the fungus (Piotrowski et al. 2004), and were representative of thermal conditions at the site where *C. ornatus* were collected. Surface temperature of frogs located at this site during the wet season range from 19.2-24ºC (Chapter 6).

During the trial, *C. ornatus* resisted initial infection and rapidly cleared infections once they were acquired. Under similar laboratory conditions, many species that have not suffered from declines in the wild are highly susceptible to *Bd* (Berger et al. 1999a; McDonald and Alford 1999; Woodhams and Alford 2005). Only a single individual Australian microhylid has been found infected in the wild, a *C. ornatus* sampled by Kriger and Hero (2006a), despite extensive sampling of 595 individuals across nine microhylid species (Chapter 3). This suggests that if infections occur in the field, they are extremely rare, and the results of the present study

indicate that this rarity is likely to be caused by a combination of low rates of initial infection and high rates of clearance.

There are relatively few reported cases of frog species that can eliminate *Bd* infections. Murray et al. (2009) showed that individuals of the species *Litoria pearsoniana* gained and lost infections during a mark-recapture study in south-east Queensland. Similarly, Kriger and Hero (2006c) observed that wild individuals of *Litoria wilcoxii* lost their infections in southern Queensland. Experimentally infected *L. chloris* cleared their infections after being held at 37ºC, whilst frogs held at lower temperatures (<24ºC) died (Woodhams et al. 2003). Similarly, infected *Pseudacris triseriata* cleared infection when they were held at 32ºC (Retallick and Miera 2007), and approximately 50% of infected *Mixophyes fasciolatus* held at 27ºC lost their infections in the laboratory (Berger et al. 2004). Kenyon (2008) also found that *L. genimaculata* housed under constant laboratory conditions at 23ºC were capable of clearing infections. Like other vertebrates, amphibians have well-developed immune defenses, including both acquired and innate responses that may confer protection against *Bd* (Duellman and Trueb 1994; Carey et al. 1999; Apponyi et al. 2004). The different responses shown by *C. ornatus* and *L. wilcoxii* in my experiment could be due to differences in their immune systems. Peptides secreted from skin glands onto the skin surface can kill *Bd in vitro* (Rollins-Smith and Conlon 2005), and the effectiveness of AMP skin secretions in Australian frog species is significantly correlated with resistance to chytridiomycosis (Woodhams et al. 2007a). Population trends associated with chytridiomycosis in Australian rainforest frogs have also been correlated with the effectiveness of AMP defenses against *Bd* (Woodhams et al. 2005). It is possible that innate defenses such as AMPs prevented *Bd* infection in my experiment at the lower doses of 100, and 1000 zoospores. The frogs' defenses may simply have been overwhelmed by successive doses of 10,000 zoospores. Similarly, *Mixophyes fasciolatus* juveniles exposed to varying levels of *Bd* responded only after exposure to large numbers of zoospores. Exposure to 10 zoospores did not cause infections, exposure to 100 zoospores caused 100% mortality 35-47 days post-exposure, and exposure to 1000 zoospores caused 100% mortality 23-38 days post-exposure (Berger et al. 1999b).

In addition to innate immune responses, there may also be some element of acquired immune response occurring in *L. wilcoxii*, as two of the four individuals that became infected in trial 3 of the experiment lost the infections, and did not become re-infected in trial 4. This could be due to acquired immunity or to an increase in levels of innate immune defenses such as AMPs in response to infection. The *L. wilcoxii* individuals used in the experiment had been used in previous experiments (Kenyon 2008). It is possible that some of the animals had previously been exposed to *Bd*, and were displaying acquired immune responses during this trial. Frogs with previous exposure to *Bd* are known to survive second infections better than

immunologically naïve frogs (Richmond et al. 2009), and it is possible that they also can clear or resist second infections completely.

Natural peptide mixtures produced by microhylids are effective against *Bd* (Chapter 5). However, AMPs from microhylids are similar to peptides produced by hylid and myobatrachid species vulnerable to *Bd* infections in the field. This suggests that other immune mechanisms, or other factors such as interspecific behaviour, may be responsible for the apparently high resistance of *C. ornatus* to *Bd* infection in nature. Species that are less susceptible to the disease may behave in a manner that is not conducive to *Bd* transmission, growth or survival (Rowley and Alford 2007a). A fully terrestrial mode of life, including terrestrial breeding, which characterises the Australian Microhylidae, may contribute to the apparent immunity of these species to chytridiomycosis in nature.

The majority of population declines associated with *Bd* have occurred in species that inhabit aquatic environments, and fewer population declines have been noted in terrestrially-breeding species. The reduced responses of terrestrially-breeding species to chytridiomycosis may be due to lower rates of transmission of *Bd* (Lips et al. 2006). *Batrachochytrium dendrobatidis* zoospores swim less than 2 cm before they encyst, so the pathogen is likely to be spread among individuals in close contact during aggregation or mating (Piotrowski et al. 2004). Transmission may occur by direct contact with infected individuals or indirectly via contaminated substrates or water (Berger et al. 1998; Daszak et al. 1999; Rachowicz and Vredenburg 2004; Lips et al. 2006; Richards-Zawacki 2009). This has been demonstrated in the laboratory (Davidson et al. 2003), and in field mesocosms (Parris and Cornelius 2004). Because zoospores survive in water, riparian species are more susceptible to transmission of the disease (Piotrowski et al. 2004), and completely terrestrial species such as the Microhylidae may simply have a reduced probability of coming into contact with *Bd* in nature.

However, completely terrestrial species have been found infected with the fungus, supporting the hypothesis that *Bd* may be transmitted via the environment (Burrowes et al. 2004; Lips et al. 2006). Lips et al. (2006) and Richards-Zawacki (2009) found *Bd* on moist environmental substrates such as rocks, sticks, and leaves during epidemics in Panama. This suggests that Australian microhylids should not completely escape infection through a lack of opportunity for transmission. They inhabit areas where stream-associated, aquatic-breeding species have suffered population declines, and do not actively avoid riparian areas; they are commonly found within a few meters of water bodies, including creeks (pers. obs.; Kriger and Hero 2006a). This should expose them to at least some risk of transmission through contact with other species of frogs, or contact with substrates recently occupied by infected individuals (Lips et al. 2006).

Another aspect of innate immunity that may be important in providing protection from *Bd* is symbiotic cutaneous anti-fungal microbiota. *C. ornatus* produce clutches of eggs in

terrestrial nests, and adults exhibit egg brooding behaviour (Zweifel 1985). Studies show that when egg-brooding adults desert, or are removed from the developing egg clutch, embryos often succumb to fungal infections (Forester 1979; Townsend et al. 1984; Simon 1993). Brooding may prevent fungal attack of eggs due to the transfer of antimicrobial microbiota or substances produced by the microbiota onto the developing eggs (Simmaco et al. 1998; Rollins-Smith et al. 2002b; 2005; Harris et al. 2006). Cutaneous bacterial symbionts can inhibit the growth of a range of bacteria and fungi, including *Bd* (Austin 2000; Harris et al. 2006; Lauer et al. 2007; Banning et al. 2008). It is possible that species that brood their embryos have evolved to maintain a mutualistic antifungal skin microbiota in order to protect their embryos, and thus they are less likely to succumb to chytridiomycosis than species that do not attend nests (Lauer et al. 2007).

It appears that a combination of life-history and behavioural traits coupled with an effective immune response is likely to account for the absence of clinical signs of infection in *C. ornatus*, and their ability to eliminate infection over time. Thus, the threat of chytridiomycosis to wild populations of *C. ornatus* may not be severe. Further laboratory experiments will be needed to elucidate the mechanisms responsible for resistance. For example, it is not clear whether animals kept in stable laboratory environments accurately reflect the environmental conditions encountered in the field, and therefore the susceptibility of animals in nature to *Bd*. The composition of the skin microbiota may change during captivity, and it may be necessary to carry out experiments immediately after animals are removed from the wild to capture how they respond to challenges by *Bd*. Experiments comparing recently captured animals whose microbiota has been removed using antibiotics to control animals with unmanipulated levels of microbiota, would provide a first step towards determining the importance of microbiota in disease resistance. Knowledge gained from these investigations will be useful in understanding the general determinants of susceptibility to chytridiomycosis in amphibians, and may be useful in designing probiotic treatments to prevent or moderate the effects of infection in other taxa.

CHAPTER 5: ANTI-FUNGAL SKIN PEPTIDES OF AUSTRALIAN MICROHYLIDS

Abstract

Chytridiomycosis is an emerging infectious disease of amphibians caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), and is responsible for causing mass mortality, population declines and extinctions of amphibian species world-wide. Susceptibility to *Bd* infection varies greatly among the host species, depending on combinations of environmental conditions that affect the biology of host and pathogen. Multiple innate defense mechanisms appear to be involved in the resistance of amphibians to chytridiomycosis, however, one that may be particularly important is antimicrobial peptide (AMP) secretion. AMPs are produced and released from granular glands in the dermal layer of amphibian skin, and each species produces its own unique AMP repertoire that may act against a variety of pathogens.

From 2005 through 2007, the skin secretions of 81 microhylids from six species were collected and assayed to determine if they contained AMPs that could inhibit the growth of *Bd*. Secretions containing skin peptides were collected by norepinephrine induction, and used in growth inhibition assays to measure their effectiveness against *Bd*. Percentage inhibition of *Bd* growth was calculated for each sample, and for those that produced complete inhibition, the minimum inhibitory concentration (MIC; the lowest concentration of peptide sample at which no *Bd* growth was detectable), the total peptide secreted in relation to surface area, and the overall protection based on surface area were approximated.

Fifty of the 81 (62%) microhylids sampled produced AMPs with at least some activity against *Bd*; these results are similar to the 60% of samples with activity against *Bd* that Kenyon (2008) found for two sympatric hylid species. 14 microhylid samples (17%) showed 100% inhibition of *Bd* growth. Mean total protein secretion (μg) per cm^2 of microhylids was similar to the two species of hylids examined by Kenyon (2008), which suggests that microhylids produce AMPs in quantities similar to other frogs. The average MICs of three out of four non-microhylid species reported by Woodhams et al. (2007a) fell within the range that was found for microhylids. This suggests that the AMPs of microhylids are effective at concentrations similar to those of other Australian frogs. The mean protection of microhylids was significantly lower than that of the two species of hylids examined by Kenyon (2008), suggesting that microhylids have less AMP protection against *Bd* than do some other species.

The AMPs of microhylids do not appear to be any more effective against *Bd* than those of other Australian species. AMPs are not the only innate immune response in amphibians, and other epithelial defenses, such as regulation of skin sloughing and interactions with symbiotic microbiota may influence *Bd* zoospore survival and encystment rates. AMPs may contribute to

the very low susceptibility of Australian microhylids to chytridiomycosis, but cannot be solely responsible for it.

Introduction

Infectious agents can control biodiversity, and are important drivers of community dynamics (Anderson and May 1986; McCallum and Dobson 1995; Cleaveland et al. 2001; Hudson et al. 2001). Chytridiomycosis is an emerging infectious disease of amphibians caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), and is responsible for causing mass mortality, population declines and extinctions of amphibian species world-wide (Berger et al. 1998; Daszak et al. 1999; Lips 1999; Longcore et al. 1999). Susceptibility to *Bd* infection and its pathological effects vary greatly, as the nature of population losses has varied amongst host species and regions of the world (Wake 1991; Fisher and Shaffer 1996; McDonald and Alford 1999). Some species have declined to the point of extinction, some have declined and then recovered, and others appear completely unaffected by the fungus (Blaustein and Wake 1990; Berger et al. 1998; Retallick et al. 2004; Lips et al. 2006). Several northern Queensland frog species persist in populations that show stable infections with *Bd* but do not suffer at the population level from the effects of chytridiomycosis. For example, *L. genimaculata* shows high prevalence of *Bd* infection, but few or no individuals succumb to the disease, suggesting that a balance between the host and pathogen has been achieved (Speare 2000). Another species, *Taudactylus eungellensis,* suffered from extensive population declines during initial outbreaks of chytridiomycosis, but remnant populations of frogs persist in association with *Bd* (Retallick et al. 2004). These observations indicate that amphibian species have varying levels of resistance to this pathogen.

Resistance to the negative effects of disease at the population level can occur for several reasons. Individuals may be resistant to acquiring or maintaining infections, the pathogen may not be effectively transmitted between hosts, or individual hosts may not be susceptible to its pathogenic effects. All of these forms of resistance can have multiple causes due to combinations of environmental conditions associated with the biology of host and pathogen. *Batrachochytrium dendrobatidis* infects epidermal cells via motile, reproductive zoospores which attach to the keratinised outer layers of an amphibian host. The zoospores encyst, insert cytoplasm into an epidermal cell, form a thallus, and eventually form a zoosporangium which produces up to 300 infectious zoospores (Pessier et al. 1999). At maturity, the contents of the zoosporangium are released to the exterior of the skin, and the zoospores frequently infect nearby areas of the skin of the same host (Berger et al. 1998; Longcore et al. 1999; Pessier et al. 1999).

Many aspects of the ecology and physiology of amphibians may kill *Bd*, prevent infection, or impede growth and the ability to cause disease (Carey et al. 1999; Pessier et al. 1999; Woodhams et al. 2005). Multiple innate defense mechanisms appear to be involved in the resistance of amphibians to chytridiomycosis (Woodhams et al. 2007b); however, one that may be particularly important in reducing or preventing infection is antimicrobial peptide (AMP) secretion (Rollins-Smith et al. 2002a; 2002b; Rollins-Smith and Conlon 2005; Woodhams et al. 2005). As skin is the primary site of *Bd* infection, AMPs may be a particularly important line of defense in preventing infection and progression of chytridiomycosis.

Granular glands in the dermal layer of amphibian skin produce and store an array of host-defensive, bioactive substances, including AMPs, that form an integral part of amphibian, innate immune systems (Nicolas and Mor 1995; Rinaldi 2002; Apponyi et al. 2004; Pukala et al. 2006). AMPs are small (10-46 amino acid residues), cationic, hydrophobic compounds that are active against bacteria, yeast, fungi, protozoa, and viruses (reviewed in Nicolas and Mor 1995; Rinaldi 2002; Apponyi et al. 2004). Each species produces its own unique AMP repertoire that may act individually or synergistically against a variety of pathogens (Erspamer 1994; Simmaco et al. 1998; Rinaldi 2002; Apponyi et al. 2004; Pukala et al. 2006; Woodhams et al. 2006). Little is known about the natural triggers of peptide secretion (Rollins-Smith et al. 2002c); however, alarm responses to predators or injury may activate the sympathetic nervous system (Nicolas and Mor 1995; Simmaco et al. 1998). This, in turn, stimulates adrenergic receptors, which causes granular glands to release their contents on the surface of the skin, often in large quantities (Rollins-Smith et al. 2002b; 2005). Electro-stimulation of the skin, or exposure to adrenergic agents such as epinephrine or norepinephrine, are techniques that artificially induce secretion of the contents of the granular glands in amphibians (Benson and Hadley 1969; Tyler and Stone 1992). Whilst the concentrations of AMPs present on the skin of healthy, resting amphibians are unknown, the concentrations released in skin secretions following artificial stimulation can exceed 1mg/ml (Tyler and Stone 1992). After depletion, skin peptides may be resynthesised within days to weeks (Erspamer 1994).

Over 300 amphibian AMPs have been isolated and described, and *in vitro* experiments show that a range of peptides are active against *Bd* (Rollins-Smith et al. 2002b; Woodhams et al. 2005). Natural peptide secretions of four Australian frog species (*Litoria caerulea*, *L. chloris*, *Mixophyes fasciolatus* and *Limnodynastes tasmaniensis*) have inhibited *Bd* growth *in vitro*, and the effectiveness of AMPs was significantly correlated with species' resistance to chytridiomycosal infection (Woodhams et al. 2007a). Population trends associated with chytridiomycosis in Australian rainforest frogs have also been correlated with the effectiveness of skin peptide defenses against *Bd*. Natural peptide mixtures from five stream-associated Queensland species (*Nyctimystes dayi*, *Litoria genimaculata, L. leseuri, L. nannotis,*

and *L. rheocola*) inhibited growth of *Bd in vitro*, and effectiveness of peptides was significantly negatively correlated with the degree of decline of species (Woodhams et al. 2005).

In addition to physiological factors, interspecific behaviour is an aspect of host biology that may explain why frogs of some species succumb to chytridiomycosis while others do not. Microhabitat use and thermoregulatory behaviour may alter the extent or likelihood of initial contact with *Bd* or may affect the growth rates of pathogen populations on infected hosts, and may thus alter susceptibility to chytridiomycosis (Woodhams et al. 2003; Rowley and Alford 2007a; 2007c). Frogs with life-histories that involve direct development of terrestrial eggs into froglets, excluding a tadpole stage, have generally suffered less from extensive population declines than have aquatic-breeding species (Lips 1999; McDonald and Alford 1999; Hero et al. 2005; Woodhams and Alford 2005; Lips et al. 2006; Puschendorf et al. 2006a). The tadpole stage may be important in disease transmission, as tadpoles stand a high chance of being exposed to the fungus' aquatic zoospores, and they act as reservoirs for the pathogen (Marantelli et al. 2004; Rachowicz and Vredenburg 2004). Adults of species that deposit aquatic eggs must also, of necessity, come into contact with water. In addition to the absence of a tadpole stage, direct-developing species commonly exhibit some form of parental care of the developing egg clutch. Brooding adults may prevent fungal attack of eggs by spreading anti-microbial skin secretions around the eggs, or by providing antimicrobial substances produced by their skin microbiota (Duellman and Trueb 1994; Crump 1995; Harris et al. 2006). Studies have shown that deserted nests of brooding species, often contain eggs that succumb to fungal infections (Forester 1979; Townsend et al. 1984; Simon 1993). Possession of antifungal secretions that protect developing egg clutches may predispose terrestrial breeders to stronger resistance against *Bd* than more aquatic species.

While research has focused on measuring the AMP effectiveness of species that have been affected by *Bd*, little work has been carried out on species that have not appeared to suffer from declines associated with chytridiomycosis. Examining the effectiveness of AMPs in species that have not undergone population declines is necessary to determine whether resistance in these species is conferred by this aspect of the innate immune system. Australian frogs of the family Microhylidae inhabit environments that are favourable to *Bd*, and occur where sympatric species have suffered from population declines associated with chytridiomycosis (Richards et al. 1993; McDonald and Alford 1999; Richards and Alford 2005), however, they appear to be unaffected by the disease. A multi-year survey of 595 individuals in wild populations of nine microhylid species found none with *Bd* infections (Chapter 3), and no microhylids appear to have suffered from population declines in northern Queensland, as numbers appear to be stable (Chapter 2; Richards and Alford 2005; Williams 2007). In laboratory experiments, at least one microhylid species (*Cophixalus ornatus*) has been successfully infected with *Bd*, but infection levels were low, and frogs appeared to clear

infections relatively rapidly (Chapter 4). One possible explanation for all of these facts is that Australian microhylids possess AMPs that are highly effective inhibitors of *Bd*. It is thus of interest to compare AMP secretions and measures of AMP effectiveness between microhylids and other species that are more susceptible to this disease. This study aims to determine whether Australian microhylids possess innate immune mechanisms, in the form of AMPs, that are highly effective against *Bd*.

Methods

Animals

From 2005 through 2007, four 500 m, high-elevation (≥ 800 m) transects were surveyed for microhylids within the Wet Tropics (WT) bioregion of northern Queensland, Australia (Table 5.1). Individual frogs were located via their mating calls, and were captured in inverted, labeled, 20x20cm plastic bags containing a handful of leaf litter. Calling sites were individually marked, to allow release of individuals at the point of capture. A sterile medical cotton swab (Medical Wire & Equipment Co. Bath Ltd., Wiltshire, UK) was run over the dorsal and ventral surfaces of each frog, including the forelegs, and hindlegs, according to methods described by Skerratt et al. (2008). These swabs were analysed for the presence of *Bd* DNA at James Cook University (JCU), using the real-time TaqMan polymerase chain reaction (qPCR) diagnostic assay developed by Boyle et al. (2004), with each sample run in triplicate. To minimise contact and disturbance, males were released at their calling sites as soon as possible after measuring, identification, and peptide collection had taken place (usually <3hr). Individuals were weighed to the nearest 0.01g and snout vent length (SVL) was measured (to the nearest 0.1mm). To ensure that *Bd* was not transferred among frogs or field sites, strict hygiene protocols were followed (Speare et al. 2004).

Table 5.1 Location of collection sites within the WT region of northern Queensland.

Label	Location	Latitude S	Longitude E	Elevation (m)
AU10	Charmillin Ck	17°40.86'	145°31.19'	1000
BK	Mt Bellenden Ker	17°15.84'	145°51.31'	1550
CU8	Mt Lewis	16°35.38'	145°17.35'	800
Pal	Paluma	19°00.42'	146°12.43'	900

Collection of skin peptides

Secretions containing skin peptides were collected by norepinephrine induction as described by Rollins-Smith et al. (2005). Frogs were weighed to the nearest 0.1 g and injected with 0.01 ml per gram body weight of norepinephrine bitartrate salt (Sigma N-5785, St. Louis, USA) at 10nM concentration in phosphate buffered saline. Animals were placed in a disposable

250ml specimen jar with 50 ml of collecting buffer (50 mM sodium chloride, 25 mM sodium acetate, pH 7.0), and remained largely submerged for 15 min while skin secretions accumulated. Animals were then removed, and the collecting buffer solution was acidified with 1ml of 50% HCl in order to inactivate endoproteases that are also secreted by the skin (Resnick et al. 1991). The acidified collection buffer with peptides was passed over C-18 Sep-Pak cartridges (Waters Corporation, Milford, MA, USA), and the Sep-Paks were stored under moist conditions in a sealed vial containing a small amount of 0.1% HCl until they could be further processed at JCU herpetology laboratory.

Peptides bound to Sep-Paks were eluted with 70% acetonitrile, 29.9% water, 0.1% triflouroacetic acid (TFA; v/v/v) and concentrated to remove the acetonitrile using a rotary evaporator. Samples were then concentrated to dryness by freeze drying. A MicroBSA Assay (Pierce, Rockford, IL, USA) was used to determine the total concentration of skin peptides recovered after Sep-Pak separation. The assay was followed according to manufacturer's directions except that bradykinin (RPPGFSPFR; Sigma Chemical, St. Louis, MO) was used to establish a standard curve (Rollins-Smith et al. 2002c; Woodhams et al. 2005). Peptide samples were reconstituted to a known concentration using HPLC water to either 1mg/mL or 5mg/mL depending on the volume of the sample. This mixture was then purified using a sterile 0.22 μm filter (Millex GV, Millipore Corp., Bedford, MA, USA), and transferred into labeled, sterile, microtubes. Serial dilutions were created using sterile HPLC water as the diluting agent to determine the minimum concentration of peptide solution that would cause an inhibition of *Bd* growth in the challenge assay. Dilutions of 1:1, 1:2, 1:4, and 1:8 were prepared, with each sample having a minimum of one and a maximum of four dilutions.

***Bd* growth inhibition assay**

A *Bd* type isolate (GibboRiver-L.lesueuri-00-LB-1) was cultured following the protocol of Longcore et al. (1999) except that it was grown in 50% nutrient tryptone-gelatine hydrolysate-lactose (TGhL) broth medium (8 g tryptone, 2 g gelatin hydrolysate, 1 g lactose, 1000ml distilled water).

Zoospores were harvested and plated with or without peptides as described by Rollins-Smith et al. (2002a). In summary, samples for testing comprised five replicates of $1x10^6$ zoospores/mL in 50 μl of broth, plated in 96-well flat bottom microtiter plates (Costar 3596, Corning Inc., NY, USA) with the addition of 50μl of serially diluted peptide mixture in sterile HPLC-grade water (Rollins-Smith et al. 2002a; 2002b; 2002c). To determine maximal growth (positive control), some wells received $1x10^6$ zoospores/mL in 50 μl of broth, plated with 50μl of HPLC-grade water without peptide. To determine the value for maximal inhibition of *Bd* (no growth), a negative control was included comprising $1x10^6$ heat-killed zoospores/mL (treated at 60 ºC for 30 min) in a volume of 50 μl broth, plated with 50μl of HPLC-grade water without peptide.

Plates were sealed with Parafilm®, and incubated at 23°C. Growth at each of days 0-10 was measured as optical density at 492nm (OD) with a spectrophotometer. The OD of wells was recorded daily until the positive control samples reached a growth plateau, which usually occurred within 7-9d and did not exceed 10d. In comparison to negative controls, wells with higher OD readings were interpreted a cell growth, and readings lower than positive controls were interpreted as an indication of inhibitory peptide bioactivity. Any contaminated wells were removed from the analysis.

The percentage inhibition of *Bd* growth in challenge assays was determined by comparing OD readings of the positive control wells on each plate on the day of maximum *Bd* growth (Dx), with the OD readings of the positive control wells at day zero (D0). Dx was determined by plotting the positive control readings from each plate against time and determining the day where the OD reading reached a plateau. Once this day was determined, the following equation was calculated using the equation formulated by Kenyon (2008):

$$\% \text{ inhibition} = \{1-[(Dx - D0)/(Dx - D0) + (\text{mean positive control OD})]\} \times 100$$

Inhibition of *Bd* growth was also observed manually by viewing plates under a light microscope and looking for wells that had limited or complete loss of *Bd* growth. When wells were observed where the fungus showed no signs of growth, the sample was recorded as having 100% inhibition.

The standard statistic considered in medical and toxicological literature is the LC50, 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, as a frog surrounded by a concentration of AMPs that reached the LC50 for *Bd* zoospores could presumably still become infected and possibly die as a result of chytridiomycosis. I therefore calculated and analysed the Minimal Inhibitory Concentration (MIC). The MIC in μg/ml is defined as the lowest concentration of peptide sample at which no *Bd* growth was detectable. That is, the OD was not significantly greater than that observed for negative control wells.

Total peptide secreted by each individual frog per unit surface area was approximated using the following equation (Woodhams et al. 2005):

$$\sum \text{protein secretion } (\mu g/cm^2) = \text{Protein quantity } (\mu g) / \text{Surface area of frog } (cm^2)$$

The concentration of peptides in each sample was determined using the MicroBSA protein assay. The surface area for each frog was approximated using a mathematical equation developed by McClanahan and Baldwin (1969), which is calculated using body weight. The equation is:

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

To determine the overall protection that was afforded to an individual frog, the effectiveness of skin peptide secretions was calculated and compared. This was carried out using an equation that was based on the MIC value of peptide samples and the amount of peptides per surface area (Kenyon 2008).

$$\text{Overall protection (ml at MIC per cm}^2\text{)} = \frac{\text{Total mass of peptides}}{\text{Surface area (cm}^2\text{)}} \times \frac{1}{\text{MIC}}$$

Statistical analysis

Some samples did not show complete inhibition of *Bd* growth at any tested AMP concentration. These are not commensurate with samples that did. I analysed all data in two stages; initially, I compared the proportion of samples that did and did not show complete inhibition at any tested concentration among microhylid species using Fisher's exact tests carried out in StatXact 4.0 (Cytel Software Corp. 1998).

To determine whether protein secretion was correlated with body mass, I regressed the amount of peptide secretion (μg) on body mass (g). To compare total peptide secretion among microhylid species I conducted a one-way ANOVA on total peptide secretion (μg/cm^2) of individuals with complete inhibition of *Bd* growth. I compared the average total protein secretion in microhylids with average results for Queensland rainforest frog species *Litoria genimaculata* and *L. rheocola,* as reported by Kenyon (2008), using a Student's t-test.

The MIC values (μg/ml) of peptide samples that caused 100% inhibition of *Bd* growth were compared among microhylid species using one-way ANOVAs. Mean MIC equivalents of microhylids were also compared with those of four southeastern Queensland frog species reported by Woodhams et al. (2007a), using a Student's t-test. To test for differences in the effectiveness of skin peptides, the overall protection conferred to each individual frog whose peptides showed 100% inhibition of *Bd* was calculated as MIC (μg/ml) equivalents per cm^2 surface area. Overall protection was compared among species using a one-way ANOVA. Mean protection values for microhylids were also compared to values for other Queensland rainforest frog species reported by Woodhams et al. (2005) and Kenyon (2008) using a Tukey boxplot.

Results

Inhibition of growth of *Bd*

Natural mixtures of skin peptides were collected during 2005-07 from 81 individual microhylids belonging to six species. qPCR analyses of skin swabs indicated that all frogs were negative for the presence of *Bd* DNA. The potency of skin peptides against *Bd* was examined, and results of challenge assays for each of the skin peptide samples are shown in Appendix 2. Thirty one of the 81 (38.3%) skin peptide samples showed no inhibition of the growth of *Bd*, 36

samples (44.4%) showed partial inhibition of *Bd* growth, and 14 samples (17.3%) showed complete inhibition of growth at the concentrations tested. These results are summarised in Table 5.2. The proportion of peptide samples that caused total, partial or no inhibition of the growth of *Bd* did not differ significantly among species (Fisher statistic = 6.63, exact P = 0.75).

Table 5.2 Skin peptide assay results showing inhibition against *Bd* growth.

Species	*N*	No inhibition	Partial inhibition	Complete inhibition	Mean % inhibition
A. fryi	7	3	3	1	34.64
A. pluvialis	3	1	2	0	12.19
A. robusta	21	9	9	3	40.16
C. aenigma	2	1	1	0	12.69
C. neglectus	25	7	14	4	42.35
C. ornatus	23	10	7	6	54.62
Total	81	31	36	14	32.78

In each species that was sampled, some individuals produced peptide secretions that caused at least some inhibition of the growth of *Bd*. No samples taken from *Austrochaperina pluvialis* or *Cophixalus aenigma* produced complete inhibition of growth, however, sample sizes for these species were low (*N*=3 and *N*=2 respectively). AMPs that completely inhibited *Bd* growth occurred in four species at four locations (Table 5.3). For these samples, the calculated percentage inhibition of *Bd* growth ranged from 92-100% in challenge assays (see Appendix 2), but inhibition was determined to be at 100% by visual observation. In total, 50 of the 81 samples assayed (62%) showed complete or partial inhibition of the growth of *Bd*. This value is very similar to the proportion that Kenyon (2008) found using the same techniques. In her results 124 of 206 samples (60%) taken from the hylids *Litoria genimaculata* and *L. rheocola* showed some evidence of *Bd* inhibition (chi-squared homogeneity test, chi-squared = 0.06, 1 d.f., P = 0.81)

Table 5.3 Detailed results for skin peptide samples that showed complete inhibition of *Bd* growth in challenge assays.

Species	Date	Site	Protein assay (μg)	Calculated % inhibition	MIC (μg/ml)	SA (cm2)	Peptide secretion (mg/mL)	Protection
A. fryi	21/01/26	CU8	1223.5	92.94	500	15.86	77.14	0.15
A. robusta	24/01/07	Pal	3320	94.69	1000	9.90	335.35	0.34
A. robusta	24/01/07	Pal	1693	100	500	10.34	163.80	0.33
A. robusta	24/01/07	Pal	1959.5	95.31	1000	11.66	168.01	0.17
C. neglectus	7/12/05	BK	902.5	100	250	12.24	73.75	0.30
C. neglectus	7/12/05	BK	1537.5	97.01	250	14.84	103.61	0.41
C. neglectus	7/12/05	BK	1290.5	100	250	9.45	136.58	0.55
C. neglectus	7/12/05	BK	1167.5	99.10	250	11.57	100.95	0.40
C. ornatus	8/03/07	AU10	1644	100	500	8.68	189.49	0.38
C. ornatus	23/01/07	Pal	2814.5	100	1000	9.04	311.38	0.31
C. ornatus	24/01/07	Pal	1327.5	99.11	100	8.74	151.94	1.52
C. ornatus	24/01/07	Pal	1411.5	92.01	500	9.73	145.03	0.29
C. ornatus	25/01/07	Pal	1946.5	98.08	1000	7.91	246.05	0.25
C. ornatus	25/01/07	Pal	1185.5	100	500	9.33	127.03	0.25

Within *C. ornatus*, the proportion of peptide samples that did and did not completely inhibit the growth of *Bd* differed significantly among sites. The proportion of samples from *C. ornatus* at Paluma that inhibited *Bd* (83.3%) was significantly greater than the proportion of samples from site AU10 (6.7%; Fisher statistic = 0.58, exact $P < 0.01$).

Total skin peptides secreted

For peptide samples that produced partial or complete inhibition of *Bd* growth (*N*=50), the total amount of peptides secreted (ignoring species identity) was not correlated with body mass (r^2=0.01; p=0.49). Separate correlation analyses within species with more than 5 samples showing complete inhibition also showed no significant correlations (*A. robusta* r^2=0.04; *C. neglectus* r^2=0.02; *C. ornatus* r^2=0.17, all $P > 0.05$).

Protein secretion (μg) per cm^2 for individuals with AMPs that completely inhibited *Bd* growth differed significantly among species (ANOVA, $F_{3,14} = 5.41$, $P = 0.01$). *A. robusta* had the highest average protein secretion per unit area, followed by *C. ornatus*, *C. neglectus*, and *A. fryi* (Figure 5.1; Table 5.4). The average total protein secretion (μg) per cm^2 of samples from two species of Queensland rainforest hylid frogs reported by Kenyon (2008) fell within the range of results I obtained for microhylids (Table 5.4).

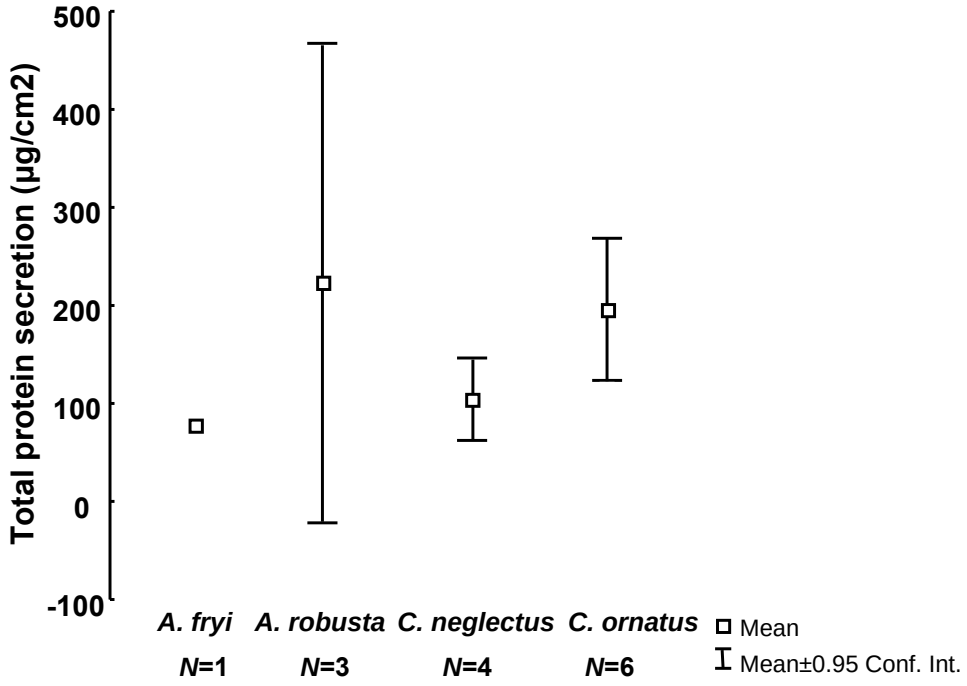

Figure 5.1 Total protein secretion (µg) per surface area (cm^2) of microhylids that produced complete inhibition of *Bd* growth. Differences among species are significant (ANOVA, $F_{3,14}$ = 5.41, P = 0.01).

Table 5.4 Average total protein secretion (µg) per surface area (cm^2) for microhylids and for northern Queensland rainforest hylids (*Litoria* spp.) as reported by Kenyon (2008).

Species	*N*	Total secretion (µg) per cm^2
Austrochaperina fryi	1	77
Austrochaperina robusta	3	222
Cophixalus neglectus	4	104
Cophixalus ornatus	6	195
Litoria genimaculata	160	130
Litoria rheocola	56	185

Effectiveness of AMPs (MIC)

For samples that inhibited *Bd*, the concentration of peptides necessary to completely inhibit growth of the fungus ranged from 100-1000 µg/ml (Table 5.3). The most potent natural peptide mixture occurred in a secretion from *C. ornatus* that inhibited zoospore growth at a concentration of 100 µg/ml. *A. robusta* had the highest mean MIC value of 833 µg/ml, followed by *C. ornatus* (mean MIC = 600 µg/ml), *A. fryi* (mean MIC = 500 µg/ml), and *C. neglectus* (mean MIC = 250 µg/ml; Table 5.5). Mean MIC values did not differ significantly among microhylids (ANOVA, $F_{3,10}$ = 2.69, P = 0.10). Three of the four overall mean MICs of four non-microhylid species reported by Woodhams et al. (2007a) fell within the range I found for microhylids; the other was lower (*Limnodynastes tasmaniensis;* Table 5.5; Figure 5.2).

Table 5.5 Mean MIC (μg/ml) values for microhylid species, and south-eastern Australian species as found by Woodhams et al (2007a).

Species	*N*	Mean MIC (μg/ml)
Austrochaperina fryi	1	500
Austrochaperina robusta	3	833
Cophixalus neglectus	4	250
Cophixalus ornatus	6	600
Limnodynastes tasmaniensis	20	133
Litoria caerulea	20	271
Litoria chloris	20	261
Mixophyes fasciolatus	20	272

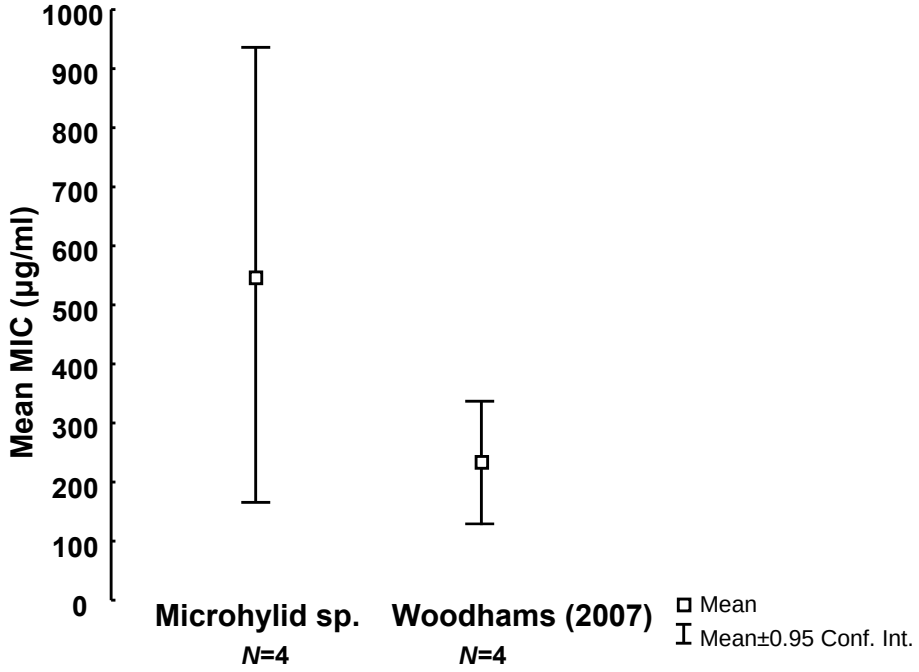

Figure 5.2 Mean MIC (μg/ml) values for microhylid species, and four south-eastern Australian species reported by Woodhams et al (2007a). Three of the four mean values for microhylids were higher than any of those reported by Woodhams et al (2007a), but the difference in means was not significant ($t_6 = 2.48$, $P = 0.09$).

Overall protection of species

The average level of protection of microhylids (mL of solution at MIC per cm^2 surface area) did not differ significantly among microhylid species (ANOVA, $F_{3,14} = 0.42$, $P = 0.74$). Mean protection of microhylid species were lower than, but not significantly different from other, non-microhylid Queensland frog species ($t_9 = 1.71$, $P = 0.12$; Table 5.6; Fig 5.3; Woodhams et al. 2005; Kenyon 2008). However, Woodhams et al (2005) did not inject frogs with norepinephrine, and thus may not have measured the total quantity of peptides available.

Kenyon (2008), on the other hand, used the same sampling technique I used. Comparing my results with hers suggested that microhylids may be less protected than the hylids sampled by Kenyon (2008). A t-test comparing the means for my four species with that for her two species indicated that hers had significantly higher levels of overall protection ($t_4 = 7.5$, $P = 0.002$).

Table 5.6 Mean overall protection (mL of AMPs at MIC/cm^2) microhylid species, and Australian rainforest frog species as found by Woodhams et al (2005) and Kenyon (2008).

Species	*N*	Protection	Researcher
Austrochaperina fryi	4	0.15	Hauselberger
Austrochaperina robusta	3	0.28	Hauselberger
Cophixalus neglectus	18	0.41	Hauselberger
Cophixalus ornatus	14	0.50	Hauselberger
Litoria genimaculata	160	1.19	Kenyon
Litoria rheocola	56	1.35	Kenyon
Litoria genimaculata	20	0.31	Woodhams
Litoria leseuri	20	0.48	Woodhams
Litoria nannotis	20	0.35	Woodhams
Litoria rheocola	20	0.5	Woodhams
Nyctymistes dayi	20	0.8	Woodhams

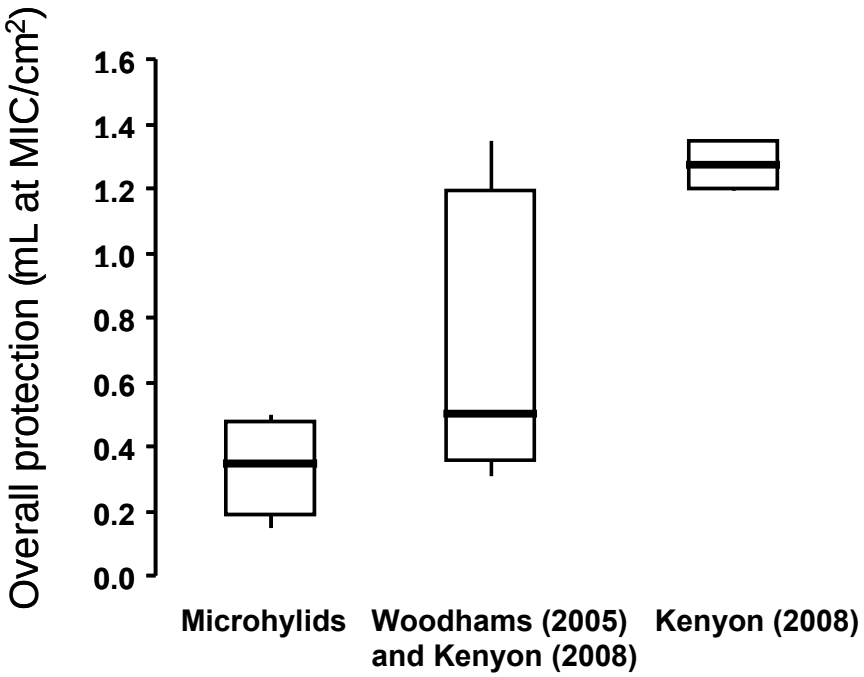

Figure 5.3 Tukey (1977) boxplot illustrating average overall protection of microhylid species from the present study, other Australian rainforest frog species as measured by Woodhams et al (2005) and Kenyon (2008), and only the two species measured by Kenyon (2008) using a technique identical to that used in the present study. Vertical bars indicate ranges, horizontal bar indicates median, boxes indicate upper and lower quartile boundaries.

Discussion

Most amphibian species express an array of peptides as part of their innate immune function (Vanhoye et al. 2003), and a more complete understanding of the effectiveness of the AMP repertoire of each species can be reached by examining the activity of natural mixtures of skin peptides. In this study, 50 of 81 (62%) individuals sampled produced AMPs with at least some activity against *Bd*, and 14 (17%) showed 100% inhibition of *Bd* growth at the levels tested.

Since it is not known exactly how AMPs act to inhibit *Bd*, I examined all possible effects by using natural mixtures of peptides in frog skin secretions, and calculating several potential measures of AMP effectiveness (total peptide secretion, MIC, and protection per unit surface area). I also compared various measures of AMP effectiveness for microhylids to those calculated for other Queensland frog species, to determine whether AMPs might be responsible for the far lower prevalence of *Bd* infection that microhylids experience in the WT.

The proportions of peptide samples that completely and partially inhibited *Bd* did not differ significantly among microhylid species, although the sample size of AMPs that produced complete inhibition against *Bd* was small. This suggests that the level of activity of AMPs is similar among microhylid species. When the samples that completely inhibited *Bd* growth were compared among species, there were significant differences in total protein secretion per cm^2 of skin surface, but the average protein secretion for microhylids did not differ significantly from results for *L. genimaculata* and *L. rheocola* (Kenyon 2008). Thus, microhylids apparently produce AMPs in quantities per unit area similar to sympatric Queensland rainforest frogs.

Microhylid species did not differ significantly in terms of the lowest concentration of peptides that caused 100% inhibition of *Bd* growth (MIC). The mean species MIC (μg/ml) for three of four Australian rainforest frog species (*Limnodynastes tasmaniensis*, *Litoria caerulea*, *Lit. chloris*, and *Mixophyes fasciolatus*) reported by Woodhams et al. (2007a) fell within the range of species mean MIC values I measured for microhylids. The AMPs of Australian microhylids thus do not appear to be effective at lower concentrations than those of other Queensland frogs.

The average level of protection of microhylids (mL of solution at MIC per cm^2 surface area) did not differ significantly among microhylid species, nor was it different from the average level of protection found for seven Queensland rainforest hylid species as measured by Woodhams et al. (2005) and Kenyon (2008). However, the average level of protection of microhylids was significantly lower than for the species *L. genimaculata* and *L. rheocola* examined by Kenyon (2008), who used the same norepinephrine injection technique I used to collect peptides. The only difference between my sample collection and analysis and that of Kenyon (2008) was that she included results for peptide samples in which no concentrations of

the actual peptides tested produced 100% inhibition of *Bd*. In these samples, extrapolation was used to predict the peptide concentrations where 100% inhibition would occur. If this introduced any bias, it should have been to reduce overall protection levels, as it allowed Kenyon to include data for samples that required very high peptide concentrations to achieve 100% inhibition of *Bd*. Despite this bias, my results indicate that overall protection levels of the four species of microhylids I examined were lower than those measured for hylids by Kenyon (2008).

Although the concentrations of peptides used in challenge assays were consistent with those used in other studies of AMPs in Australian frog species, in most cases (83%), peptide samples from microhylids did not completely inhibit the growth of *Bd in vitro*. Although less than complete inhibition could provide protection from the development of chytridiomycosis in infected frogs, and thus provide immunity to the disease, it should not by itself provide immunity against infection by the pathogen, which is the feature that distinguishes microhylids sampled in nature from other Queensland rainforest frogs. Microhylid species appear to produce peptides in amounts comparable to at least two other sympatric frog species (*L. genimaculata* and *L. rheocola*), as determined by Kenyon (2008). The proportion (62%) of samples from microhylids that showed at least some activity against *Bd* was similar to Kenyon's samples (60%), however, the overall protection of microhylid frogs provided by AMPs was significantly lower than for these two species. The protection levels for microhylid frogs was also towards the low end of the range shown for four additional Queensland frog species as found by Woodhams et al. (2007a).

Thus, although the proportion of individual microhylids that produce AMPs with some anti-*Bd* activity are similar to atleast two other species of Hylid frogs, the AMPs of microhylids do not appear to be more effective against *Bd* than those of other rainforest species. AMPs may reduce the growth and reproduction of the pathogen, but it appears that the low prevalence of infection by *Bd* in Australian microhylids is likely to be due to some factor. It is possible that AMPs combined with other defenses may account for the very low susceptibility of Australian microhylids to chytridiomycosis in nature.

Experimental evidence suggests that at least one species of microhylid (*C. ornatus*), can be infected by *Bd* but is highly resistant to the development of chytridiomycosis (Chapter 4). Experimental exposures of *C. ornatus* to high levels of *Bd* zoospores produced infections, but no individuals developed chytridiomycosis, and all individuals cleared infections, as shown by qPCR assay, at five weeks post exposure. This indicates that a different mechanism enables microhylids to clear themselves of *Bd* infections.

AMPs are not the only innate immune response in amphibians. Other epithelial defenses, such as regulation of skin sloughing and commensal microbiota, may influence *Bd* zoospore survival and encystment rates (Woodhams et al. 2008). Some bacteria that live on the

epidermis of amphibians produce antimicrobial agents effective against pathogenic fungi, including *Bd* (Austin 2000; Harris et al. 2006; Lauer et al. 2007; Banning et al. 2008; Brucker et al. 2008a; 2008b). Some species of amphibians that lay eggs in terrestrial nests that are brooded or tended by adults, possess anti-fungal microbiota that can inhibit the growth of fungi that are pathogens of embryos (Austin 2000; Lauer et al. 2007; Banning et al. 2008). These species may not suffer from severe population declines associated with chytridiomycosis because they may be more likely to harbor bacteria that inhibit the growth of *Bd* (Harris et al. 2006). It is possible that microhylids possess antimicrobial skin flora that inhibits *Bd* growth, and confers at least partial resistance to chytridiomycosis. More research is needed to determine the ability of microhylids to resist infection by *Bd*, as it appears that this family's AMPs may not be their most important defense against *Bd*. Additional studies to determine the levels at which AMPs are normally present on microhylid skin and to further understand the microbial ecology of amphibian skin are needed to determine if other innate immune responses are responsible for the apparent resistance of microhylids to chytridiomycosis.

CHAPTER 6: MICRO-ENVIRONMENTAL CONDITIONS EXPERIENCED BY MICROHYLIDS AND THEIR IMPLICATIONS FOR VULNERABILITY TO CHYTRIDIOMYCOSIS

Abstract

Temperature and moisture profoundly affect many aspects of the biology of terrestrial amphibians, including their susceptibility to disease. Both can vary over fine spatial scales, and the environments experienced by individual amphibians can thus be strongly influenced by microhabitat selection. The disease chytridiomycosis, caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) has caused declines and extinctions of amphibians in many areas of the world. The outcome of *Bd* infections is strongly influenced by temperature and moisture, and therefore depends on the microenvironmental conditions encountered by infected individuals. Although chytridiomycosis has caused many declines and extinctions of frogs in the Australian Wet Tropics region, it has not affected microhylid populations. Laboratory experiments (Chapter 4) have shown that Australian microhylids can become infected by *Bd*, however a large-scale survey (Chapter 3) failed to detect any infected individuals in the field. These results suggest that microhylids are not innately immune to *Bd* infection, but rarely if ever become (or remain) infected in the field. One possible explanation for their lack of susceptibility to infection is the microenvironmental conditions they encounter; if they frequently experience conditions hostile to the pathogen, they may be protected from infection. To determine whether this might explain their apparent immunity to infections in the field, I measured the microenvironmental conditions experienced by microhylids in retreat sites. During surveys, I recorded frogs' surface temperatures and the temperatures of their calling and retreat sites. I used permeable and impermeable (plastic-coated) agar models with embedded temperature probes to collect longer-term data on the thermal and hydric regimes encountered by microhylid frogs, by placing pairs of models in frog retreat sites. Models were placed in the field for periods of 62-112h between 27 November 2005 and 10 March 2006. Model temperatures were recorded hourly, and models were weighed twice daily to measure water gain or loss.

Permeable models either gained or lost weight when compared to impermeable models, depending on the presence of rainfall at sites. Models never lost more than 20% of weight even after 96h at sites without rainfall, suggesting that retreat sites provide a buffered environment that slows dehydration. Models produced an accurate outline of the thermal envelope of active and resting frogs, as of the 61 frogs that were examined during the period in which models were placed in the field, only six (9.8%) had surface temperatures that were outside of the maximum and minimum values of models. Surface temperature readings of 197 frogs were within the growth range for *Bd*, and no frog was ever found with a surface

temperature that could inhibit the growth of *Bd*. Thermal data from models indicated that two transects were within the optimal growth range of *Bd*. However, one transect had temperatures that were below the lower boundary of the optimal range for growth 76.6 percent of the time, and another transect had temperatures that were above 28ºC at least 11.9 percent of the time, which could result in the inhibition of *Bd* growth. This suggests that the thermal and hydric environments used by microhylids may affect the overall dynamics of the *Bd* host-pathogen system, and ultimately their susceptibility to infection by *Bd*.

Introduction

Transdermal water uptake, gas exchange via the skin, and convective, conductive, and radiant heat transfer are fundamental aspects of amphibian biology (Laurance 1996; Donnelly and Crump 1998). The temperature and moisture of the immediate environment thus have strong effects on the biology of amphibians (Carey and Alexander 2003). Anuran hydro- and thermoregulation are intrinsically linked, as temperature can be affected by rates of evaporative water loss (EWL), and the ability to thermoregulate can be affected by the availability of water (Tracy 1976; Preest and Pough 1989; Wygoda and Williams 1991).

The majority of amphibians have relatively little physiological control over heat transfer mechanisms or EWL from the integument, so that hydro- and thermoregulation are accomplished by behavioural changes (O'Connor 1999). These behavioural adaptations enable some species to maintain relatively constant body temperatures and moisture levels despite fluctuations in ambient conditions, and include traits such as basking, nocturnal activity, fossorialilty, the use of water conserving postures, and the use of particular microhabitats in the environment such as burrows and retreat sites (Brattstrom 1970; Shoemaker and Nagy 1977; Huey 1982; Shoemaker et al. 1992; Toledo and Jared 1993).

The use of refuges is a critical component of the ecology of many terrestrial amphibians, allowing them to avoid adverse environmental conditions. Amphibians use naturally occurring cavities (Stewart and Pough 1983), burrows (Hoffman and Katz 1989; Felton 1999), or “forms”, which are shallow depressions created by removing surface vegetation, allowing direct contact between the animal’s ventral surface and the soil (Dole 1965). A moist refuge consisting of leaf litter or soil creates a layer of still air around an animal, and impedes diffusion by lengthening the diffusive path (O'Connor et al. 2006). This decreases rates of evaporation, and can extend the amount of time that animals can be away from a water source (Tracy 1976; Pough et al. 1983; O'Connor et al. 2006). Laboratory experiments (Helmuth 2002), and field trials (Schwarzkopf and Alford 1996) show that the actual temperature and humidity experienced by an amphibian in a refuge microclimate may differ substantially from the

ambient environment (Hutchinson and Dupré 1992; Seebacher and Alford 2002). Refuge selection thus allows amphibians to buffer themselves against the negative effects of temperature or moisture on physiological performance (Tracy 1976; Pough et al. 1983; Stewart and Pough 1983; Schwarzkopf and Alford 1996).

A variety of methods have been used to investigate the temperature and moisture relations of amphibians. Body temperatures have been measured directly, using thermometers, thermistors, or thermocouple probes inserted into the cloaca or held against the skin, or using infrared thermometers (Bakken 1992; Rowley and Alford 2007b). EWL has been measured directly by determining the mass of water vapour leaving an animal using hygrometric flow-through techniques (Withers et al. 1984; Christian and Parry 1997). Temperature and EWL have also often been estimated using models or artificial animal replicas (Spotila and Berman 1976; Bradford 1984; Wygoda and Williams 1991). Models produce an approximation of body temperature for an animal in steady-state, but as many amphibians possess variable rates of EWL and evaporative cooling, the design and use of models is more difficult than for other ectotherms (Shoemaker and Nagy 1977). Models must mimic the hydric properties of amphibians, and therefore they must be hydrated. Rates of EWL can then be determined by periodically weighing models to determine moisture loss or gain (Bartelt and Peterson 2005). Rates of EWL and temperature have been estimated in amphibians by measuring changes in weight of moistened, porous plaster moulds (O'Connor and Tracy 1987; Wygoda and Williams 1991; Wygoda and Garman 1993), hollow copper tubes covered in wet fabric (Bradford 1984; Bartelt and Peterson 2005), dead or immobilised amphibians (Wygoda 1988; Seebacher and Alford 2002), and agar casts of animals (Schwarzkopf and Alford 1996; Navas and Araujo 2000; Rowley and Alford 2010).

While a number of studies have used models to predict temperature and EWL in active amphibians, there is little literature on temperature and EWL for animals in retreat sites. Schwarzkopf and Alford (1996) used agar models to measure desiccation rates of cane toads (*Bufo marinus*); models were placed in retreat sites that had been previously used by toads. They found that desiccation rates varied dramatically among retreat-site types. They were lowest in burrows, and highest in fully exposed sites used by toads. Rowley and Alford (2009) produced an accurate outline of the thermal envelope of body temperature for the Australian hylid frog *Litoria lesueuri*. By placing pairs (one with zero resistance to EWL, one with near perfect resistance to EWL) of agar models with embedded temperature dataloggers in retreat and activity sites, they characterised the thermal and hydric conditions available to frogs.

A detailed understanding of the micro-environments selected by frogs may improve our understanding of interactions between frogs and the epidemic disease chytridiomycosis (Berger et al. 1998). This disease is caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), which requires a moist environment to reproduce, and has a thermal

optimum of 17-25°C (Piotrowski et al. 2004). The fungus shows inhibition of growth at 28°C and death occurs at 30°C (Longcore et al. 1999; Piotrowski et al. 2004). Major determinants of disease outbreaks and pathogen emergence may include environmental factors such as temperature and moisture (Dobson and Carper 1993; Epstein 1999; Daszak et al. 2001; Brem and Lips 2008); this is apparent in the epidemiology of outbreaks of chytridiomycosis. The pathogen's relatively low range of optimal temperatures range and high moisture requirement are obvious in the spatial patterns of epidemic outbreaks. These occur most often and most severely in high elevation species that occupy moist environments (Speare 2000; Lips et al. 2005), increase in the cooler months (Berger et al. 2004; Retallick et al. 2004; McDonald et al. 2005; Woodhams and Alford 2005; Kriger and Hero 2007a), and increase in the wet season in tropical environments (Berger et al. 2004; Lips et al. 2006). Variation in thermoregulatory opportunities and behaviour of amphibians are likely to contribute to the differences in disease prevalence and severity observed among host species, populations, and regions.

The microhylid frogs of Australia are an example of a group of amphibians that can exist in terrestrial environments without access to standing water. They rely on their external surroundings for water, using the condensation of moisture on vegetation to hydrate (Williams and Hero 2001). They produce terrestrial, direct-developing eggs, and thus do not need access to water for reproduction. Behavioural adaptations to their terrestrial life history include being nocturnally active, retreating in shelters such as under logs and leaf litter, and constructing burrows for deposition of terrestrial eggs (Zweifel 1985; Hoskin 2004; Felton et al. 2006). Regional patterns of diversity in microhylids are strongly related to consistent moisture levels throughout the year, and are limited by low rainfall in the dry season (Williams and Hero 2001). Although many species live at high elevations in the Wet Tropics (WT), none have been observed to decline in abundance during mass declines and extinctions of other species caused by chytridiomycosis (Richards et al. 1993; McDonald and Alford 1999; Chapter 2). Laboratory studies have confirmed that the most common species, *Cophixalus ornatus*, can become infected by *Bd* (Chapter 4), and the antimicrobial peptide (AMP) defenses of microhylids are no more effective against the pathogen than are those of hylid species (Chapter 5), many of which have declined in association with epidemic outbreaks of chytridiomycosis. However, a survey of 595 individuals of nine species failed to find any infected individuals in nature (Chapter 3). This suggests that some factor other than the innate defense provided by AMPs must protect microhylids from infection in nature. One possible mechanism is their choice of microenvironments. *Batrachochytrium dendrobatidis* has a relatively narrow range of tolerance for temperature and moisture conditions, and if individuals or species choose to occupy environments outside that envelope, they may be protected from infection. The present study was designed to gain an insight into the effects of microenvironment on the body temperature

and hydric regimes of microhylid frogs, and to determine whether their habitat preferences are a factor in their resistance to infection by *Bd* in nature.

Methods

Microhabitat use of microhylids

To measure the microhabitats used diurnally and nocturnally by microhylids, individuals were sampled along ten transects within the WT region of northern Queensland (Table 6.1). Sampling was carried out during wet seasons (September-March) from 2004 through 2008. Nocturnal sampling began after sunset (19:00h), and continued until the required data were collected or until males ceased calling. Male frogs were found by walking along transects and listening for advertisement calls; females, immatures and non-calling males were located opportunistically. Sites were sampled up to approximately 50m each side of the transect line. Diurnal sampling consisted of log turning along transects during the day. This involved walking up to 50m either side of transects, lifting large pieces of wood or logs that were encountered and examining the habitat underneath them for frogs.

The locations of all frogs encountered in surveys were marked with fluorescent flagging tape at or above eye level, and labeled with unique numbers. Substrate type, height above ground, and degree of exposure of the individual were recorded. Frogs located during diurnal surveys were always in retreat sites and completely unexposed. The substrate type of diurnal retreat sites was categorised as: under log, under rock, in burrow or under recorder box. Frogs located during nocturnal surveys were categorised as: exposed, partially covered, or completely covered. The categories for height above ground were: 0-5, 6-10, 11-20, 21-40, 41-60, 61-80, 81-100, 101-120, 121-140, 141-160, and above 160cm.

Table 6.1 Locations and dates of microhabitat surveys.

Label	Location	Latitude S	Longitude E	Elevation (m)	Sampling Date
AU2	Meena Ck	17°40.01'	145°52.60'	200	7-10/2/2005
AU4	Henrietta Ck	17°36.92'	145°45.32'	400	9-12/12/2005
AU6	South Johnston	17°40.09'	145°43.25'	600	9-12/12/2005
AU8	Ravenshoe	17°36.17'	145°37.85'	800	6-9/3/2007
AU10	Charmillin Ck	17°40.86'	145°31.19'	1000	26/11/2004 - 17/2/2005 6/11/2005 - 13/12/05 20/1/2006 - 3/4/2006 8/3/2007
BK	Mt Bellenden Ker	17°15.84'	145°51.31'	1550	26/11/2004 – 17/2/2005 8/11/2005 – 9/12/2005
CU8	Mt Lewis	16°35.38'	145°17.35'	800	9/12/2005 - 24/2/2006
CU10	Mt Lewis	16°35.21'	145°16.36'	1000	24/01/2006
CU12	Mt Lewis	16°30.56'	145°16.35'	1200	12/12/2005
Pal	Paluma	19°00.42'	146°12.43'	900	7-10/3/06 20-24/1/07

Direct measurements of environmental and body temperatures

To determine the thermal regimes experienced by microhylids, individuals were sampled during diurnal and nocturnal visits to four transects (AU10, BK, CU8 and Pal); transect CU8 was sampled twice (Table 6.2). Body temperature was measured prior to capture using a non-contact infrared (IR) thermometer (Raytek ST80 Pro-Plus Non-contact laser thermometer; Rowley and Alford 2007b). The IR thermometer was placed approximately 20cm away from the frog aiming at the lower dorsal area. Emissivity on the thermometer was set at 0.95. Surface temperature of the environmental substrate where the frog was located was also recorded from within 10cm.

Table 6.2. Locations of thermal regime transects and periods of deployment of physical models.

Transect	Latitude	Longitude	Elevation	Hours of data	Start date	End date
AU10	17°40.86'	145°31.19'	1000	112	27/11/2005	30/11/2005
BK	17°15.84'	145°51.31'	1550	89	5/12/2005	9/12/2005
CU8 1	16°35.38'	145°17.35'	800	62	9/12/2005	12/12/2005
CU8 2	16°35.38'	145°17.35'	800	74	21/01/2006	24/02/2006
Pal	19°00.42'	146°12.43'	900	69	7/03/2006	10/03/2006

Measurements using physical models

To provide longer-term data on the thermal and moisture regimes encountered by frogs in retreat sites, permeable and impermeable agar models were created and used following methods described by Rowley and Alford (2009). Using these methods, data are collected on the temperatures of permeable agar models, which have zero resistance to EWL, and

impermeable (plastic coated) agar models, with perfect resistance but otherwise identical to the permeable models. These are used to define the lower and upper boundaries, respectively, of the range of body temperatures available to frogs with variable or unknown rates of EWL. Permeable models are periodically weighed, and their changes in weight are used as indices of relative water stress among the sites at which models are deployed.

Physical models used in the present study were based on a preserved *C. ornatus* ('ornate nursery frog'), with a snout-vent-length (SVL) of 25.6mm and weight of approximately 1.4g. This specimen had been collected from Kirrama (Qld) in 1997, and was preserved and stored at James Cook University. An exact imprint of the specimen was made in dental moulding material (Palgaflex Quick; D-8031, Praparate GMBH & Co, Germany) by gently pressing the preserved frog into a small container of the moulding material. After the initial mould dried, plaster of paris was poured into the impression and a 3cm long nail was inserted into the wet plaster at the rear end of the cast. The plaster was left to set for 24 hours, and was removed from the original mould, leaving a plaster cast of the frog with a nail protruding from the posterior end. Liquid latex (Shamrock Pty. Ltd., Blackburn, Victoria) was then painted over the plaster cast of the frog and nail, and left to dry for 2-3 hours. 3-4 coats of latex were applied to the cast and nail to ensure a sufficient coating. Once dry, the latex was peeled off the plaster model, resulting in a latex mould of a microhylid frog with a hole in the posterior end. Six identical latex moulds were fashioned in this way from the same original plaster mould.

Permeable models were made by using agar mixed with water at a ratio of 3g per 100mL. Molten agar was poured into the latex casting shells, and a temperature probe was inserted into the model using the channel left by the nail at the posterior end of the original plaster casting, and leaving the lead protruding from the posterior end. Once dry, the latex shell was removed from the agar, leaving the completed model (Figure 6.1). Because the temperature probe weighed substantially more than the agar model and was connected to an external datalogger, using these models to measure water loss by monitoring changes in weight would have been complicated by the need to subtract the mass of the temperature probe. To simplify data collection, models were also constructed without temperature probes. These models were made in the same way as for those with temperature probes, except that the hole in the latex casts was covered with masking tape when the liquid agar was poured into the mould. Half of the models of each type were subsequently made impermeable by coating them with liquid plastic (Plasti Dip®, clear, Plasti Dip International Inc., Blaine, Minnesota, USA).

These models are compact and self contained, allowing them to be placed in the actual shelter sites used by the study species without altering their structure, unlike bulkier physical models (e.g., Bartelt and Peterson 2005). The SVL of all models ranged between 25-26mm, the weight of permeable models without temperature probes ranged from 3-6g, and the weight of impermeable models without probes ranged from 3.5-5.5g.

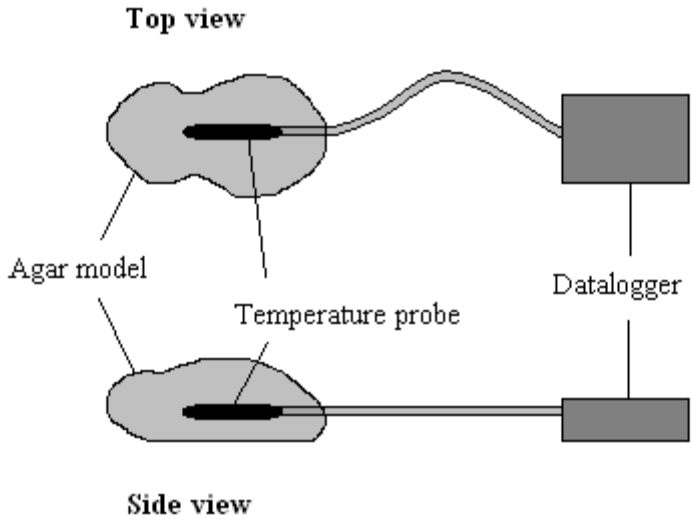

Figure 6.1 Setup of model with an attached temperature probe.

Deployment of models in the field

To prevent desiccation before deployment, completed models were wrapped in moist paper towel and stored refrigerated in plastic containers from immediately after creation until they were used in the field. Prior to being placed in the field, models fitted with temperature probes were attached to HOBO dataloggers (HOBO®XT, Onset computer corporation, USA) which were set to record temperature readings at hourly intervals. Dataloggers were housed in plastic containers containing silica gel to protect them from the elements.

At each transect, three locations were used for model trials, beginning at the 0m mark and spaced approximately 100m apart. At each location, two models with internal temperature probes (one permeable and the other impermeable) and four models without probes (two permeable and two impermeable) were used (Figure 6.2). All models were placed under logs or leaf litter where frogs had previously been located.

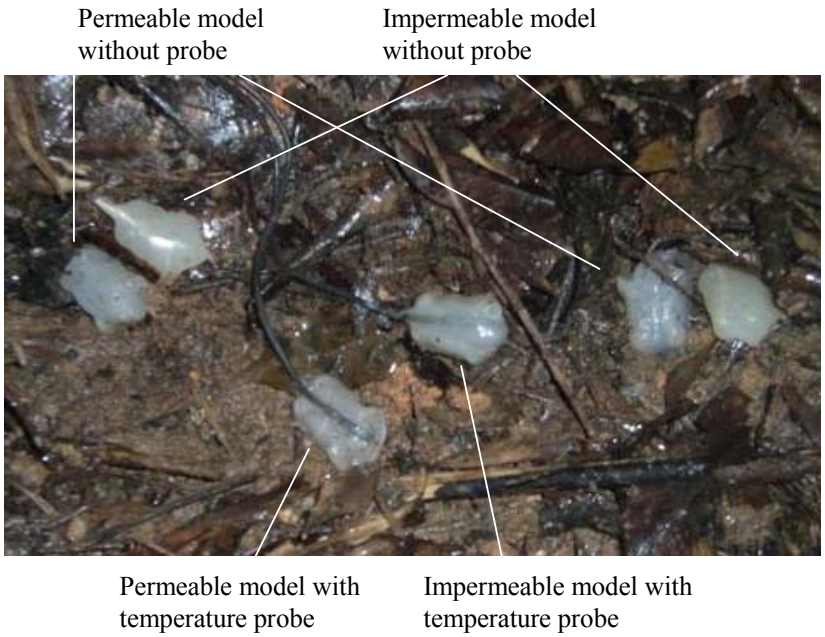

Figure 6.2 Setup of models at each of three positions along each transect. Note: models were always covered by a log or leaf litter.

Models without probes were weighed twice daily (at approximately 12hr intervals) over 3-5 day periods to measure moisture uptake or loss. They were weighed directly on a portable electric balance to 0.01g. A 50mm rain gauge was placed at each transect where models were located in order to record the amount of rainfall at sites. Substrate temperature was also recorded twice daily (when models were weighed) at each site where models were placed, using an infared thermometer (Raytek ST80 Pro-Plus non-contact laser thermometer; RAYST80).

Statistical analysis

Microhabitat use of active frogs of each species was examined using a histogram showing the frequency of frogs in each category for height above ground. The significance of differences among species in level of exposure of calling individuals, height above ground of calling sites, and substrate in retreat sites was tested using contingency tests with exact hypothesis testing in StatExact 4.0 (Cytel Software Corp. 1998). This procedure allows robust tests on tables with sparse data. To measure the relative hydric regimes and moisture conditions experienced by microhylids in retreat sites, the percent weight change of each model without a probe was calculated for each weighing after the first. The percentages for the two models of each type at each site were averaged for the time of each weighing.

For each pair of models with internal dataloggers (permeable and impermeable), maximum and minimum hourly temperature readings were calculated and averaged for each

transect, and the median value was also was recorded. This was examined in conjunction with the mean percentage weight change of permeable and impermeable models over time.

The proportion of the time that each of the thermal models was in the optimal range for *Bd* growth (17-25ºC), and the proportion that they were above or below this range was also calculated for each transect and examined using pie charts. The mean differences of hourly temperature readings in permeable and impermeable models containing temperature probes were compared using a one-way ANOVA. The body temperatures of active frogs (found during nocturnal surveys) and resting frogs (found during diurnal surveys) were compared across transects using a one-way ANOVA. Relationships of frog surface temperature to substrate temperature at each transect were analysed by regressing active and resting frog surface temperatures on substrate temperature. At sites where frogs were located both diurnally and nocturnally, frog surface temperatures for diurnal and nocturnal frogs were compared using t-tests.

Surface temperatures of frogs were also compared with the hourly temperature readings of models with internal dataloggers. The surface temperatures of frogs were compared to the hourly minimum and maximum model temperatures recorded during the time interval in which the frog temperatures were measured. The overall distribution of frog surface temperature values collected on each transect (including temperatures measured when models were not deployed) were also compared to the overall distributions of temperatures for models. The percentages of recorded surface temperature values that fell within the limits of the maximum and minimum model temperatures were calculated for active and resting frogs.

Results

Posture, exposure, and substrate use

One hundred and fifty one active frogs of seven species were located during nocturnal surveys between 2004 and 2007 and had their micro-environments recorded and characterised. The calling positions of frogs in terms of height above ground are shown in Figure 6.3. A chi-squared contingency test examining the distributions of numbers of individuals calling at each height by species (carried out in StatXact 4.0 using Monte Carlo hypothesis testing, and therefore robust to the large number of empty cells in the data) was highly significant (chi-squared = 143.0, 60 d.f., Monte Carlo $P < 0.0001$). Most of the difference was between *C. ornatus* and the other species; removing *C. ornatus* from the data set and reanalysing produced a suggestive, but not significant result (chi-squared = 17.74, 10 d.f., Monte Carlo $P = 0.0516$) suggesting that there are only minor differences among the remaining six species. All species except *C. ornatus* and *C. neglectus* had calling sites less than 20cm above ground level, with the

greatest frequency of calling sites occurring between 0-5cm above ground level. *C. ornatus* was most commonly found calling at a height of 21-40cm above ground, and one individual was found calling from a perch in a tree at approximately 250cm above ground. A single *C. neglectus* was also located calling from a tree at a height of approximately 160cm above ground.

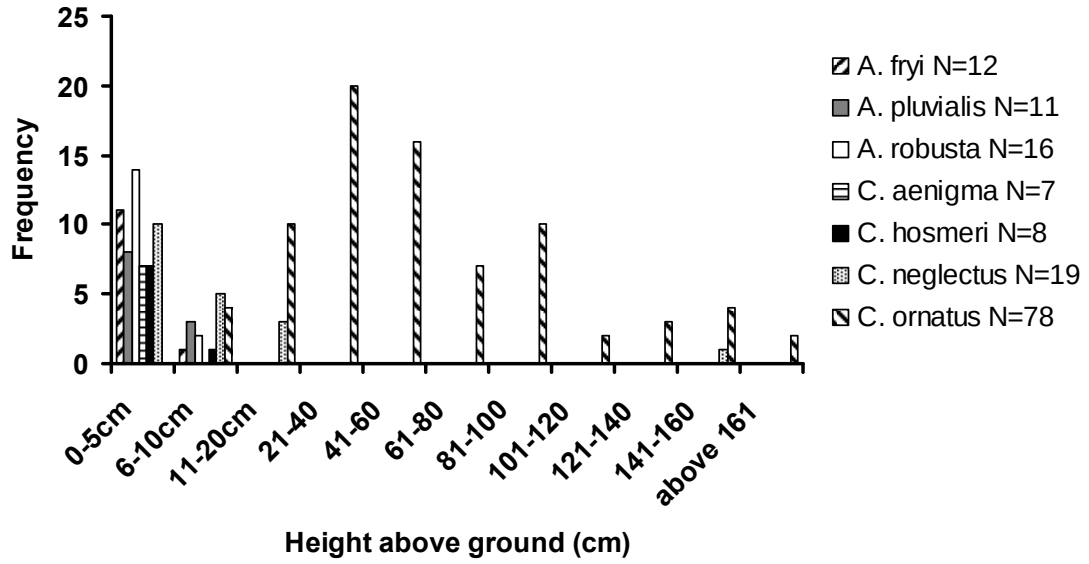

Figure 6.3 Calling position of seven species of microhylid frogs in terms of height above ground.

Habitat use by active and resting frogs found during nocturnal and diurnal surveys is shown in Table 6.3. The degree of exposure of calling males during nocturnal surveys differed significantly among species (chi-squared = 122.2, 12 d.f., Monte Carlo P < 0.0001). All species except for *C. ornatus* were found completely covered by leaf litter or vegetation between 74 and 100% of the time. In contrast, calling *C. ornatus* were found completely exposed 85% of the time. One hundred and thirty-five individuals of two species were found in retreat sites during diurnal surveys. The substrate that was most commonly used for retreat sites was logs, as 86% of *A. robusta* and 94% of *C. neglectus* individuals were found under logs in surveys. These two species differ significantly in substrates that are utilised as retreat sites, as *C. neglectus* are more specific to logs than *A. robusta* (chi-squared =11.99, 4 d.f., Monte Carlo P = 0.0074). Individuals were also found associated with other substrates such as rocks (*A. robusta* 9%; *C. neglectus* 1%), and burrows (*C. neglectus* 5%). One *A. robusta* was found underneath a recorder box that I had placed in the field.

Table 6.3 Habitat used by active frogs during nocturnal calling and by frogs located in diurnal retreat sites.

Species	Nocturnal surveys - calling position				Diurnal surveys - retreat site				
	Totally covered (%)	Partially covered (%)	Exposed (%)	*N*	Log (%)	Burrow (%)	Recorder box (%)	Rock (%)	*N*
A. fryi	75	25	-	12	-	-	-	-	-
A. pluvialis	81.82	18.18	-	11	-	-	-	-	-
A. robusta	87.5	6.25	6.25	16	86.36	-	4.55	9.09	22
C. aenigma	100	-	-	7	-	-	-	-	-
C. hosmeri	100	-	-	8	-	-	-	-	-
C. neglectus	73.68	21.05	5.26	19	93.81	5.31	-	0.88	113
C. ornatus	2.56	12.82	84.62	78	-	-	-	-	-
Total	41.72	13.25	45.03	151	92.59	4.44	0.74	2.22	135

Moisture environments in retreat sites

Models were placed in frog retreat sites at four different field sites between 27 November 2005 and 10 March 2006. One site (CU8) was sampled twice. Models were left in the field for between 62 and 112h (Table 6.2). Mean weight change of permeable and impermeable models for the five transects sampled is shown in Figure 6.4. At AU10, BK, and CU8 1 no rainfall occurred during the sampling period, and permeable models lost weight over time. Permeable models retained at least 80% of their initial weight after periods of 60hr at CU8 1, and 96h at AU10 and BK. At transect CU8 2, rainfall was recorded at 12, 24, 36, 60 and 72h, and permeable models increased in weight at these intervals. At Pal, rainfall was recorded between 12-48h, and at 72h, and permeable models increased in weight at each of these intervals. The weights of impermeable models remained relatively constant throughout the study.

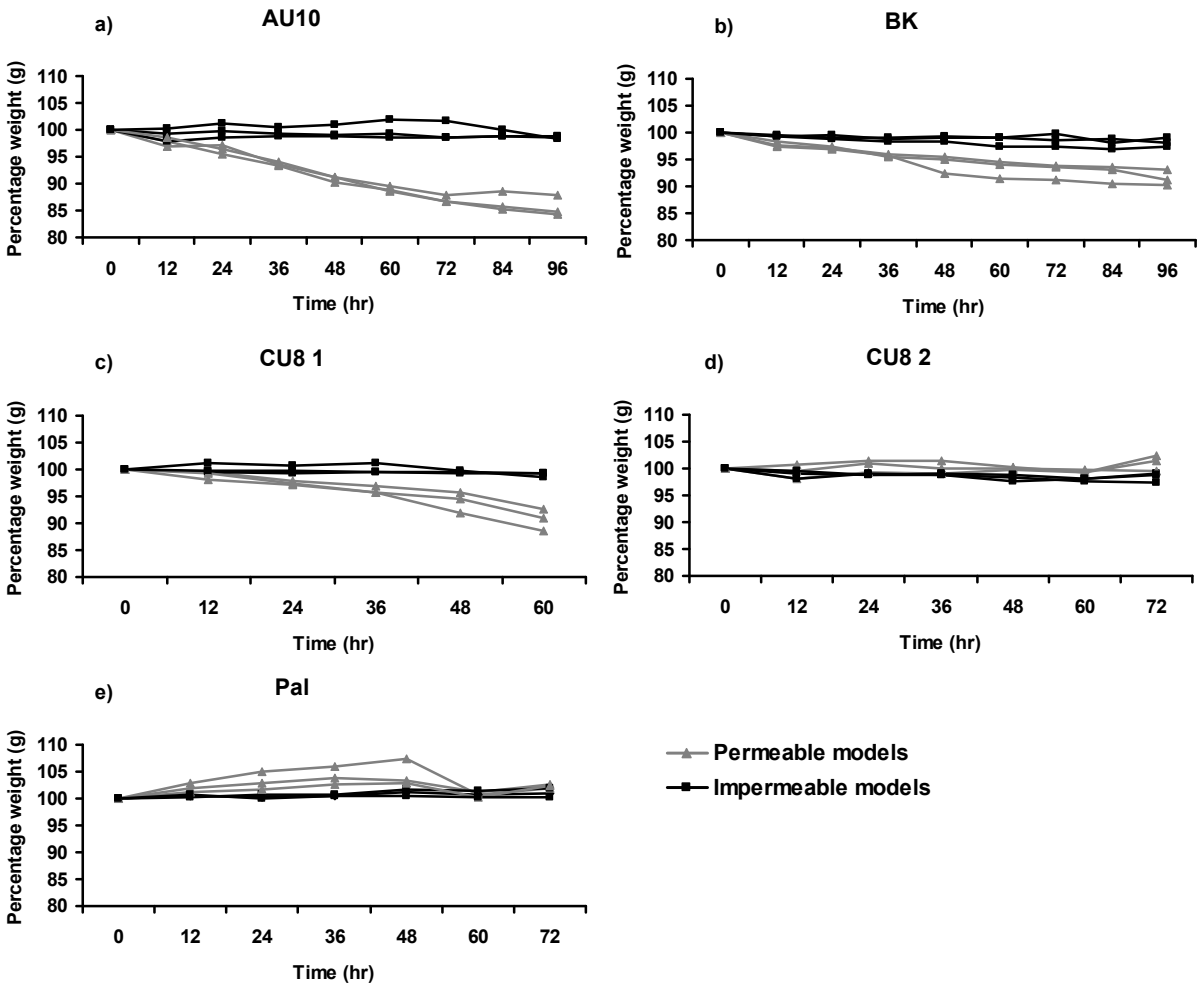

Figure 6.4 Mean weight change of permeable and impermeable models at field sites. No rainfall was recorded for **a)-c)**, Rainfall was recorded for **d) – e)**.

Model temperature data

Hourly datalogger readings at all four transects from models with inserted temperature probes are shown in Appendix 3. Results of the maximum and minimum hourly temperature readings of models are shown in Table 6.4 and Figure 6.5. The proportions of time that models were within and outside of *Bd*s optimal growth range are also shown for each transect in Table 6.4 and Figure 6.6.

Model temperatures ranged from 18.3-29.4ºC at AU10; 12.5-20.0ºC at BK; 19.6-22.0ºC at CU8 1; 21.4-23.2ºC at CU8 2; and 20.9-22.7ºC at Pal. Temperature variation was the greatest at AU10 and BK (11.1ºC and 7.5ºC respectively); temperatures were more constant at CU8 and Pal with all measurements on each model type falling within a range of 2.5ºC.

Table 6.4 Average minimum, maximum and median values for permeable and impermeable models at transects, and the proportion of time models were within *Bd*s optimal growth range.

Transect	Model type	Hours re-corded	Min temp	Max temp	Median temp	% within 17-25º	% below 17º	% above 25º	% above 28º
AU10	Permeable	112	18.32	29.42	20.37	75.60	0	24.4	11.90
AU10	Impermeable	112	18.50	28.11	20.6	76.35	0	23.65	16.55
BK	Permeable	89	12.49	18.48	14.88	17.57	82.43	0	0
BK	Impermeable	89	13.20	19.96	15.41	29.23	70.77	0	0
CU8 1	Permeable	62	19.64	21.72	20.55	100	0	0	0
CU8 1	Impermeable	62	20.26	22.02	20.84	100	0	0	0
CU8 2	Permeable	74	21.42	22.86	21.89	100	0	0	0
CU8 2	Impermeable	74	21.69	23.28	22.27	100	0	0	0
Pal	Permeable	69	20.99	22.06	21.46	100	0	0	0
Pal	Impermeable	69	20.76	22.66	21.58	100	0	0	0

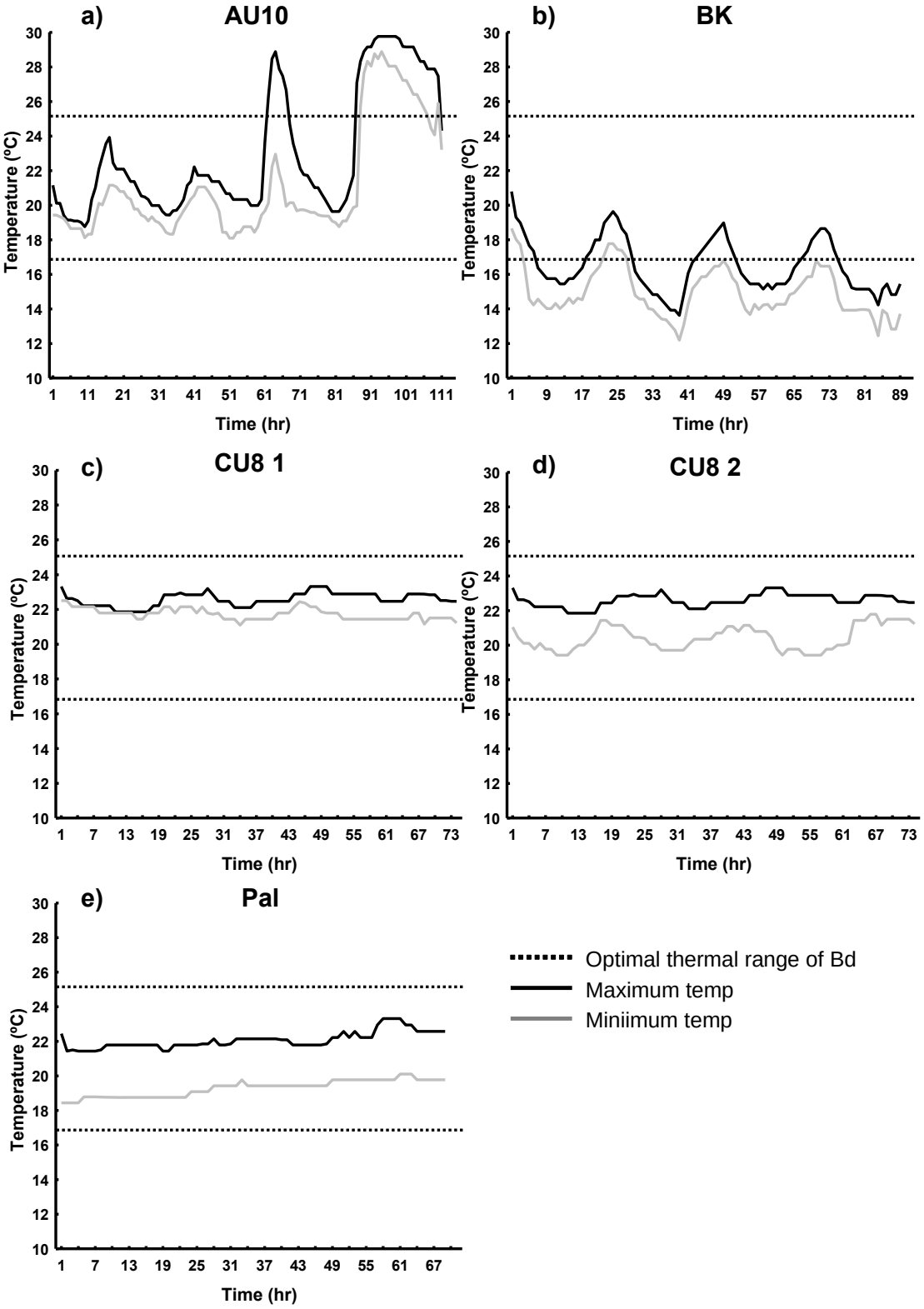

Figure 6.5 Maximum and minimum temperature readings of models with internal probes at: **a)** AU10; **b)** BK; **c)** CU8 1; **d)** CU8 2; **e)** Pal, with the optimal growth range of *Bd* for reference.

At the CU8 and Paluma transects, all model temperatures were within the optimal range for *Bd* growth over the entire measurement period. At BK the model readings were within the thermal tolerance of *Bd*, but were below the lower boundary of the optimal range for growth 82.4% of the time for permeable and 70.8% of the time for impermeable models. At AU10 model readings were within the range for optimal growth 75.6 and 76.4% for permeable and impermeable models respectively. They were above 25ºC 24.4% and 23.4% of the time, and were above 29ºC 11.9% and 16.6% of the time (Figure 6.6). At these temperatures the fungus does not grow and will die if exposure is prolonged.

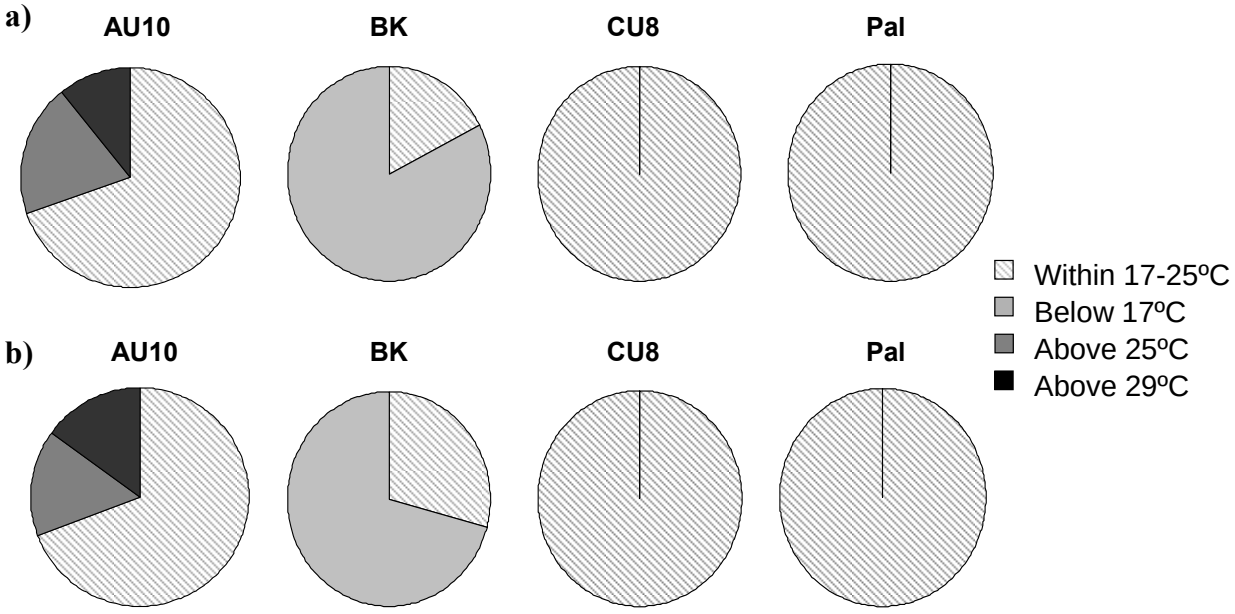

Figure 6.6 Proportions of time that **a)** permeable, and **b)** impermeable models with internal temperature probes were within the optimum temperature range for *Bd* growth (17-25ºC).

Mean differences in the hourly temperature readings between permeable and impermeable models are shown in Figure 6.7. The mean difference in temperature between permeable and impermeable models was 0.73°C for AU10, 0.64°C for BK, 0.55°C for CU8, and 0.37°C for Pal; these results did not differ significantly between sites (ANOVA, $F_{3,111} = 0.07$, p=0.78). The greatest differences between permeable and impermeable models recorded were: 3.4°C at AU10, 3.11°C at BK, 2.19°C at CU8, and 1.96°C at Pal (Figure 6.7).

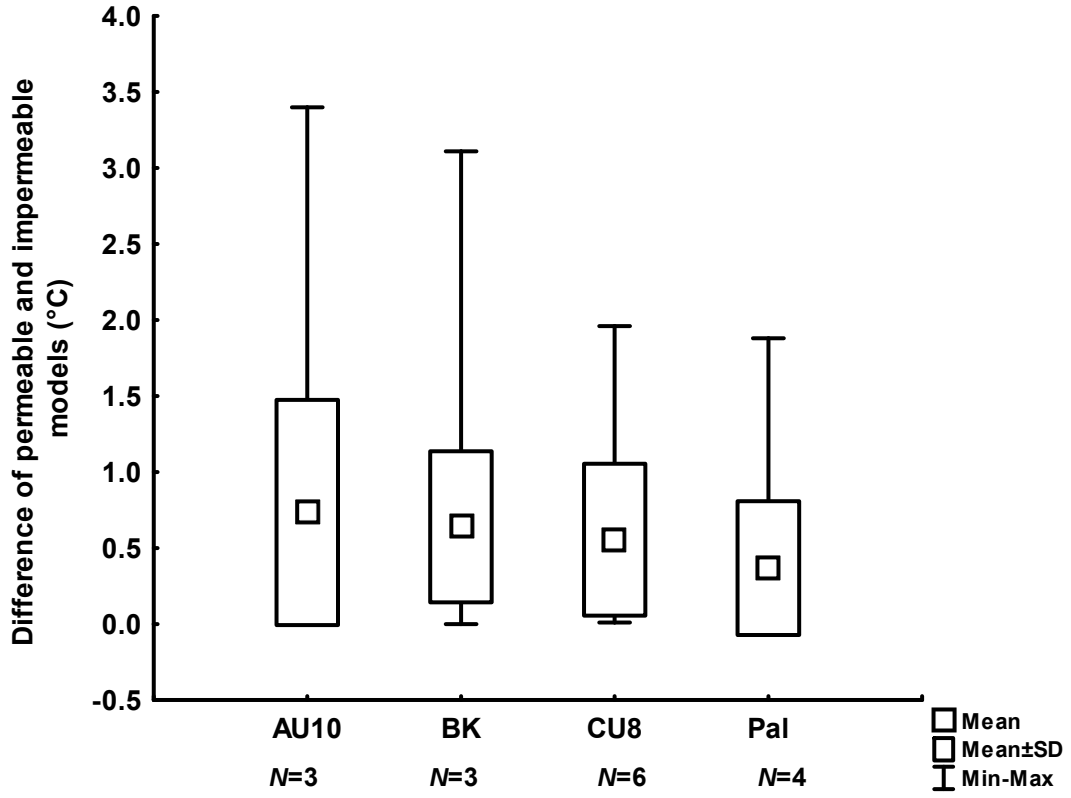

Figure 6.7 Difference in temperature between impermeable and permeable models with temperature probes. AU10, BK, and Pal had three sets of models of with inserted temperature probes; CU8 had six sets of models.

Surface temperature data

A total of 197 frog surface temperatures were recorded, including those of 87 active frogs and 110 frogs that were found at retreat sites (Table 6.5). The results of comparisons of frog surface temperature with substrate temperature are shown in Figure 6.8. None of the 95% confidence intervals for the difference between frog and substrate temperature include zero, indicating that frog surface temperatures were always significantly higher than substrate surface temperatures. Differences between frog and substrate ranged from 0.53-1.47ºC and were greatest for active frogs at AU10, and smallest for active frogs found at CU8. Differences between frog and substrate temperatures did not differ significantly among sites for active frogs (ANOVA, $F_{3, 86} = 2.44$, p=0.07), or for frogs found in retreat sites (ANOVA, $F_{1,109} = 0.07$, p=0.78).

Table 6.5 Surface temperatures of frogs, recorded at transects where models were deployed.

	Active				Retreat site				Total		
Transect	*N*	Range	Mean frog	Mean substrate	*N*	Range	Mean Frog	Mean substrate	*N*	Mean frog	Mean substrate
AU10	53	17.2-23.8	19.7	18.7	0	-	-	-	53	19.7	18.7
BK	11	12.1-19.9	14.7	13.9	93	10.8-20.4	15.7	15.1	104	15.6	15.0
CU8	12	20.6-21.9	21.1	21.6	0	-	-	-	12	21.1	21.6
Pal	11	21.8-22.8	22.6	21.3	17	19.2-23.1	21.2	20.5	28	21.7	20.8
Total	87	12.1-23.8	19.5	18.9	110	10.8-23.1	18.5	17.8	197	19.6	19.0

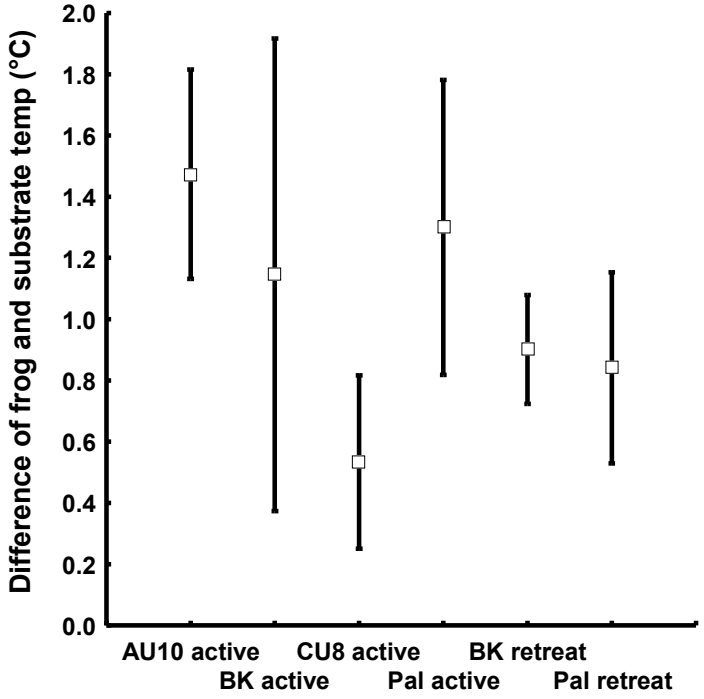

Figure 6.8 Absolute mean differences between frog and substrate surface temperatures for active and inactive individuals at each site. Error bars are 95% confidence limits.

Regressions of surface temperatures of frogs on substrate temperature for each of the four transects indicated that surface temperatures of active frogs are significantly positively correlated with substrate temperature at BK (N=11, r=0.86, r^2=0.74, p<0.01) and AU10 (N=53, r=0.40, r^2=0.16, p<0.01; Figure 6.9a and 6.9b). No significant correlation occurred at CU8 (N=12, r=-0.07, r^2=0.004, p>0.05) or Pal (N=11, r=-0.03, r^2=0.00, p>0.05), however sample sizes were relatively small at these sites (Figure 6.9c and 6.9d). Active frog temperature at AU10, was correlated with substrate temperature, but frog temperatures were often higher than the substrate. At BK, active frog temperature was correlated to substrate temperature, but was often higher than substrate temperature. Active frogs tended to be below the substrate temperature at CU8, and above the substrate temperature at Pal. At both sites where frogs were found in retreat sites, frog surface temperature was significantly positively correlated with substrate temperature, (BK N=93, r=0.76, r^2=0.57, p<0.01, and Pal N=17, r=0.90, r^2=0.82, p<0.01; Figure 6.9e and 6.9f). Resting frogs had similar temperatures to the substrate, but tended to be slightly higher (Figure 6.9).

At two sites (BK and Pal), the surface temperatures of both diurnal and nocturnal frogs were recorded. At BK, surface temperatures of resting frogs were higher than those of active frogs, but they were not significantly different (Figure 6.10a). However, at Paluma, active frogs had significantly higher temperatures than those found within retreat sites (Figure 6.10b).

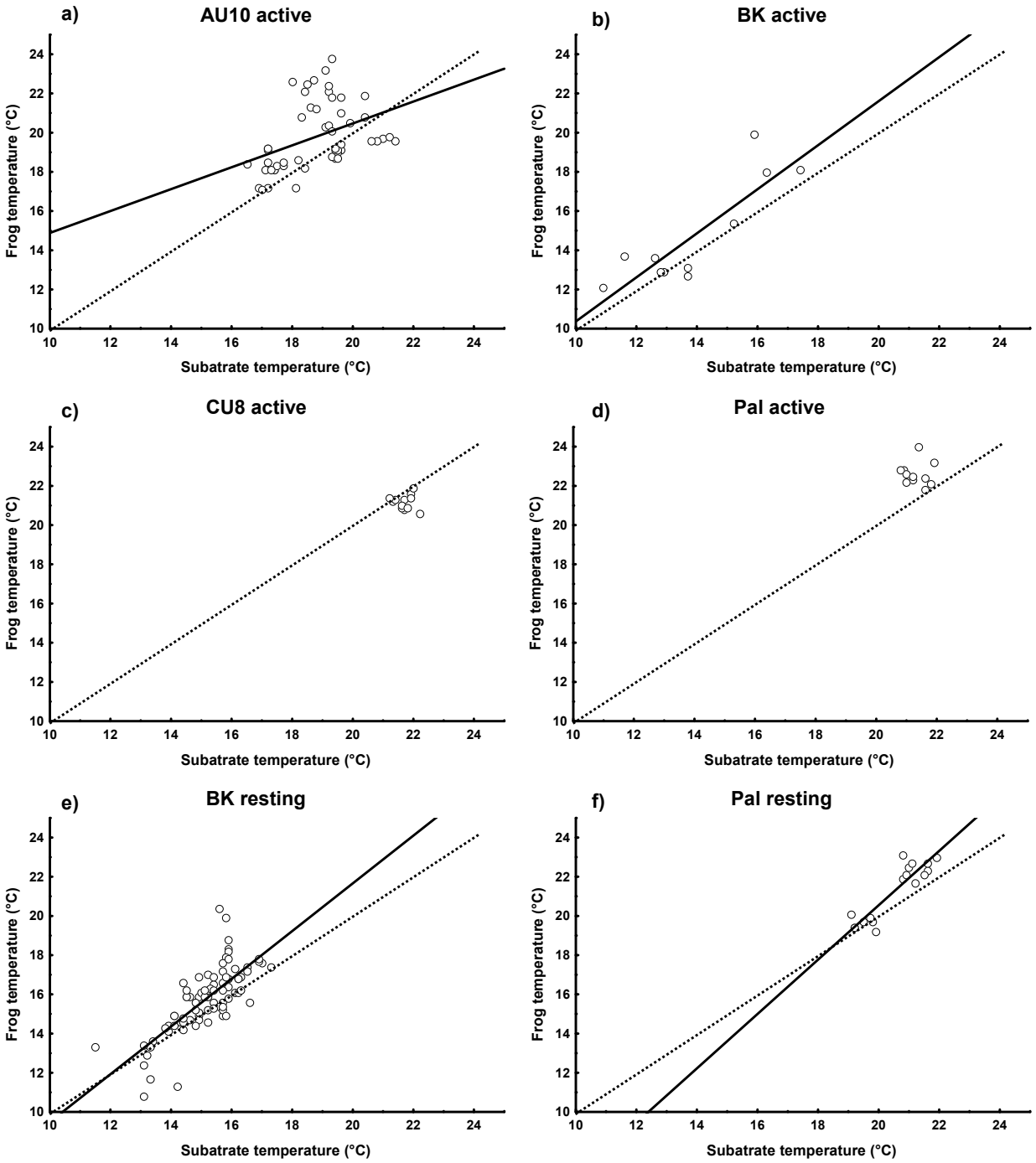

Figure 6.9 Regression analyses for surface temperature of frogs and substrate in active frogs at **a)** AU10, **b)** BK, **c)** CU8, and **d)** Pal; and resting frogs at **e)** BK and **f)** Pal. Dotted line indicates expected results if surface and substrate temperatures were identical. AU10 active $r=0.40$, $p<0.01$; BK active $r=0.86$, $p<0.01$; CU8 active $r=-0.07$, $p=0.83$; Pal active $r=-0.03$, $p=0.93$; BK resting $r=0.76$, $p<0.01$; Pal resting $r=0.90$, $p<0.01$.

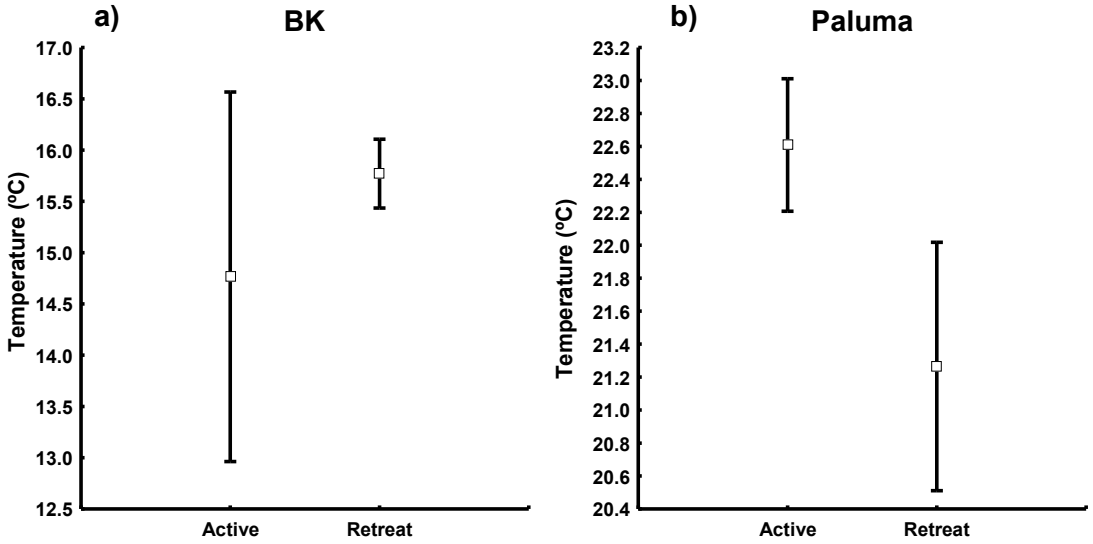

Figure 6.10 Comparison of surface temperatures of active and resting frogs from **a)** BK and **b)** Paluma. Error bars are 95% confidence limits. Temperatures did not differ significantly at BK (t-test t_{102} = -1.79, p=0.08), but significant for Pal (t-test, t_{26} = 2.87, p=0.008).

For each transect site, Figure 6.11 shows the maximum and minimum temperatures recorded by models with internal data probes, plotted with the surface temperatures of individual frogs that were located whilst models were deployed in the field and the optimal thermal range of *Bd*.

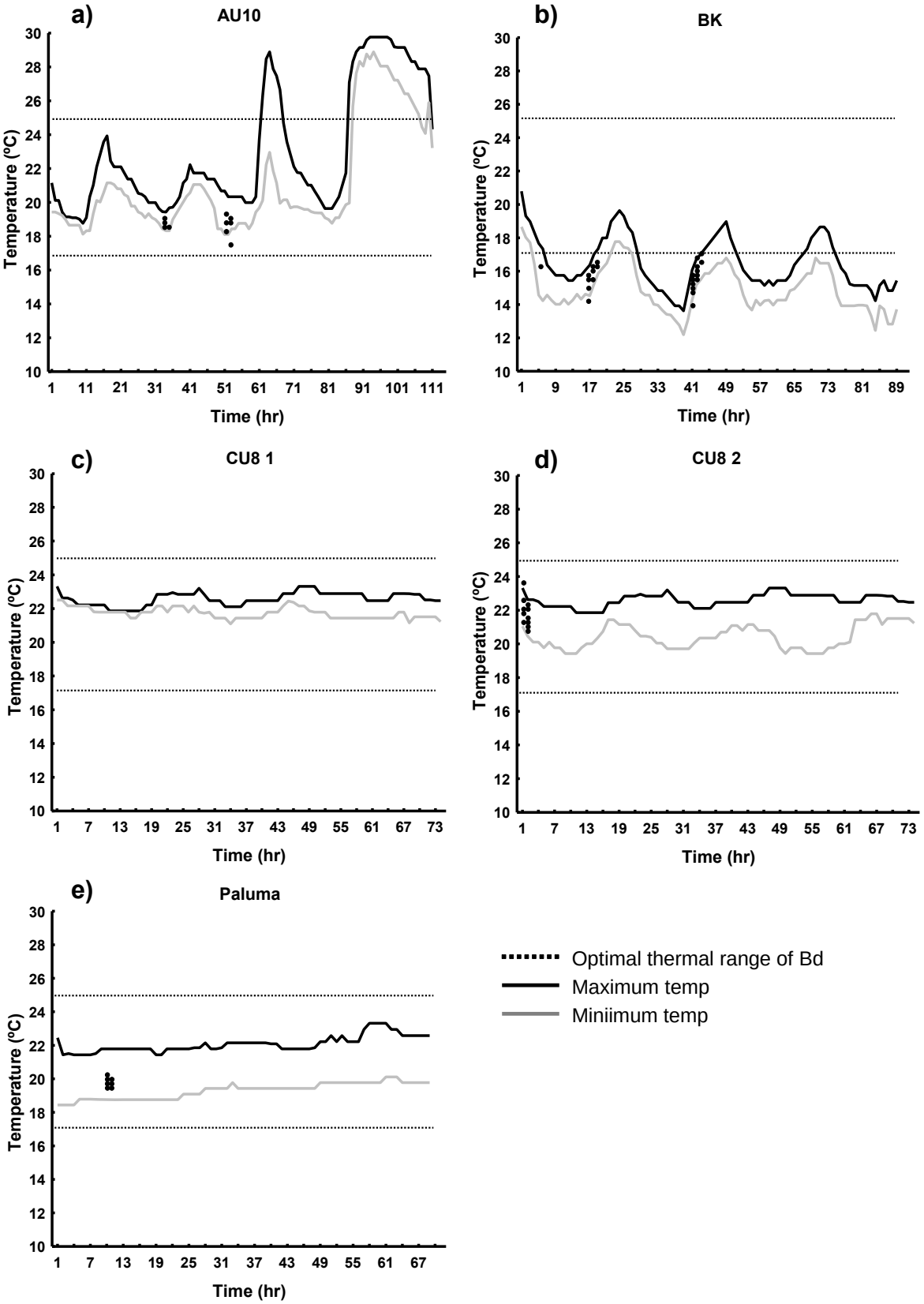

Figure 6.11 Mean hourly maximum and minimum temperature readings of models with internal probes, and the thermal optimum of *Bd* growth (17-25ºC). Surface temperatures of microhylid frogs collected at transects within the sampling period are presented as black dots: **a)** n=11; **b)** n=31; **c)** n=0; **d)** n=12; **e)** n=7.

Of the 61 frog temperatures that were measured while models were in place in the field, only 6 (9.8%) had surface temperatures that were outside of the hourly minimum and maximum model temperature readings range (Table 6.6; Figure 6.11). Nineteen out of 23 (82.61%) active frogs had surface temperatures that were within the limits of the maximum and minimum temperatures of models, and 36 out of the 38 (94.74%) frogs found within retreat sites had surface temperatures that were within the temperature limits of the models that were recording along the transect at the same time as the frogs were found (Table 6.6). At AU10, the three frogs that were outside the model limits were active, and they had surface temperatures that were below the range of models. At BK, the two frogs that were outside the model limits were resting, and they had surface temperatures that were also below the range of models. At CU8 2, the frog that was outside the model limits was active, and it had a surface temperature that was above the range of models.

Table 6.6 Proportion of surface temperature readings of frogs that were within maximum and minimum boundaries of thermal envelopes defined by models in resting sites. Frogs sampled whilst models were deployed at transects are in the first category, and the entire set of surface temperature readings of frogs (including those that were taken whilst models were not employed) are in the second category.

Sample		Active frogs			Retreat site frogs			Total		
	Site	Readings in range	*N*	%	Readings in range	*N*	%	Readings in range	*N*	%
Frogs sampled when models deployed	AU10	8	11	72.72	-	-	-	8	11	72.72
	BK	-	-	-	29	31	93.55	29	31	93.55
	CU8	11	12	91.67	-	-	-	11	12	91.67
	Pal	-	-	-	7	7	100	7	7	100
	Total	**19**	**23**	**82.61**	**36**	**38**	**94.74**	**55**	**61**	**90.16**
Total Sample of all frogs	AU10	49	53	92.45	-	-	-	49	53	92.45
	BK	10	11	90.91	92	93	98.92	102	104	98.08
	CU8	12	12	100	-	-	-	12	12	100
	Pal	10	11	90.91	17	17	100	27	28	96.43
	Total	**81**	**87**	**93.10**	**109**	**110**	**99.09**	**190**	**197**	**96.45**

When the entire set of frog surface temperatures were compared to model output (not just the frogs that were collected whilst models were recording temperature data), 190 of 197 (96.45%) frogs had temperature that fell within the limits of model temperatures (Table 6.6). Eighty one of 87 (93.1%) active frogs had temperatures that were within the model limits, and 109 of the 110 (99.09%) frogs that were found at retreat sites had temperatures that were within model limits.

Discussion

Temperature is known to affect the occurrence of disease in humans and wildlife (Berger et al. 2004; Pascual and Dobson 2005). This may be due to a variety of factors including thermal requirements of the pathogen, changes in host community, or variation in the density and abundance of hosts (Pascual and Dobson 2005). Changes in the thermoregulatory opportunities that are available to rainforest frogs due to large-scale global warming may have contributed to widespread population declines in association with the emerging infectious disease, chytridiomycosis (Pounds et al. 2006). Outbreaks of chytridiomycosis in Costa Rica have been attributed to increasing temperatures, which are shifting towards the growth optimum of the fungal pathogen *Bd*. *Batrachochytrium dendrobatidis* has also caused declines and disappearances of frog species within the WT region of northern Queensland.

Studies of the biology of *Bd in vitro* indicate that growth and reproduction occur within the range of 4-25°C, but the best conditions for growth are between 17-25°C (Berger et al. 1998; Longcore et al. 1999; Piotrowski et al. 2004). Optimal growth occurs at 23°C, growth slows above 25^{o}C and ceases at 28°C, and death occurs at 30°C and above (Longcore et al. 1999; Piotrowski et al. 2004). Differences in interspecific thermoregulatory behaviour and aspects of natural history such as microhabitat associations are known to affect susceptibility to infection by *Bd* (Woodhams et al. 2003; Berger et al. 2004; Richards-Zawacki 2009). The thermal and hydric micro-environments used by amphibians effect their susceptibility to chytridiomycosis-related declines, as susceptible species use microenvironments that are more favourable for *Bd* growth and survival (Rowley 2006).

Male microhylids typically emerge from under logs or within subterranean burrows at night, and call at large distances from permanent or ephemeral water bodies (Brooke 1996; Hauselberger and Alford 2005; Felton et al. 2006). Calling activity usually begins after dusk, and depending on rainfall, will continue until the early hours of the morning (Brooke 1996; Hauselberger and Alford 2005). Some species tend to call in response to rainfall, or on nights where rainfall has occurred during the day while others call throughout the wet season regardless of rainfall (pers. obs.). These results corroborate those of Brooke et al. (2000); Hoskin (2004); Hauselberger and Alford (2005); and Williams (2007). In the present study, five species were found calling at ground level (<20cm above ground), a single *Cophixalus neglectus* was found calling at a height of approximately 160cm, and the majority of *C. ornatus* individuals called from perches from 20-160 cm in height. This suggests that most microhylid species typically call under the cover of leaf litter, and are located close to the forest floor, with the exception of *C. ornatus*. *Cophixalus ornatus* also tended to be exposed whilst they were calling, whereas all other species were either partially or totally concealed by leaf litter or vegetation. Two microhylid species were located during diurnal surveys, and were most

commonly found in retreat sites under logs. In terms of retreat sites, *C. neglectus* were more specific to logs than *A. robusta*. It seems likely that these species actively seek out diurnal shelter sites that may slow rates of dehydration and prevent desiccation (Hoskin 2004).

Pairs of permeable and impermeable models placed in frog retreat sites demonstrated that even in these sites frogs should often experience EWL. The weights of permeable models were significantly different from those of impermeable models at almost all sites. They gained weight when rainfall was recorded, and lost weight when it was not. Rates of water loss appeared to be low, however, and permeable models never lost more than 20% of weight due to moisture loss over a period of up to 96h. Where rainfall was recorded, permeable models either increased weight, or remained at the same weight for up to 36 h after the rainfall event. This suggests that whilst frogs do lose water during the wet season when no rainfall occurs, retreat sites provide an insulated environment that slows rates of dehydration and allows frogs to remain hydrated for extended periods of time. This result has also been observed in the retreat sites used by cane toads (*Bufo marinus*), which suffered significantly less EWL as measured by agar models in closed retreat sites (Schwarzkopf and Alford 1996). As rainfall is a regular event in the wet season throughout the WT, microhylids are not likely to be affected by dessication when they are located within retreat sites. This is not surprising, as microhylids lay terrestrial eggs which undergo direct development, and these eggs are anamniotic, surrounded only by jelly capsules that must remain fully hydrated (Zweifel 1985). Thus, high substrate moisture levels are necessary to maintain eggs throughout development.

Agar models have been used successfully in a variety of studies of amphibian water relationships, as they experience the same evaporation rate as frogs with very low cutaneous resistance to EWL (Spotila and Berman 1976; Wygoda 1984; 1988). In a comparison of EWL between live frogs and agar models, Young et al. (2005) found that the microhylid *Austrochaperina adelphi* lost water at the same rate as models, indicating that some microhylids have very low skin resistance to EWL. No other species have been measured, leaving the possibility open that some species may have higher or variable skin resistance. Regardless of this, the temperatures recorded by the pairs of agar models with internal temperature probes placed at retreat sites (one with no resistance to EWL and the other with complete resistance), should define an envelope that includes the body temperatures experienced by frogs. Models were placed at four transect sites which were located in dense vegetation, and the ground cover did not receive substantial amounts of direct sunlight. Mean differences between impermeable and permeable models did not differ significantly among transects, and ranged from 0.37-0.73°C. This suggests that permeable models experience very little cooling caused by EWL, so that evaporation has little effect on the body temperatures of microhylids in retreat sites.

Across four transects, the surface temperatures of 197 frogs were recorded; these were similar to the temperature of their surrounding substrate. Differences between frog and substrate

temperature ranged from 0.53-1.47ºC, and were not significantly different among transects. At two of the four transects where active frogs were found (AU10 and BK) and at both transects where retreat site frogs were found, frog surface temperature was significantly positively correlated to substrate temperature. The two sites that did not show correlations of surface and substrate temperature, experienced narrow ranges of temperatures, so that only small variation in frog temperatures at these sites may result in regressions which were not significant.

Active frogs may have been thermoregulating to some extent. At transect AU10, at substrate temperatures between 18 and 21^{o}C, frog temperatures tended to be 2-4 degrees higher than substrate temperatures. All temperatures of active frogs measured at Paluma were warmer than the corresponding substrate. Because frogs are active nocturnally, it is also possible that these differences may reflect different rates of evaporative water loss; most of the frogs measured were *Cophixalus ornatus*, which is the most arboreal species and may have the ability to reduce rates of EWL, while most substrates were moist and may have been cooling at higher rates than frogs. In general frog temperatures were similar to substrate temperatures, a result that has also been observed in other terrestrial anuran species (Navas 1996).

At two transects, the surface temperatures of both diurnal and nocturnal frogs were recorded. At BK, surface temperatures of active and resting frogs were not significantly different, but at Pal, diurnal, resting frog temperatures were significantly lower than those of nocturnal, active frogs. From the model data, daily and nightly temperatures appear to be relatively constant at the Pal transect. This suggests that moist retreat sites may provide a cooler microclimate during the day, than when frogs are active at night. At BK, the daily and nightly temperatures fluctuate, with nightly temperature being substantially cooler than daytime temperature. The cooler nighttime temperatures experienced by active frogs may be more comparable to temperatures that occur within retreat sites during the day. This may explain why differences between active and resting frogs occurred at Pal but why there was no difference in active and resting frogs.

The physical models used in this study produced an accurate outline of the thermal envelope for both active and resting frogs as measured in the field. Of the 61 frog temperatures that were measured while models were deployed, only 6 (9.8%) had surface temperatures that were outside the mean range of the models containing temperature probes. Of the entire set of 197 individual surface temperature recorded at the four field sites, 190 (96.45%) fell within the limits of model temperature readings. Even though models collected data for relatively short periods, they did do a good job of capturing the range of temperatures available to frogs during the wet season. Models were a better predictor of the thermal range of microhylids in retreat sites, as the surface temperatures of frogs within retreat sites fell within model limits 94.7% of the time for frogs found whilst models were recording temperature and 99% of the time for the entire set of frog surface temperature readings. However, models also gave relatively accurate

windows of the temperature range of active frogs, with active, nocturnal surface temperatures of frogs falling within model limits 82.6% of the time of the time for frogs found whilst models were recording temperature and 93% of the time for the entire set of frog surface temperatures. This probably reflects the fact that nocturnally active frogs have little opportunity to thermoregulate, but suggests that active frogs may experience a slightly different temperature regime than those in retreat sites.

Due to the fact that only three pairs of models with temperature probes were used at each transect site for periods of 62-112h, they clearly did not sample the entire range of thermal conditions that frogs might experience in retreat sites. The model record is a small sample of the conditions that frogs could experience at each site, and the results depend very strongly on the time of year and the environmental conditions when readings were taken. For this reason, some frogs were inevitably outside the range experienced by models. In order to improve the defined thermal envelopes for microhylids, more models could be employed at each site, with some being placed in positions where active, nocturnal frogs have previously been found calling.

The results from thermal models are in accordance with Rowley and Alford, (2009), who used similar models to characterise the thermal regime of an Australian hylid frog. Models were placed in the field for periods of up to eight days, and they found that they provided an accurate outline of the thermal envelope of body temperature for *Litoria lesueuri*. Body temperature measurements of frogs fell within the envelope of model temperatures 83% of the time, and were within a wider envelope produced by expanding the measured range upward and downward by 0.5^{o}C 97% of the time.

The data on microhabitat use of microhylids suggests that in nature, these species occupy retreat sites that usually present conditions that will support the growth of *Bd*. Retreat sites remain moist throughout the wet season due to high levels of humidity and rainfall, and permeable models did not exhibit high desiccation rates. For this reason, it is unlikely that *Bd* growth or reproduction would be inhibited based on moisture levels. However, the temperature data suggest that high temperatures may sometimes inhibit *Bd* growth in retreat sites used by frogs.

Surface temperature readings of active and resting frogs at all transects were within the growth range for *Bd*, and no frog was ever found with a surface temperature that could inhibit the growth of *Bd*. At two of the four transects surveyed (CU8 and Pal), model temperature levels were within the optimal range for *Bd* growth the entire time that models were employed to record thermal data. However, at BK, whilst model readings were within the thermal tolerance of *Bd*, they were below the lower boundary atleast 70 percent of the time. Models were used in summer, and the temperature at this site would be expected to be substantially lower during other times of the year. This suggests that the transect at Mt Bellenden Ker may not be an ideal environment for the growth of the fungus. At transect AU10,

model temperatures reached levels that were above the range for optimal growth of *Bd* at least 24.1 percent of the time. Model temperatures also reached levels above 28ºC at least 11.9 percent of the time; at these temperatures *Bd* stops growing. No temperatures recorded by any models reached 30ºC, which is a level that can kill *Bd*. This suggests that on two transects, environmental conditions experienced by frogs in retreat sites were outside the optimal growth range of *Bd* for substantial periods of time, and at one site, conditions could have resulted in inhibition of growth of the pathogen. This could equate to an impaired ability of *Bd* to cause disease in frogs who inhabit these retreat sites. This suggests that at least some microhylids may not be susceptible to chytridiomycosal infection, based on the environments in which they exist.

A similar study of the thermal behaviour of three species of WT hylid frogs found that microenvironmental conditions are likely to affect the susceptibility of species to chytridiomycosis (Rowley 2006). During the wet season, temperature readings of all three species (that are susceptible to *Bd* in the wild), were outside the optimal growth range of *Bd* for substantial amounts of time. More that 50% of individual temperature measurements for *Litoria lesueuri*, and between 25 and 50% of temperature measurements for *L. serrata* and *L. nannotis* were above the optimum range for *Bd* growth, which is comparable to the temperature readings for microhylids that were outside the optimum growth range recorded at transect AU10. Approximately 15% of measurements were above the thermal tolerance of *Bd* ($30^{\circ}C$) for *L. serrata,* and a substantial number of outliers were above the threshold for *L. lesueuri.*

This suggests that even frog species that are susceptible to *Bd*, and have suffered from population declines associated with the fungus, spend substantial amounts of time at temperatures that are outside the optimal growth range of *Bd*, and small periods of time at temperatures which may be high enough to kill the fungus. It also suggests that whilst some microhylids may exist in habitats that do not favour the growth and reproduction of *Bd* (such as the summit of Mt. Bellenden Ker), the temperature regime of microhylids found in this study is not likely to strongly affect the susceptibility of these species to chytridiomycosis.

This study highlights the fact that environmental conditions experienced by amphibians depend not only on climatic variables, but on species-specific patterns of microhabitat use. By placing models in specific retreat sites where frogs were previously located, a detailed picture of the range of body temperature and hydric regime of EWL for microhylids was determined. Retreat sites were found to be particularly important to these terrestrial frogs as a means of providing buffering of the external environment, especially in terms of EWL. Because the outcome of *Bd* infection is strongly influenced by temperature and moisture, the specific microenvironments used by microhylids are likely to affect the overall dynamics of the host-pathogen system. However, microhylids are not likely to be less susceptible to the effects of chytridiomycosis infection because of thermal constraints on the growth of *Bd* in the environments that they inhabit. Knowledge gained from the use of models in determining the

microhabitat of microhylids is useful in understanding host-parasite interactions, and further work is required to determine the thermal regimes of retreat sites year round.

CHAPTER 7: GENERAL DISCUSSION

Disease is an important factor affecting survival of hosts, and pathogenic organisms have been implicated in declines and extinctions of a wide variety of wildlife (Warner 1968; Berger et al. 1998; Cunningham and Daszak 1998; Daszak and Cunningham 1999). In recent years a number of new infectious diseases have emerged, causing sudden declines of host species and representing a substantial threat to global biodiversity (Daszak et al. 2000). The emerging amphibian disease chytridiomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), has been implicated in mass mortalities, population declines, and extinctions of amphibian species in many regions of the world (Berger et al. 1998; Lips 1999; Longcore et al. 1999; Pessier et al. 1999; Weldon et al. 2004; Lips et al. 2006; Rachowicz et al. 2006). Chytridiomycosis causes mortality across a broad taxonomic range of amphibian species, however the extent of its pathological effects varies greatly among species. Some species are highly susceptible, and become extirpated in initial epidemic outbreaks of chytridiomycosis; others are less susceptible, declining in numbers but not reaching local extinction, some species tolerate *Bd* infections without developing clinical signs of chytridiomycosis, and, finally, some appear completely resistant to infection (Berger et al. 1998; Daszak et al. 2003; Lips et al. 2006). Several studies have suggested that some proportion of this variation among species is associated with ecological and life-history traits of host species (Williams and Hero 1998; Lips et al. 2003; Retallick et al. 2004; Woodhams and Alford 2005).

World-wide, the presence of *Bd* in terrestrial amphibians with direct development is low, and very few examples of population crashes have been recorded (Bell et al. 2004; Burrowes et al. 2004; Lips et al. 2006; Brem and Lips 2008). One possible explanation for this could be that a terrestrial life-style reduces contact with fungal zoospores, reducing transmission rates. Microenvironments selected by amphibian species can also affect the growth and survival of *Bd*, as this organism is highly influenced by thermal and hydric conditions (Longcore et al. 1999; Berger 2001; Johnson and Speare 2003; Woodhams et al. 2003; Berger et al. 2004; Piotrowski et al. 2004; Carey et al. 2006). Studies of the biology of *Bd in vitro* indicate that it requires a moist environment to reproduce, and that growth and reproduction occur within the range of 4-25°C. The optimal conditions for growth are between 17-25°C. Dramatically reduced or complete cessation of growth occurs at 28°C, and death occurs at 30°C (Berger et al. 1998; Longcore et al. 1999; Piotrowski et al. 2004).

Australian microhylids are terrestrial, direct-developing frogs belonging to 19 species. Most species occur in the Wet Tropics (WT) region of Queensland (Cogger 1996), where extensive declines of other species have occurred in association with epidemic outbreaks of chytridiomycosis, and infections with *Bd* are now endemic (Berger et al. 2004; McDonald et al.

2005; Woodhams and Alford 2005). Although they do not breed aquatically, microhylids inhabit cool, moist habitats; environments that should be favourable for *Bd*. They also have ecological traits that have been suggested to characterise declining species, such as occurring at high elevations and having low reproductive output and restricted distributions (Laurance et al. 1996; Lips 1998; McDonald and Alford 1999; Murray and Hose 2005). There is a lack of information regarding long-term population trends in microhylids, although anecdotal evidence suggests that population densities appear to be high (Hauselberger and Alford 2005; Williams 2007). The fully terrestrial mode of life, including terrestrial breeding that characterises the Australian Microhylidae, may be responsible for the apparent lack of effects of chytridiomycosis on these species in nature.

Population trends of microhylids at one site within the WT

Temporal calling patterns of two microhylid species (*A. robusta* and *C. ornatus*) were examined, using automated recordings of calling activity made every night during five different wet seasons spanning an 11-year period. This enabled me to look for changes in relative population sizes over time. Environmental variables were recorded over this period, allowing me to examine correlations between calling and weather conditions.

Every season, there was a rapid increase in mean and maximum numbers of calling individuals, followed by a plateau in calling activity, after which numbers of callers dropped off rapidly. This pattern was stronger in *C. ornatus* than in *A. robusta*. The onset time and length of the plateau varied across seasons, but the date of the end of the calling season was very consistent. There was slight variation among years in the height of the plateau of calling activity in *C. ornatus*, but calling activity of *A. robusta* was more variable within years than among them. Calling activity by both species was significantly positively correlated with temperature, humidity and rainfall, although relationships with environmental variables were stronger in *A. robusta*. Weather variables accounted for approximately 43% of the variation in calling in *A. robusta*, and 7% for *C. ornatus*. Overall, there was no evidence for a systematic change in the numbers of individuals or the start date or end date of calling activity in either species over the 11-year period, suggesting that populations have not declined and seasonal patterns of calling have not been affected by climate change.

My results indicate that these tropical, prolonged breeding species show strong responses to weather, but have thus far shown no systematic changes in calling phenology in response to global climate change and that their population sizes have not been affected by the emergence of chytridiomycosis.

Prevalence of *Bd* infection in microhylids

The results of surveys of wild microhylids for the presence of *Bd* infection support the idea that they may be resistant to infection. None of 595 frogs from nine species tested using quantitative PCR (qPCR) and histological methods was positive for *Bd* infection, indicating that the prevalence of *Bd* in WT microhylids is very low in nature, and suggests that they have low susceptibility to infection by the pathogen. If *Bd* infections do occur in microhylids, they occur at prevalences well below those documented for stream-associated Australian frogs (Berger et al. 2004; Kriger and Hero 2007a; Kriger et al. 2007; Kenyon 2008; Murray et al. 2009). The absence, or extremely low prevalence, of infections by *Bd* in microhylids may reflect the biology of these frogs.

Although it is possible that the terrestrial habits of microhylids could be responsible for the low prevalence of *Bd* infection, terrestrial frogs became infected during epidemics in Panama (Lips et al. 2006). Although reduced opportunities for transmission may contribute to the extremely low prevalence of *Bd* infection from Australian microhylids, it seems likely they also have a high degree of resistance to infection by the pathogen.

Experimental infection of a microhylid species with *Bd*

When *Cophixalus ornatus* individuals were exposed to high but ecologically relevant dosages of zoospores in the laboratory, all individuals eventually became infected. Four of five *Litoria wilcoxii* positive controls also became infected. Thus, *C. ornatus* can become infected by *Bd*.

However, the number of zoospore equivalents detected on swabs taken from *C. ornatus* was always relatively low, and all *C. ornatus* had cleared their infections by five weeks post-exposure, whereas two of the five *L. wilcoxii* retained relatively intense infections. The *L. wilcoxii* that became infected also developed significantly more intense infections, with mean numbers of zoospore equivalents 51 times higher than those in *C. ornatus*. My experimental results thus suggest that although they are not entirely immune to infection by *Bd*, infected Australian microhylids, as represented by *C. ornatus*, do not go on to develop the clinical symptoms of chytridiomycosis, and rapidly clear infections even when environmental conditions favour the fungus. Experimental environmental conditions were within the range thought to be optimal for *Bd* growth (Piotrowski et al. 2004), and were representative of thermal conditions at the site where *C. ornatus* were collected.

The low level of susceptibility to chytridiomycosis observed in *C. ornatus* may be due to innate immune defenses. Amphibian species that lay eggs in terrestrial nests that are brooded or tended by parents may have particularly effective innate immune defenses such as antifungal skin secretions or microbial symbionts as a means to control fungal infestations that would otherwise overwhelm egg clutches (Austin 2000; Lauer et al. 2007). Peptide mixtures secreted

by the skin of a range of amphibian species can inhibit *Bd* growth (Rollins-Smith et al. 2002b; Woodhams et al. 2005), and the effectiveness of antimicrobial peptide (AMP) skin secretions in Australian frog species is significantly correlated with resistance to chytridiomycosis (Woodhams et al. 2007a).

Antimicrobial peptides

Natural peptide mixtures produced by microhylids were not unusually effective against the growth of *Bd*. Microhylid species produced peptides in amounts comparable to at least two other sympatric frog species (Kenyon 2008). The average quantity of protein secretion by microhylids did not differ significantly from two hylid species (Kenyon 2008). Although microhylids did not secrete greater quantities of proteins or AMPs than other species, greater effectiveness against fungi might provide greater protection against chytridiomycosis.

My results did not support the possibility that microhylid AMPs are more effective than those of other species. Microhylid mean species minimum inhibitory concentrations (MIC: μg/ml) of AMPs were within the range of three of four Australian rainforest frog species (*Litoria caerulea*, *L. chloris*, and *Mixophyes fasciolatus*; Woodhams et al. 2007a). Microhylid AMPs thus exhibit similar effectiveness to AMPs produced by hylid and myobatrachid species that are vulnerable to *Bd* infections in the field. In most cases (83%), peptide samples from microhylids did not completely inhibit the growth of *Bd in vitro*. AMPs may participate in reducing the growth and reproduction of *Bd*, but my results suggest that AMPs are not the primary factor responsible for the low prevalence of infection in microhylid species in nature. It is possible that other factors such as host behaviour, including microhabitat use, may account for the very low susceptibility of Australian microhylids to chytridiomycosis.

Microhabitat of microhylids

Interspecific differences in thermoregulatory behaviour and microhabitat associations can affect susceptibility to chytridiomycosis (Woodhams et al. 2003; Berger et al. 2004; Rowley 2006; Richards-Zawacki 2009). I investigated the thermal and hydric conditions available to microhylids in retreat sites using permeable and impermeable agar models with internal temperature probes placed at four sites within the WT to gain an understanding of how environmental conditions might affect the progress of chytridiomycosis.

Microhylid retreat sites provided a consistently highly moist environment, and it is thus unlikely that moisture conditions would result in the inhibition of growth of *Bd*. Agar models produced an accurate outline of the thermal envelopes of active and resting frogs, containing more than 90% of actual frog surface temperature readings. All surface temperatures of active and resting frogs were within the growth range for *Bd*, and no frog was ever found with a surface temperature high or low enough to inhibit fungal growth. At two of the four study areas,

in the Carbine uplands and Paluma, the thermal envelopes defined by model temperatures were also consistently within the optimal range for *Bd* growth. However, at Bellenden Ker, models were below the lower boundary of the optimal range for *Bd* growth at least 70% of the time. At the Atherton uplands site, model temperatures were above the range for optimal growth of *Bd* at least 24% of the time. Model temperatures also reached levels above 28ºC 11.9% of the time. A comparable study on the thermal behaviour of three species of WT frogs susceptible to *Bd* showed that between at least 25 and 50% of temperature measurements were above the optimum range for *Bd* growth, and up to 15% of measurements were above the thermal tolerance of *Bd* in one species. This suggests that the environmental conditions experienced by microhylids in this study are unlikely to contribute to limiting the growth and reproduction of *Bd*, and impairing its ability to cause disease.

Conclusions

The results of this study increase our understanding of the interactions between Australian microhylid frogs and the amphibian chytrid fungus. Despite inhabiting environments within the WT where *Bd* has caused mass declines and extinctions in many other frog species (Richards et al. 1993; McDonald and Alford 1999), microhylids are not known to have suffered from population declines associated with chytridiomycosis. The present study provides quantitative evidence that numbers of two species did not decline between 1995-96 and 2006-07 (Chapter 2).

Extensive sampling failed to find any infected individuals in nature (Chapter 3), and laboratory studies confirmed that the most common species, *Cophixalus ornatus* can become infected by *Bd*, but disease progression and development of chytridiomycosis did not occur, and infections were cleared over time (Chapter 4). Skin peptide analysis showed that the AMP defenses of microhylids are no more effective against the pathogen than are those of hylid species (Chapter 5), many of which have declined in association with epidemic outbreaks of chytridiomycosis. This suggests that some factor other than the innate defense provided by AMPs must protect microhylids from infection in nature. One possible mechanism is their choice of microenvironments. Because *Bd* has a relatively narrow range of tolerance for temperature and moisture, the microenvironments used by microhylids could affect the overall dynamics of the host-pathogen system. At two of four sites surveyed, environmental conditions in retreat sites were outside the optimal growth range of *Bd* some of the time, and at one of these sites, conditions would have substantially inhibited the growth of the pathogen. This suggests that at least some retreat sites may limit the growth and reproduction of *Bd*, impairing ability of the fungus to cause disease.

It appears that the amphibian chytrid fungus may not have severe conservation implications for Australian microhylids. In addition to the lack of apparent effects of *Bd* on

microhylids, the threat from this disease may have decreased within the WT, as *Bd* has become endemic to the region (McDonald et al. 2005). Management initiatives are not likely to be required for this family of frogs in regards to *Bd*.

In contrast, however, other threats, such as habitat modification and climate change, are likely to increasingly threaten Australian microhylids, particularly those with narrow habitat requirements. Global climate change is expected to have a large impact on many species including amphibians, and macro-environmental modeling suggests that the distributions of many ectothermic species will be dramatically altered (Thomas et al. 2004). Bioclimatic modeling has recently emphasised the importance of understanding the effects of changes in climate on microhylids. If we assume that their present-day distributions are at equilibrium, so that they are controlled entirely by bioclimatic factors, then models show that small changes in climate could have serious effects on the distribution of these species, causing large reductions in geographical range and increased range fragmentation. Using these models, an increase in mean temperature of 1°C is predicted to reduce the area of the core habitats of four microhylid frogs (*C. hosmeri, C. neglectus, C. exiguus,* and *C. monticola*) by at least 50% (Williams and Hilbert 2006), and will result in complete loss of the current range and core habitat of *C. concinnus* (Williams et al. 2003).

In addition to direct effects on frogs, changes in the thermoregulatory opportunities that are available to rainforest frogs caused by large-scale global warming may have already contributed to widespread population declines in association with chytridiomycosis (Pounds et al. 2006). Outbreaks of chytridiomycosis in Costa Rica have been attributed to increasing temperatures, which are shifting towards the growth optimum of *Bd*.

Results from the microhabitat section of my thesis could be used to provide further insights into the way that climate change is likely to affect Australian microhylid species. The majority of studies that predict the effect of climate change on species distributions (including those described above) consider mean ambient environmental conditions. These approaches are limited by the fact that the present distributions of many species may not reflect their fundamental climatic requirements (Parmesan *et al*. 2005). As shown in this thesis, the micro-environmental conditions experienced by individual species can differ from ambient conditions. For this reason, there is a need for the use of fine-scale micro-environmental conditions when predicting the effects of habitat alteration (such as climate change), and when observing the effects of *Bd*. Detailed species-specific data are essential for accurately predicting responses to climate changes, and for designing management plans for species.

Future directions

AMPs are not the only innate immune response in amphibians, and other epithelial defenses, such as regulation of skin sloughing and symbiotic skin microbiota are also likely to

have a major role in determining the susceptibility of frogs to chytridiomycosis (Harris et al. 2006; 2009; Brucker et al. 2008a; 2008b; Woodhams et al. 2008). Microhylids produce clutches of eggs in terrestrial nests, and adults brood eggs (Zweifel 1985). The adaptations that normally serve to protect developing egg clutches may predispose terrestrial breeders to lower susceptibility to *Bd* infection. Amphibians that lay eggs in terrestrial nests that are brooded or tended by adults are likely to have anti-fungal microbiota that can inhibit the growth of fungi that are pathogens of embryos, and may also inhibit the growth of *Bd* (Austin 2000; Harris et al. 2006; Lauer et al. 2007; Banning et al. 2008). These species may not suffer from severe population declines associated with chytridiomycosis because they are more likely to harbour bacteria that inhibit the growth of *Bd* (Harris et al. 2006). More research is needed to determine the ability of microhylids to resist chytridiomycosal infection, via investigations of the microbial ecology of amphibian skin.

Further laboratory experiments may also be needed to elucidate mechanisms responsible for the resistance of microhylids to chytridiomycosis. For example, the constant environmental conditions used in the laboratory experiment (Chapter 4) in which I demonstrated that *C. ornatus* are not constitutively immune to infection by *Bd* did not reflect conditions experienced in the field. It would be useful to conduct additional experiments under conditions mimicking those I document for field retreat sites (Chapter 6). The composition of the skin microbiota of microhylids has not been explored at all, and initial work to evaluate the role that bacterial metabolites play in protecting microhylids from chytridiomycosis needs to be performed. The composition of the microbiota may change during captivity, and experiments comparing recently captured animals whose microbiota has been removed using antibiotics to control animals would provide another step towards determining the importance of microbiota in disease resistance. Knowledge gained from these investigations will be useful in understanding the general determinants of susceptibility to chytridiomycosis in amphibians, and may be useful in designing probiotic treatments to prevent or moderate the effects of infection in other taxa.

Future effort should also be placed into monitoring programs for vulnerable species of microhylids that have restricted range sizes and populations. Species with restricted ranges are likely to be more sensitive to the effects of future habitat modification and climate change, and monitoring programs should be put in place to ensure the persistence of these species. Monitoring should specifically incorporate the use of thermal models to describe in more detail the thermal and hydric conditions available to target species across their environmental range.

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APPENDIX 1. GLOBAL RECORDS OF *Bd* INFECTION IN TERRESTRIAL, DIRECT-DEVELOPING FROG SPECIES.

Family	Species	Location	Reference
Leptodactylidae	*Craugastor azueroensis*	Panama	(Lips et al. 2006)
	C. bransfordii	Panama	(Lips et al. 2006)
		Costa Rica	(Puschendorf et al. 2006a)
	C. bufoniformis	Panama	(Lips et al. 2006)
	C. cerasinus	Panama	(Lips et al. 2006)
	C. crassidigitus	Panama	(Lips et al. 2006)
		Costa Rica	(Puschendorf et al. 2006a)
	C. fitzingeri	Costa Rica	(Puschendorf et al. 2006a)
	C. gollmeri	Panama	(Lips et al. 2006)
	C. megacephalus	Panama	(Lips et al. 2006)
	C. noblei	Panama	(Lips et al. 2006)
	C. podiciferus	Panama	(Lips et al. 2006)
		Costa Rica	(Puschendorf et al. 2006a)
	C. punctariolus	Panama	(Lips et al. 2006)
	C. tabasarae	Panama	(Lips et al. 2006)
	C. talamancae	Panama	(Lips et al. 2006)
		Costa Rica	(Puschendorf et al. 2006a)
	Eleutherodactylus aurilegulus	Honduras	(Puschendorf et al. 2006b)
	E. emcelae	Panama	(Speare and Berger 2004a)
	E. caryophyllaceus	Panama	(Lips et al. 2006)
	E. coqui	Puerto Rico	(Burrowes et al. 2004)
		Hawaii	(Beard and O'Neill 2005)
	E., cruentus	Panama	(Lips et al. 2006)
			(Speare and Berger 2004a)
	E. diastema	Panama	(Lips et al. 2006)
	E. karlschmidti	Puerto Rico	(Burrowes et al. 2004)
	E. melanostictus	Costa Rica	(Lips et al. 2003)
	E. museosus	Panama	(Lips et al. 2006)
	E. ridens	Panama	(Lips et al. 2006)
	E. saltator		(Speare and Berger 2004a)
	E. vocator	Panama	(Lips et al. 2006)
	Gastrotheca cornuta	Panama	(Lips et al. 2006)
Leiopelmatidae	*Leiopelma archeyi*	New Zealand	(Speare and Berger 2004a)
Microhylidae	*Cophixalus ornatus*	Australia	(Kriger and Hero 2006a)
Myobatrachidae	*Assa darlingtoni*	Australia	(Kriger and Hero 2007b)

APPENDIX 2. CHALLENGE ASSAY RESULTS OF PEPTIDE SAMPLES FOR THE PERCENTAGE INHIBITION OF GROWTH OF *Bd*; n=81.

Frog ID	Date	Species	Location	Elevation (m)	% inhibition	Conc. (µg/ml)
AU807Co1A	9/03/2007	*C. ornatus*	Ravenshoe	800	-34.74	1000
AU1005Co22A	20/01/2006	*C. ornatus*	Charmillan ck	1000	-40.80	1000
AU1005Co23B	20/01/2006	*C. ornatus*	Charmillan ck	1000	-84.89	1000
AU1005Co24C	20/01/2006	*C. ornatus*	Charmillan ck	1000	-51.58	1000
AU1005Co25D	20/01/2006	*C. ornatus*	Charmillan ck	1000	13.33	1000
AU1005Co26E	20/01/2006	*C. ornatus*	Charmillan ck	1000	-3.85	500
AU1005Co28F	20/01/2006	*C. ornatus*	Charmillan ck	1000	15.35	500
AU1005Co29G	20/01/2006	*C. ornatus*	Charmillan ck	1000	5.11	500
AU1005Co30H	20/01/2006	*C. ornatus*	Charmillan ck	1000	12.96	500
AU1005Co31I	3/04/2006	*C. ornatus*	Charmillan ck	1000	20.52	1000
AU1005Ar1I	20/01/2006	*A. robusta*	Charmillan ck	1000	-9.40	500
AU1007Co1A	8/03/2007	*C. ornatus*	Charmillan ck	1000	-72.93	1000
AU1007Co2B	8/03/2007	*C. ornatus*	Charmillan ck	1000	**100**	500
AU1007Co3C	8/03/2007	*C. ornatus*	Charmillan ck	1000	24.07	1000
AU1007Co4D	8/03/2007	*C. ornatus*	Charmillan ck	1000	0.33	500
AU1007Co5E	8/03/2007	*C. ornatus*	Charmillan ck	1000	-35.50	1000
BK05Cn6Q	8/11/2005	*C. neglectus*	Bellenden Ker	1500	16.91	500
BK05Cn35A	7/12/2005	*C. neglectus*	Bellenden Ker	1500	**100**	250
BK05Cn36B	7/12/2005	*C. neglectus*	Bellenden Ker	1500	**97.01**	250
BK05Cn37C	7/12/2005	*C. neglectus*	Bellenden Ker	1500	**100**	250
BK05Cn38D	7/12/2005	*C. neglectus*	Bellenden Ker	1500	**99.10**	250
BK05Cn39E	7/12/2005	*C. neglectus*	Bellenden Ker	1500	7.68	500
BK05Cn40F	7/12/2005	*C. neglectus*	Bellenden Ker	1500	-46.49	500
BK05Cn41G	7/12/2005	*C. neglectus*	Bellenden Ker	1500	-41.51	500
BK05Cn53 H	7/12/2005	*C. neglectus*	Bellenden Ker	1500	-77.74	500
BK05Cn54I	7/12/2005	*C. neglectus*	Bellenden Ker	1500	7.88	1000
BK05Cn55J	7/12/2005	*C. neglectus*	Bellenden Ker	1500	31.73	1000
BK05Cn3K	8/11/2005	*C. neglectus*	Bellenden Ker	1500	30.13	1000
BK05Cn42L	7/12/2005	*C. neglectus*	Bellenden Ker	1500	27.76	1000
BK05Cn44M	7/12/2005	*C. neglectus*	Bellenden Ker	1500	73.02	250
BK05Cn46N	7/12/2005	*C. neglectus*	Bellenden Ker	1500	39.41	1000
BK05Cn47O	7/12/2005	*C. neglectus*	Bellenden Ker	1500	-26.82	100
BK105Cn52P	7/12/2005	*C. neglectus*	Bellenden Ker	1500	18.17	1000
BK05Cn50R	7/12/2005	*C. neglectus*	Bellenden Ker	1500	18.49	1000
BK05Cn51S	7/12/2005	*C. neglectus*	Bellenden Ker	1500	-13.72	1000
BK05Cn48T	7/12/2005	*C. neglectus*	Bellenden Ker	1500	-7.97	500
BK05Cn49U	7/12/2005	*C. neglectus*	Bellenden Ker	1500	-25.33	500
BK05Cn21A	30/11/2005	*C. neglectus*	Bellenden Ker	1500	31.94	500
BK05Cn32E	6/12/2005	*C. neglectus*	Bellenden Ker	1500	11.32	500
BK05Cn33F	6/12/2005	*C. neglectus*	Bellenden Ker	1500	19.77	1000
BK05Cn34G	6/12/2005	*C. neglectus*	Bellenden Ker	1500	32.05	500
CU806Af1A	21/01/2006	*A. fryi*	Mt Lewis	800	7.93	1000
CU806Af2B	21/01/2006	*A. fryi*	Mt Lewis	800	-0.18	500
CU806Af3C	21/01/2006	*A. fryi*	Mt Lewis	800	**92.94**	500
CU806Af4D	21/01/2006	*A. fryi*	Mt Lewis	800	7.06	250
CU806Af5E	21/01/2006	*A. fryi*	Mt Lewis	800	30.61	1000
CU806Ca1F	21/01/2006	*C. aenigma*	Mt Lewis	800	12.69	1000

CU806Ap1G	21/01/2006	*A. pluvialis*	Mt Lewis	800	20.06	1000
CU806Ap2H	21/01/2006	*A. pluvialis*	Mt Lewis	800	-7.61	1000
CU806Ap3I	21/01/2006	*A. pluvialis*	Mt Lewis	800	4.33	500
CU806Co1J	21/01/2006	*C. ornatus*	Mt Lewis	800	29.52	500
CU806Co2K	21/01/2006	*C. ornatus*	Mt Lewis	800	-10.59	500
CU1006Af1A	24/01/2006	*A. fryi*	Mt Lewis	1000	-3.58	1000
CU1006Af2B	24/01/2006	*A. fryi*	Mt Lewis	1000	-8.48	1000
CU1006Ca1C	24/01/2006	*C. aenigma*	Mt Lewis	1000	-10.39	1000
Pal06Ar1A	7/03/2006	*A. robusta*	Paluma	900	-14.10	1000
Pal06Ar2B	7/03/2006	*A. robusta*	Paluma	900	36.35	1000
Pal06Ar3C	7/03/2006	*A. robusta*	Paluma	900	2.12	1000
Pal06Ar4D	8/03/2006	*A. robusta*	Paluma	900	-7.81	1000
Pal06Ar5E	8/03/2006	*A. robusta*	Paluma	900	43.62	1000
Pal06Ar6F	8/03/2006	*A. robusta*	Paluma	900	32.36	1000
Pal06Ar7G	9/03/2006	*A. robusta*	Paluma	900	18.64	1000
Pal07Ar1A	23/01/2007	*A. robusta*	Paluma	900	-8.089	1000
Pal07Ar2B	23/01/2007	*A. robusta*	Paluma	900	-7.15	1000
Pal07Ar3C	23/01/2007	*A. robusta*	Paluma	900	3.77	1000
Pal07Ar4D	23/01/2007	*A. robusta*	Paluma	900	-7.81	1000
Pal07Co1E	23/01/2007	*C. ornatus*	Paluma	900	**100**	1000
Pal07Ar6F	24/01/2007	*A. robusta*	Paluma	900	**94.69**	1000
Pal07Ar8H	24/01/2007	*A. robusta*	Paluma	900	12.57	1000
Pal07Ar9I	24/01/2007	*A. robusta*	Paluma	900	23.91	500
Pal07Ar10J	24/01/2007	*A. robusta*	Paluma	900	-45.35	100
Pal07Ar11K	24/01/2007	*A. robusta*	Paluma	900	-35.46	500
Pal07Ar12L	24/01/2007	*A. robusta*	Paluma	900	-31.49	500
Pal07Ar13M	24/01/2007	*A. robusta*	Paluma	900	18.58	500
Pal07Ar14N	24/01/2007	*A. robusta*	Paluma	900	**100**	500
Pal07Ar15O	24/01/2007	*A. robusta*	Paluma	900	**95.31**	1000
Pal07Co2P	24/01/2007	*C. ornatus*	Paluma	900	-26.94	1000
Pal07Co3Q	24/01/2007	*C. ornatus*	Paluma	900	**99.11**	100
Pal07Co4R	24/01/2007	*C. ornatus*	Paluma	900	**92.01**	500
Pal07Co5S	25/01/2007	*C. ornatus*	Paluma	900	**98.08**	1000
Pal07Co6T	25/01/2007	*C. ornatus*	Paluma	900	**100**	500

Samples where 100% inhibition was observed are highlighted in **bold**

Note: Percentage inhibition results with a negative value show positive growth of *Bd* in comparison to controls

APPENDIX 3. DATALOGGER READINGS OF AGAR MODELS WITH INTERNAL TEMPERATURE PROBES.

AU10

AU10		0m	0m	100m	100m	200m	200m
Time	Date	agar	plastic	agar	plastic	agar	plastic

TABLES OF RAW DATA HAVE BEEN REMOVED

	Max	**29.62**	**29.76**	**28.89**	**29.58**	**29.76**	**24.99**
	Min	**18.12**	**18.43**	**18.09**	**18.32**	**18.76**	**18.76**
	Median	**20.35**	**20.46**	**20.8**	**21.37**	**19.96**	**19.97**

BK

BK		**0m**	**0m**	**100m**	**100m**	**200m**	**200m**
Time	**Date**	**agar**	**plastic**	**agar**	**plastic**	**agar**	**plastic**

TABLES OF RAW DATA HAVE BEEN REMOVED

Max	**18.66**	**19.64**	**21.15**	**20.8**	**18.69**	**19.43**
Min	**12.45**	**13.63**	**12.83**	**13.13**	**12.19**	**12.83**
Median	**14.83**	**16.07**	**14.93**	**15.23**	**14.87**	**14.93**

CU8 1

CU8 Time	Date	0m agar	0m plastic	100m agar	100m plastic	200m agar	200m plastic

TABLES OF RAW DATA HAVE BEEN REMOVED

Max	**21.86**	**22.45**	**21.86**	**21.76**	**21.44**	**21.86**
Min	**19.43**	**20.67**	**19.77**	**20.01**	**19.71**	**20.11**
Median	**20.46**	**21.37**	**20.46**	**20.35**	**20.74**	**20.8**

CU8 2

CU8		**0m**	**0m**	**100m**	**100m**	**200m**	**0m**
Time	**Date**	**agar**	**plastic**	**agar**	**plastic**	**agar**	**plastic**

TABLES OF RAW DATA HAVE BEEN REMOVED

Max	**22.88**	**23.32**	**22.82**	**23.21**	**22.88**	**23.32**
Min	**21.09**	**21.82**	**21.73**	**22.11**	**21.44**	**21.15**
Median	**21.79**	**22.47**	**22.09**	**22.47**	**21.8**	**21.86**

Paluma

Paluma Time	Date	0m agar	0m plastic	100m agar	100m plastic	200m agar	200m plastic

TABLES OF RAW DATA HAVE BEEN REMOVED

Max	**21.8**	**23.32**	**22.15**	**22.45**	**22.22**	**22.22**
Min	**20.39**	**20.8**	**21.44**	**20.33**	**20.8**	**21.15**
Median	**21.09**	**21.51**	**21.79**	**21.73**	**21.51**	**21.51**