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Dynamics of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) in isolated patches of lowland rainforest

Thesis submitted by

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CONTRIBUTION OF OTHERS

My MSc thesis was supervised by Lin Schwarzkopf, Ross Alford and Robert Puschendorf. All aspects of the project from the initial stage of project design and methods, through to editing and the completion of the final thesis have been strongly influenced by their expertise. Funding for this project was provided by Powerlink and the Australian Research Council.

Field studies were undertaken with the help of fellow researcher Sarah Sapsford and many volunteers: Marcin Skladaneic, Deborah Bower, Hunter (Alden) McCall, David Fischer, Stephen Zozaya, Ralph Manzanell, Meredith Weir, Katrin Schmidt, Kahleana Stannard, Eddie Williams, Caitlin Nielsen, and Janelle Evans. Laboratory aspects of my research were aided by Sara Bell, Mathew Vickers, Betsy Roznik, John Llewelyn, Samantha Forbes, Mick Ellison, and Arnaud Gourret. Editorial advice was given by April Reside, Deborah Bower, Richard Duffy, Heather McNab, Sandy McNab, and Lauren Sperotto. PCR diagnostics for *Batrachochytrium dendrobatidis* was undertaken by the Amphibian Disease Diagnostic Laboratory at Washington State University by Ashley McCally, Patricia Frias, and Karen Benkyo. Stream-associated invertebrates were identified by Katrin Schmidt and Paul Zborowski.

ETHICS APPROVALS AND PERMITS

The research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th Edition, 2004 and the Qld Animal Care and Protection Act, 2001. The proposed research study received animal ethics approval from the JCU Animal Ethics Committee Approval Number A1432. Permits to work on native amphibian species was provided by the Queensland Department of Environmental and Resource Management permit number WITK03070508.

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GENERAL ABSTRACT

Over the past 20 years, amphibian declines caused by *Batrachochytrium dendrobatidis* (*Bd*) have prompted a significant amount of research into the amphibian-chytrid host-parasite relationship. The complexities of the relationship have limited our ability to understand the pathogen, particularly as differences are observed in the impacts of *Bd* among different amphibian species, and the variation in the impact of the pathogen along an elevation gradient.

The significant declines of amphibians at high elevations (400+ m asl) in the Wet Tropics bioregion of north eastern Australia has focused research on endemic species, and the environmental conditions they experience. There is, however, little research on tropical lowland areas. Within tropical lowlands rainforest of the Wet Tropics bioregion, *Bd*-susceptible amphibians continue to thrive, and although *Bd* is present in the tropical lowlands, it has not been as devastating to amphibian populations.

Much of the lowland tropical rainforest of the Wet Tropics is contiguous with higher elevation areas, providing the potential for *Bd* at higher elevations to influence lowland populations as infected hosts and *Bd* zoospores move downstream. However, not all areas of lowland rainforest are contiguous with the uplands. There are isolated rainforest patches, with no connection to high-elevation streams, where *Bd* is present and experiences a different set of environmental conditions to much of the rest of the Wet Tropics. It is within these lowland areas, which are not contiguous with highland rainforest streams, that I conducted my research. The aims of my study were to investigate the ecology of the common mist frog (*Litoria rheocola*), and determine the influence of *Bd* in this system. This was done by a) investigating seasonal variation in the behaviour of uninfected *L. rheocola,* b) examining behavioural differences between *Bd* infected and uninfected *L. rheocola*, and c) investigating the potential reservoir hosts of the amphibian chytrid fungus *Bd* of various amphibian and non-amphibian species.

The common mist frog, *L. rheocola,* has similar ecological requirements to *Bd*; both share the need of moisture and relatively cool conditions. I examined seasonal changes in the thermal microenvironment, behaviour, movement, microhabitat selection and body temperatures of uninfected *L. rheocola*, using harmonic radar tracking. Warm environmental temperatures in my study area limit the suitability of the tropical lowlands for *Bd* to winter. Movements and microhabitat use of *L. rheocola* were similar year-round. My results suggested that increases in body temperatures due to seasonal increases in environmental temperatures may protect lowland populations from decline due to *Bd*.

During the winter, a large proportion of *L. rheocola* in my study area became infected. Comparing the behaviours of infected and uninfected individuals in winter suggested that uninfected individuals maintained higher body temperatures, perched further from the stream, and used microhabitats that minimized the probability of becoming infected. The behaviour of individuals during periods of high infection risk can reduce their probability of infection.

In my study areas, prevalence of *Bd* dropped to zero in *L. rheocola* in the summer. The range of amphibians that naturally become infected by *Bd* is large, but *Bd* has not been recorded in all amphibians, including species common within the distributional range of *Bd*. By swabbing all the frogs in my study area, I detected *Bd* in northern dwarf treefrogs (*Litoria bicolor*) and Australian wood frogs (*Hylarana daemeli*), in which *Bd* had not previously been detected. I also detected *Bd* on a range of stream-associated invertebrates. If *Bd* can persist on flying invertebrates, it provides a potential method for the spread and dispersal of *Bd*, an aspect of the pathogen's ecology that remains unknown. Stream-associated invertebrates provide a potential vector that could have aided in the spread of *Bd* within and between regions and countries.

The tropical lowlands of the Wet Tropics provide a refuge for many species of *Bd*-susceptible amphibians. Their persistence may permit the evolution of increased immunity, allowing recolonisation of upland areas. My study of lowland populations has provided an increased understanding of the dynamics allowing persistence of these frogs.

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CHAPTER 1: INTRODUCTION

Host ecology can be influenced by infectious disease, because it can influence individuals, population dynamics and the community in which individuals reside (Anderson and May 1979; Holmes 1996; Morens et al. 2004; Han 2008). The infection status of individuals and the host's ability to transmit infection are dependent on many factors, including the pathogen type, and its mode of transmission; host characteristics, such as age, sex, reproductive condition, genotype and behaviour; environmental factors such as habitat and season. These factors can all influence pathogen load, and the health, and survival of infected individuals (Gaines and McClenaghan, Jr. 1980).

Diseases are a natural threat to the survival of individuals and populations. Unfortunately, the threat of disease to wildlife has increased with anthropogenic modification of landscapes, and translocation of wildlife and their pathogens (Bradley and Altizer 2007). Increasingly, the pressures of disease are recognised as a serious threatening process for numerous species (Daszak et al. 1999; Brown 2004; Hamede et al. 2008). Disease can reduce population size and species distributions, for example in the case of the detrimental effects of chytridiomycosis on amphibians (Berger et al. 1998), rinderpest in wild ruminants of East Africa (Lafferty and Gerber 2002), and myxomatosis on rabbits in Australia (Dwyer et al. 1990). Studying host-pathogen interactions contributing to disease spread, host susceptibility, resistance, and survival, determine the impact of pathogens on fauna (Real and Biek 2007).

Until recently, the role of disease in population regulation was overlooked in wildlife ecology. More recently, emerging infectious diseases and wildlife declines have highlighted the impacts of disease on species and populations, causing ecosystem-level effects (Carey et al.1999).

Host movement

The vast majority of terrestrial vertebrates move for basic ecological functions, driven by their need for resources, and reproduction (Anderson and May 1979; Holmes 1996; Morens et al. 2004; Han 2008), and movement patterns are an important factor influencing disease spread and prevalence (Caley and Morris 2001; Han 2008). For example, infected social insects that move regularly and widely through a colony transmit infection more readily than if they reduce their movements and contact with others (Pie et al. 2004). Movement influences rates of contact with pathogens and transmission among hosts (Loehle 1995; Hodgkison and Hero 2002) as the movement of many pathogens is determined by host movement (Smith Jr. et al. 1996; Efford 1998, Gortázar et al. 2007). Thus, studies of host movement may directly reveal transmission rates (Sanderson 1966), and movement information may be pivotal for the design of disease control strategies.

Although studying movement is typically difficult and labour-intensive, many of the inherent complexities of movement studies have been overcome in recent years. There have been important improvements in tracking-device technology, including reduced size, and increases in the diversity of tracking methods (Langkilde and Alford 2002). Pathogen detection methods have also improved; live hosts can often be tested without detrimental effects (Keating 1991; Retallick et al. 2006).

Variation in host movement patterns can alter transmission rates within and between populations and to new areas (Lachish et al. 2009). Physiologically demanding, movement requires energy, and therefore may reduce the allocation of resources to other physiological processes such as disease resistance (Altizer et al. 2006). Alternatively, infected animals may allocate more energy to immune responses, and therefore move less (Demas et al. 1997). It is, therefore, likely that movement patterns will change when individuals are infected (Moorman et al. 1999).

Studies investigating movement of infected wildlife often find significant differences between infected and uninfected individuals (Ramsey and Cowan 2003; Farnsworth et al. 2006; Jansen et al. 2007). Infected animals may move less compared with uninfected individuals (Jansens et al. 2007). However, some diseases cause increases in movement, for example there is a 22-30% increase in the range size of possums (*Trichosurus vulpecula*) infected with tuberculosis (Ramsey and Cowan 2003). Movements may be pathogen-induced, increasing the opportunities for pathogen transmission (Berdoy et al. 2000). Many ground dwelling arthropods seek elevated locations prior to death when infected by particular pathogens (Yamakazi et al. 2004, Samson et al. 1988, cited in Hajek and St. Leger 1994 p. 301). Finally, not all pathogens influence movement (Rogers et al.1998; Ramsey and Cowan 2003; Rosatte 2006).

Seasonality

Climatic conditions may impact host–pathogen interactions by influencing the occurrence and transmission of pathogens, through seasonal changes in host movement, host contact rates, immune system function, social behavior, and birth rates and death rates, which influence the number of available susceptible hosts (Brooke et al. 2000; Dowell 2001; Nelson 2004; Altizer et al. 2006; Lips et al. 2006). Seasonal changes in environmental conditions often coincide with changes in movement patterns, for example, movements to and from breeding or feeding grounds and the expansion or contraction of home ranges, and therefore influence exposure to pathogens (Sanderson 1966; Altizer et al. 2006; Farnsworth et al. 2006; Wahlstrom and Liberg 2009). If resources become limited seasonally, individuals may be forced to come into contact with one another, influencing disease spread, especially if pathogens are abundant (Altizer et al. 2006). Seasonality is clearly a significant factor in the spread of disease (Conner and Miller 2005; Fryxell and Sinclair 2008).

Like their hosts, the abundance and population dynamics of pathogens may fluctuate seasonally (Hosseini et al. 2004; Altizer et al. 2006). For example, the human influenza virus requires specific low humidity conditions to facilitate survival and transmission; in temperate regions this results in annual winter epidemics (Tamerius et al. 2013). Seasonal variation in the rabies virus is dependent on host species, in raccoons rabies increases in prevalence during the breeding season (Rosatte 2006), whilst in big brown bats (*Eptesicus fuscus*) significant increases in bat abundance during the birthing season increases the available hosts for rabies (George et al. 2011). (George et al. 2011).

Sex and age

There is variation in infection probability between sexes and across age classes, leading to variation in the impacts of infection (Nunn et al. 2008). Ecological requirements, immunity levels, activity levels and movement patterns vary with age; altering the likelihood of infection (Miller and Conner 2005; Michel et al. 2006). For some species, infection risk is greatest in juveniles; whilst in others infection risk is greater in sub-adults or adults (Banks 1969; Strathmann et al. 2002). As juveniles may have under-developed immune systems, they may influence disease transmission within and between social groups and subpopulations differently to adults (Nunn et al. 2008). For example, Jeltsch et al. (1997) suggest that movement of sub-adults away from their birth home range into new home ranges is crucial in explaining the patterns of spread of rabies in red foxes (*Vulpes vulpes*), whereas Conner and Miller (2004) demonstrated that the dispersal of fawns, yearlings and adult mule deer (*Odocoileus hemionus*) is unlikely to contribute to the spread of chronic wasting disease, but the migration of adult mule deer does.

Sexually mature individuals of many species move further than immature individuals, increasing their likelihood of contact with and transmission of infection (Miller and Conner 2005). During periods of increased activity (e.g., in the breeding season) infection prevalence may increase (Sandell 1986; Altizer et al. 2003; Farnsworth et al. 2006). For example, during the breeding season, rabies infection increases in adult raccoons (*Procyon lotor*) (Rosatte 2006), with males further increasing disease transmission rates via injuries inflicted during fights (Root et al. 1999). Increases in the prevalence of sin nombre virus in deer mice (*Peromyscus maniculatus*) and chronic wasting disease in mule deer (*Odocoileus hemionus*) also occur during the breeding season (Root et al. 1999; Farnsworth et al. 2006). Females may have higher rates of infection than males during the breeding season because of the increased energy costs associated reproduction (Hubbard 1985). Some diseases, such as infectious keratoconjunctivitis of big horn sheep (*Ovis canadensis*), are neither sex nor age dependent (Jansens 2007).

Reservoir hosts and vectors

The presence of a pathogen within a susceptible host population, although detrimental to population health, is unlikely to cause species extinction without the presence of a reservoir host (McCallum and Dobson 1995, 2002; Lips et al. 2006). Although a susceptible host harbours a pathogen and is susceptible to infection, reservoir hosts provide a substrate for pathogen survival without the hosts' succumbing to infection, enabling the maintenance of pathogen populations when susceptible hosts are in low densities or absent (Haydon et al. 2002; Davidson et al. 2003; Blaustein et al. 2005). Reservoir host species are often a common, widespread, and abundant species with low pathogen susceptibility (Blaustein et al. 2005, Brunner et al. 2004). Harbouring of a pathogen by reservoir hosts and the successive transmission to more susceptible species is extremely detrimental to susceptible hosts (Davidson et al. 2003), and therefore, the likelihood of population declines and the threat of extinction from disease increases substantially when pathogens are not host specific, and multiple reservoir hosts are present (Cullen and Owens 2002; Haydon et al. 2002). The impacts of multiple reservoir hosts harboring a pathogen was seen in east Africa when rinderpest moved from domestic cattle into wild ungulates decimating populations (Sinclair and Arcese 1995).

Batrachochytrium dendrobatidis and amphibian declines

The amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), has been implicated as one of the most severe and devastating diseases to affect a class of organisms in recorded history (Stuart et al. 2004). Amphibian declines and extinctions have occurred at an unprecedented rate on all continents inhabited by amphibians (Stuart et al. 2004). *Batrachochytrium dendrobatidis* is a member of the family Chytridiomycota and one of only two members pathogenic to vertebrates, infecting keratinised body parts of amphibians, the mouthparts of tadpoles and the skin of adult frogs (Berger et al. 1998; Longcore et al. 1999; Kilpatrick et al. 2009). Death from *Bd* occurs due to a disruption of normal epidermal function, causing an osmotic imbalance from a loss of electrolytes (Voyles et al. 2007).

The ecological requirements and life cycle of *Bd* have been well studied (Berger et al. 1998; Piotrowski et al. 2004; Longcore et al. 1999; Voyles et al. 2007). *Batrachochytrium dendrobatidis* is temperature, pH, and moisture sensitive (Piotrowski et al. 2004). Hence, seasonal changes are significant factors influencing the survival, growth, reproduction, and prevalence of the pathogen and, in turn, its effect on amphibian populations (Piotrowski et al. 2004; McDonald, et al. 2005; Kriger and Hero 2007; 2008; Lips 1999; Woodhams et al. 2008). Growing in the laboratory at temperatures between 4-28°C, (optimally 17- 25°C), *Bd* cannot survive desiccation, and it deteriorates and dies at temperatures greater than 28°C and below 4°C (Johnson and Speare 2003; Piotrowski et al. 2004; Woodhams et al. 2008). Infection prevalence in frog populations increases during cooler months and decreases during warmer periods, probably due to *Bd's* temperature-dependent physiological processes (Bradley et al. 2002; Berger et al. 2004; Piotrowski et al. 2004; Retallick et al. 2004; McDonald et al. 2005; Woodhams and Alford 2005; Kriger et al. 2006; Murray et al. 2009; Alford 2010).

Batrachochytrium dendrobatidis has been implicated in the decline of at least 14 Australian species; and it has been recorded in at least 63 species Australia-wide (Richards et al. 1993; Laurance et al. 1996; McDonald and Alford 1999; Murray et al. 2010; McNab unpub. data). Declines in the Wet Tropics of north Queensland have been most drastic at locations above 400 m in elevation, whilst populations of the same species survived below this elevation, including, waterfall frogs (*Litoria nannotis*), common mistfrogs (*L. rheocola*), green-eyed treefrog (*L. serrata*) and Australian lace-lids

(*L. dayi*) (Richards et al. 1993; Williams and Hero 2001). Susceptibility to the pathogen varies among species with some species experiencing population declines (e.g., Australian lace-lids [*Litoria dayi*]), extinctions (e.g., gastric brooding frogs [*Rheobatrachus vitellinus*], southern day frogs [*Taudactylus diurnus*]), or remaining virtually unaffected (e.g., jungguy frogs [*Litoria jungguy* species complex]) (Retallick et al. 2004; Alford 2010). The continued survival of lowland populations appears to have occurred due to a combination of life history traits, behavioural variation among species, habitat preferences, and environmental conditions (Richards et al. 1993; Laurence et al. 1996; Williams and Hero 1998, McDonald and Alford 1999; Berger et al. 1998, Lips 1998, 1999; McDonald et al. 2005; Rowley and Alford 2013). Survival of lowland amphibian populations provides the opportunity to investigate how amphibians can survive in the presence of this devastating disease.

This study

Batrachochytrium dendrobatidis threatens many stream-associated amphibians in Australia and worldwide, an in-depth understanding of their relationship with the pathogen can provide insights into factors aiding in population and species survival. I aimed to improve our knowledge of an Endangered amphibian, the common mistfrog (*Litoria rheocola*), and its relationship with *Bd* in lowland areas, while at the same time broadening our understanding of non-amphibian reservoir hosts of *Bd*.

Common mistfrogs (*Litoria rheocola*) are an especially appropriate study organism for research into the responses of an endangered species to a deadly pathogen, because some populations of this species, occurring below 400 m elevation, survived the amphibian declines that occurred in the 1990s in tropical Australia, whereas populations occurring above this elevation were extirpated. In addition, populations of *L. rheocola* are presently recolonising high elevation areas from which they had disappeared (McDonald and Alford 1999). In addition, common mistfrogs inhabit isolated lowland fragments of the Wet Tropics, disjunct from the otherwise contiguous forests of the Wet Tropics, and isolated from any mountainous regions over 400 m above sea level (ASL), which do not share watercourses with high elevation locations. In these isolated lowland fragments, temperature and humidity are less buffered than in contiguous rainforests. I aimed to study these isolated populations, with a view to comparing my research to other work on the same species in more contiguous forests, highlighting the importance of the environment in disease dynamics.

Aims

The aim of this study is to investigate the dynamics of common mistfrogs (*L. rheocola*) and *Bd* in patches of lowland rainforest isolated from the main body of the Wet Tropics. Additionally, an examination of potential non-amphibian hosts and vectors of *Bd* was undertaken. Specifically, the aims of this study were to:

1. Investigate seasonal variation in the ecology of uninfected *Litoria rheocola* in isolated lowland patches of rainforest that contain *Batrachochytrium dendrobatidis*,

2. Examine ecological differences between *Litoria rheocola* infected by *Batrachochytrium dendrobatidis,* and uninfected individuals, and
3. Investigate potential non-amphibian reservoir hosts of the chytrid fungus *Batrachochytrium dendrobatidis*.

CHAPTER 2: SEASONAL VARIATION IN THE BEHAVIOUR OF AN ENDANGERED AMPHIBIAN SURVIVING IN TROPICAL LOWLAND RAINFORESTS

Abstract

The influence of pathogens on host health is often dependent on host behaviour and environment. At low elevations (<400 m), common mistfrogs (*Litoria rheocola*) coexist with the pathogen (*Batrachochytrium dendrobatidis*), which caused host declines at higher elevations. Using harmonic radar tracking, I examined the movement and habitat selection of *L. rheocola* surviving in the presence of *Bd* in a lowland population. I found that seasonal changes in environmental conditions coincide with changes in *L. rheocola* behaviour, body temperatures and movements. Microhabitat use and nocturnal movement did not vary seasonally, and were unlikely to influence *Bd* prevalence in lowland populations of *L. rheocola*. However, diurnal movements and body temperature did vary seasonally.

Introduction

Worldwide, serious amphibian population declines have been occurring since the late 1980's with declines and potential extinctions occurring in 2468 and 122 amphibian species, respectively (Stuart et al. 2004). Declines have been linked with chytridiomycosis, a disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) (Berger et al. 1998; Stuart et al. 2004). *Bd*-implicated declines have been most severe in stream-dwelling amphibian species at high elevations, many of which share particular life history traits (small clutch size, restricted ranges, stream breeding) (Williams and Hero 1998; Lips et al. 2003; Stuart et al. 2004; Collins and Crump 2009). These same life history traits occur in populations of declining species at low elevations, yet in some cases low elevation populations are continuing to thrive, suggesting factors such as environmental conditions and behaviour are aiding in the survival of low elevation amphibian populations.

The influence of environmental factors (temperature, salinity, pH, humidity) on *Bd* and the resulting impacts on amphibians are well studied (Johnson et al. 2003; Piotrowski et al. 2004; Stevenson et al. 2013). Yet, behaviours: microhabitat use, movement, and body temperature regulation have been thoroughly investigated in very few *Bd*-susceptible species. Much of our understanding of the behavior of tropical stream-dwelling amphibians is derived from the few, more commonly studied species (Dole and Durant 1974). Behavioral differences among species may be an important factor determining survival of *Bd*-infected stream-associated amphibians (Rowley and Alford 2007).

In the Wet Tropics bioregion of northern Australia, many high elevation (> 3400 m) rainforest hylid populations suffered severe *Bd* implicated declines, whilst populations of these species below 400 m elevation survive (Richards et al. 1993; Berger et al. 1998). The common mistfrog (*Litoria rheocola*) is a small rainforest hylid that suffered such declines (Kriger and Hero 2008). Populations of lowland *L. rheocola* fluctuate seasonally, with environmental temperatures and the prevalence of *Bd*. Although declines occur when it is cooler (i.e., in winter) populations continue to survive, suggesting behaviour may influence survival.

The objective of this study was to investigate seasonal changes in the behaviour of *L. rheocola* at low elevation. I used harmonic tracking, behavioural and microhabitat observation and measurements of body temperatures in lowland rainforest that do not share watercourses with high elevation areas. Understanding the behaviour of surviving *Bd*-susceptible amphibians can provide information about amphibians' ability to naturally manage *Bd* infection and the impact of *Bd* outside of its optimal environmental conditions.

Methods

Study sites

This study was conducted between 18 May 2009 and the 05 April 2011, with at least two fields trip in each season (summer, autumn, winter, spring) seasons. I surveyed four sites at two streams, in isolated pockets of tropical lowland rainforest (complex mesophyll vine forest) at Mena Creek, near Tully, in the Wet Tropics bioregion of North Queensland, Australia. The two rainforest streams flowed into Mena Creek (Mena 1 (17°39'03.01"S, 145°59'23.94"E) and Mena 2 (17°39'11.97'S, 145°59'06.96"E). Each stream was between 0-90 m above sea level (ASL) and did not connect to any mountainous streams at > 300 m elevation. Two four-hundred-metre transects were established along each stream. Anthropogenic disturbance in the area consisted of access roads for 132kV/275kV transmission lines (Mena Creek). Streams were narrow (1 – 6 m wide), shallow (0.05 – 1.40 m deep) and typically lit with dappled sunlight; however, width and depth of the streams varied considerably with rainfall. Streams at all sites had permanent water flow through riffles, runs and pools, with rocks of various grain sizes from pebbles to boulders, with a sandy stream-bed. The dominant understory flora was diverse, and included members of the families Arecaceae, Pandanaceae, Cyatheaceae, and Philydraceae.

Figure 2.1 Location of Mena Creek study site indicated by star, habitat of Mena Creek adjacent

Environmental conditions

Environmental conditions including air and water temperatures were recorded to the nearest 0.5°C using Livingstone^{TM} mercury glass thermometers; humidity was recorded with a Brannan^{TM} whirling hygrometer. These variables were assessed diurnally and nocturnally, at the start (0 m mark) of each transect, prior to each tracking event.

Common mistfrog (*Litoria rheocola*)

Litoria rheocola is a small (males: 23 – 39mm, females: 27-39) pale brown to brown, stream-dwelling frog endemic to the Wet Tropics (Williams and Hero 1998, 2001; Hoskins and Hero 2008) (Figure 2.2). An obligate stream breeder, male *L. rheocola* are common in streams year-round, whereas females and juveniles are less often observed (McDonald and Alford 1999). Gravid females and calling males have been recorded year-round, suggesting breeding occurs in most months (Hodgkison and Hero 2001). *Litoria rheocola* is listed as Endangered after suffering significant declines from 1989-1994 (Richards et al. 1993, Northern Queensland Threatened Frogs Recovery Team 2001; IUCN 2011).

Figure 2.2 Common mistfrog (*Litoria rheocola*) (Left), and Common mistfrog (*Litoria rheocola*) with tracking device (Right).

Harmonic diodes

I used harmonic direction-finding diodes to track frogs. These tracking devices were constructed using a surface-mounted diode (SOT-323 Surface-mount zero bias Schottky detector diode) with a 13 cm, 0.5 mm leader wire as an antenna, glued to the diode with a conductive silver epoxy (Gourret et al. 2011). The end of each antenna was given a silicone-coated colour code to allow visual identification of individuals. Diodes were coated in silicone and attached to 1-mm diameter silicone tubing, through the middle of which cotton thread was inserted, and tied around the frog's inguinal region (Figure 2.2). The cotton thread was intended to decay, to allow eventual detachment of the tracking device if the tracked individual was not recovered. The silicone tubing and cotton thread were attached so that the device would not slip off, and cut to the size of the frog's inguinal region. The total mass of the tracking device (approximately 0.15 g) never exceeded 8% of the frog's total mass, restricting tracking to adults over 2g. Hence, the movement of juveniles, sub-adults and small adults could not be investigated (Lemckert and Brassil 2000).

Capture, processing and visual surveys

Between the 18 May 2009 and the 05 April 2011 I conducted thirteen tracking sessions, seven during the wet season and six during the dry season (Table 2.1). The movements of 281 adult male frogs were recorded using harmonic diodes attached to the frogs immediately upon capture. Beginning at dusk (~18:00 in winter, ~19:00 in summer), transects were surveyed for *L. rheocola* by actively searching and listening for calling individuals in the stream and streamside vegetation. Visual surveys of untracked frogs were also undertaken, number of calling males was recorded.

Typically, frogs were captured by inverting a clip-lock bag over my hand and placing it directly over the frog. A new clip-lock bag was used for each individual, to prevent disease transmission among frogs and cross-contamination of samples, using the protocols recommended by Mendez et al. (2008) and Phillott et al. (2010). Once captured, snout-urostyle length (SUL) was measured to the nearest 0.1 mm using plastic Vernier callipers, and mass was recorded with a 10 g Pesola™ spring balance prior to the attachment of a tracking device. Only male frogs over 2 g in mass were tracked, to conform to ethical mass requirements for tracking devices. The frog's position in the environment, behaviour, microhabitat and body temperature was recorded. Body temperature and substrate temperature were recorded using a hand-held infrared thermometer (Raytek ST80 Pro-Plus Non-Contact thermometer RAYST80). All adult male *L. rheocola* were released at their point of capture.

Frogs were tracked using a harmonic direction finder (RECCO R5 transmitter-receiver Recco Rescue Systems, Lidingö, Sweden). The hand-held direction finder acts as both a transmitter and receiver, creating sound when it receives a reflected signal from a diode (Rowley and Alford 2007). To locate frogs, I slowly walked transects, rotating the receiver in all directions. Once a signal was detected, the direction finder could be focused by decreasing the gain and homing in on the tracking device. The receiver's ability to detect tracking devices declined with distance as signal strength was reduced by vegetation that absorbed radio frequencies (Sizun 2005). Individuals were relocated daily, once at night (18:00-04:00) and once in the day (08:00-17:00). Upon relocation of a frog, I recorded ecological data including the frog's position in the environment, behaviour, microhabitat and body temperature, as well as movement. Movements were recorded as direct linear measurements using a 50 m Komelon™ fibreglass tape measure, and thus were minimum distances moved; actual distances moved would be the same or greater (Secor 1994; Webb and Shine 1997; Doak 2005). Only when an individual was repeatedly located between successive tracking sessions (i.e., night to day or day to night) was movement data recorded. Movements were only recorded when an individual had moved from their location during the previous survey. Data recorded on relocated tracked frogs did not involve handling the frogs, and was done with minimum disturbance. The behaviour of tracked frogs was considered representative of the behaviours of untracked frogs (Sapsford et al. 2013).

Table 2.1 Dates of tracking and surveying periods during 2009 - 2011

Start	Finish	Calendar season	Wet/Dry season	Trip no.
18/05/2009	27/05/2009	Autumn	Dry	1
16/10/2009	22/10/2009	Spring	Dry	2
13/11/2009	20/11/2009	Spring	Wet	3
11/12/2009	16/12/2009	Summer	Wet	4
13/01/2010	19/01/2010	Summer	Wet	5
17/05/2010	20/05/2010	Autumn	Dry	6
4/07/2010	8/07/2010	Winter	Dry	7
26/07/2010	4/08/2010	Winter	Dry	8
7/10/2010	14/10/2010	Spring	Dry	9
10/01/2011	16/01/2011	Summer	Wet	10
10/02/2011	14/02/2011	Summer	Wet	11
7/03/2011	8/03/2011	Autumn	Wet	12
25/03/2011	2/04/2011	Autumn	Wet	13

Batrachochytrium dendrobatidis assessment

To determine *Bd* infection status and intensity, frogs were swabbed using sterile cotton medical swabs (Tubed Dry Swabs, Medical Wire and Equipment, Corsham, Wiltshire U.K). This detection method has the highest sensitivity (Hyatt et al. 2007). Frogs were swabbed on the front feet, hind feet, inner thighs, the ventral surface and pelvic patches. These surfaces were swabbed because they are most consistently infected by *Bd* (Berger et al. 1998; 1999; Williams et al. 2002; Woodhams and Alford 2005). All tracked individuals were swabbed upon capture prior to attachment of a tracking device. Swabs were frozen and sent to the Center for Integrated Biotechnology at Washington State University, Pullman, WA, USA. Each swab was tested in a triplicate using real-time quantitative PCR (q-PCR) assays for the presence of *Bd* zoospores (Boyle et al. 2004). Only data from uninfected males is used for this study, however, notes on infection, females and infected individuals are included where relevant.

Habitat use

Microhabitat characteristics were recorded upon relocation of any tracked individual. Substrates were recorded in seven broad categories: as vegetation, leaf litter, log, rock, under rock, soil and submerged. The distance of individuals from particular substrates (rock, leaf litter) was recorded. The position of the frog was categorised as arboreal, terrestrial or aquatic. Additionally, the section of stream to which the individual was most closely associated (riffle, run, or pool) was recorded. Riffles were distinguished as sections of a stream with shallow, fast, broken water. Runs were defined as slower-moving, unbroken, deeper water, and pools were characterised by increased water depth and extremely slow-moving water.

Analysis

Only data collected from uninfected individuals was used for analysis. Nocturnal and diurnal behaviours were analysed separately. To avoid pseudoreplication and possibly biasing in our results by weighting more heavily frogs located more frequently, individuals were used as replicates and summary statistics were calculated for each individual. Only individuals that were observed three or more times were included in analyses. Data was analysed using separate MANOVAs comparing sets of variables related body temperature, movement, and microhabitat use across seasons (summer, autumn, winter, spring). Analyses were performed using Statistica 7 (StatSoft 2004).

Results

Tracking devices had minimal impact on frog behaviour (pers. obs., Sapsford et al. 2013). Females were observed much less often than males (0.01% of observations). Although amplexus was never observed, tadpoles and metamorphs were present, indicating a successfully reproducing population. *Batrachochytrium dendrobatidis* was present on both streams (Table 2.2), the prevalence and intensity of which fluctuated among seasons, peaking during winter and reaching the lowest levels in summer (Figure 2.3 and Figure 2.4).

Table 2.2 *Batrachochytrium dendrobatidis* infection in male *L. rheocola*

Season	Number swabbed	Number infected	Mean prevalence	Mean standard deviation (+/-)	95% LCL	95% UCL
Summer	85	2	0.035	7.484	0.002	0.082
Autumn	81	2	0.074	4.447	0.003	0.086
Winter	41	22	0.536	486.675	0.374	0.693
Spring	67	5	0.070	7.016	0.024	0.165

LCL = Lower Confidence Limits; UCL = Upper Confidence Limits

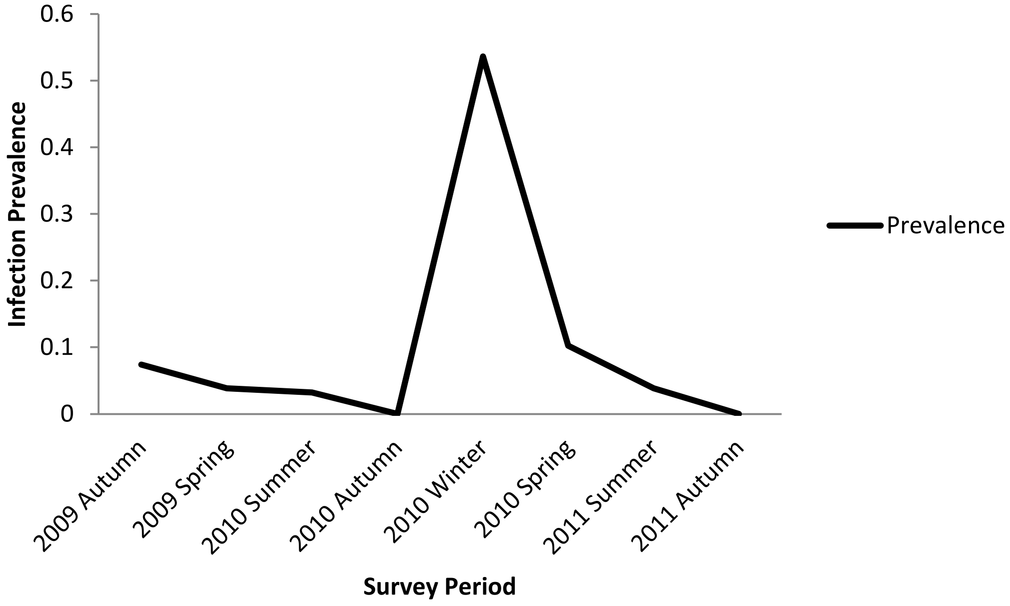


Figure 2.3 Seasonal prevalence of *Batrachochytrium dendrobatidis* in *L. rheocola* shown separately for each tracking period in my study

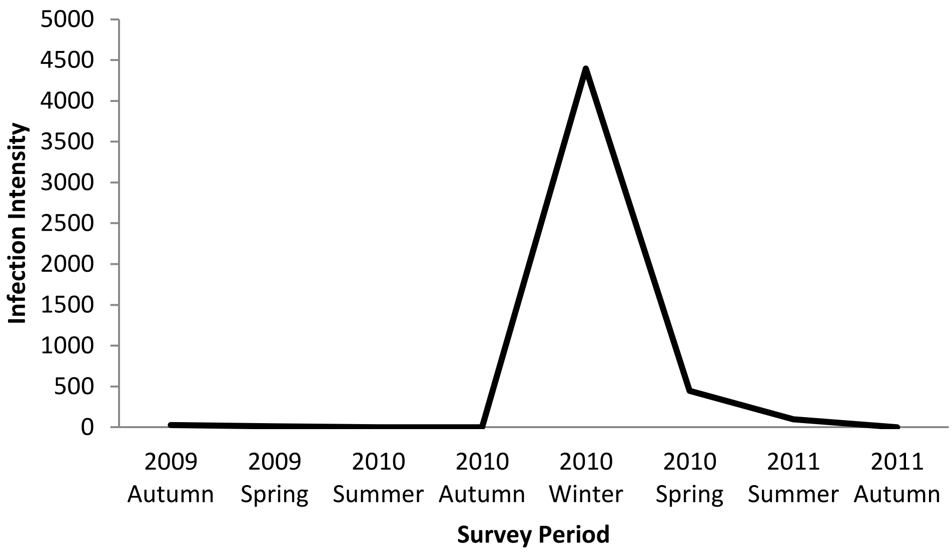


Figure 2.4 Seasonal intensity of *Batrachochytrium dendrobatidis* in *L. rheocola* shown separately for each tracking period in my study

Temperatures (*body and environmental*)

Environmental conditions fluctuated throughout the year; minimum ($F_{3,\ 24} = 22.632$, $P = 0.000$) and maximum temperatures ($F_{3,\ 24} = 31.89$, $P = 0.000$) were significantly different among seasons, except autumn and spring. The low temperatures coincided with dry winter months (June-August), whilst the higher temperatures and rainfall occurred during the summer months (December-February). Rainfall differed significantly among seasons ($F_{3,\ 24} = 6.447$, $P = 0.002$) (Figure 2.5).

Litoria rheocola experienced body temperatures over a range of at least 20.4°C (13.4°C - 33.8°C) throughout the year. The majority of body temperatures recorded were within the optimal growth range of *Bd* (15-25°C) (Stevenson et al. 2013). However, body temperatures differed significantly among seasons ($F_{3, 12} = 169.61$, $P < 0.000$); as individuals spent significantly more time with body temperatures below 15°C in winter ($P < 0.007$) and significantly more time with body temperatures above 26°C in summer ($P = 0.028$) (Table 2.3).

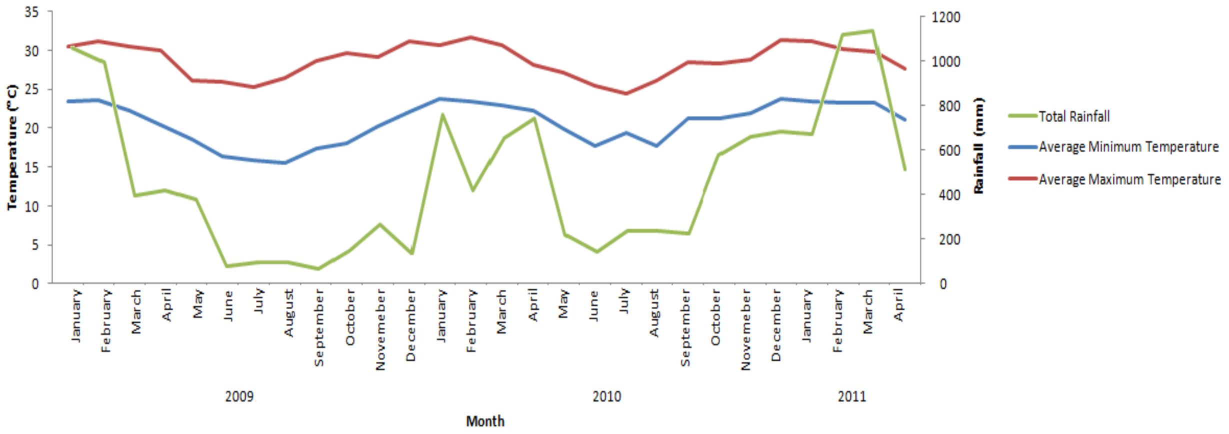


Figure 2.5 Environmental conditions during survey period recorded by BOM weather station at South Johnstone, 6 km north of Mena Creek.

Table 2.3 Proportion of individual *Litoria rheocola* found with body temperatures in temperature categories relevant to effects of temperature on the growth, reproduction and survival of *Bd*.

Season	<17	17.00-24.99	25.00-27.99	>28
Summer	0.000	0.467	0.337	0.194
Autumn	0.090	0.708	0.196	0.004
Winter	0.055	0.926	0.0183	0.000
Spring	0.008	0.887	0.0954	0.008

Behaviour

L. rheocola becomes active at dusk and begins foraging in exposed positions on rocks in the stream or on streamside vegetation. Activity levels and detection rates were highest in spring, and decreased in summer, to winter, and autumn. The proportion of males calling varied seasonally, peaking in summer (85%) followed by spring (75%), winter (62%), and autumn (55%).

Diurnally, *L. rheocola* typically assumed a water-conserving posture in a shady, sheltered retreat site. They were rarely observed active or calling diurnally (<1% of individuals). On the few occasions non-tracked individuals were observed diurnally (<1% of individuals), individuals were either basking or were moribund and showing signs of imminent death.

Movement

Movements were restricted to the stream environment; no individuals were recorded more than eight meters from the stream. *Litoria rheocola* typically displayed small displacement distances, the majority of movements were less than 4 m (82.5%). Large movements (over 8 m) were rare (2.4%), although individuals moved large distances occasionally (maximum = 38.20 m over five days). Individuals used a mean length of 3.72 m (± 3.03 m STDEV) of stream bank over 4-9 days; the largest length of stream bank used was 18 m.

Nocturnal movements did not differ significantly among seasons (MANOVA, $F_{3,\ 33} = 0.972$, $P = 0.513$), whereas diurnal movements were significantly different ($F_{3,\ 21} = 1.738$, $P = 0.021$) (Table 2.4). Also, median distance moved along the stream by individuals moved almost twice as far in winter than in autumn (Tukey-Kramer Post-hoc comparison, $P < 0.05$).

Table 2.4 Examples of mean distances moved by *Litoria rheocola* nocturnally and diurnally

Movement Variable	Autumn	Spring	Summer	Winter
Nocturnal - Median distance moved from stream (cm)	39.57	64.75	70.16	77.14
Nocturnal - Maximum distance moved from stream (cm)	94.81	101.00	99.72	135.33
Nocturnal - Median movement along stream (cm)	164.29	158.82	240.48	200.00
Nocturnal - Median vertical distance moved (cm)	77.45	78.96	86.67	92.11
Nocturnal - Median horizontal distance moved (cm)	140.88	210.87	228.62	223.89
Nocturnal - Median movement from nocturnal perch to diurnal retreat (cm)	242.88	340.00	276.73	262.31
Nocturnal - Maximum movement from nocturnal perch to diurnal retreat (cm)	347.27	389.80	388.21	386.15
Diurnal - Median distance moved from stream (cm)	64.64	49.75	44.27	47.73
Diurnal - Maximum distance moved from stream (cm)	87.10	82.17	98.38	58.00
Diurnal - Median movement along stream (cm)	1.61	2.63	2.66	1.25
Diurnal - Median vertical distance moved (cm)	129.92	119.26	83.75	96.36
Diurnal - Median horizontal distance moved (cm)	171.76	227.32	232.44	288.85
Diurnal - Median movement from diurnal retreat to nocturnal perch (cm)	173.79	288.16	252.14	254.17
Diurnal - Maximum movement from diurnal retreat to nocturnal perch (cm)	247.93	338.95	290.86	348.33

Microhabitat

Litoria rheocola preferred riffle zones (79% of observations) and arboreal habitats (>79% of observations) year-round. There were no seasonal differences among microhabitat variables used diurnally ($F_{18, 54} = 1.028$, $P = 0.460$) or nocturnally ($F_{13, 39} = 1.189$, $P = 0.253$), individuals used the dominant substrates in sheltered, exposed, wet, dry, terrestrial, arboreal, and aquatic microhabitats equally among seasons (Figure 2.6; Figure 2.7). Less than 7% of individuals used microhabitats associated with deeper water, suggesting that *L. rheocola* avoid pools. The majority of individuals remained within a single jump (~0.60 m) of particular habitat features; such as the stream (76.95%), rocks (85.16%), and leaf litter (88.44%).

Litoria rheocola used members of more than 31 plant families and seven abiotic substrates as either diurnal retreats or nocturnal perches (Table 2.5). Substrate types were often repeatedly used upon consecutive relocations (23.66% of observations); however, the exact position of an individual on a substrate differed upon relocation. Microhabitats used appeared to reflect the abundance of a substrate within the habitat.

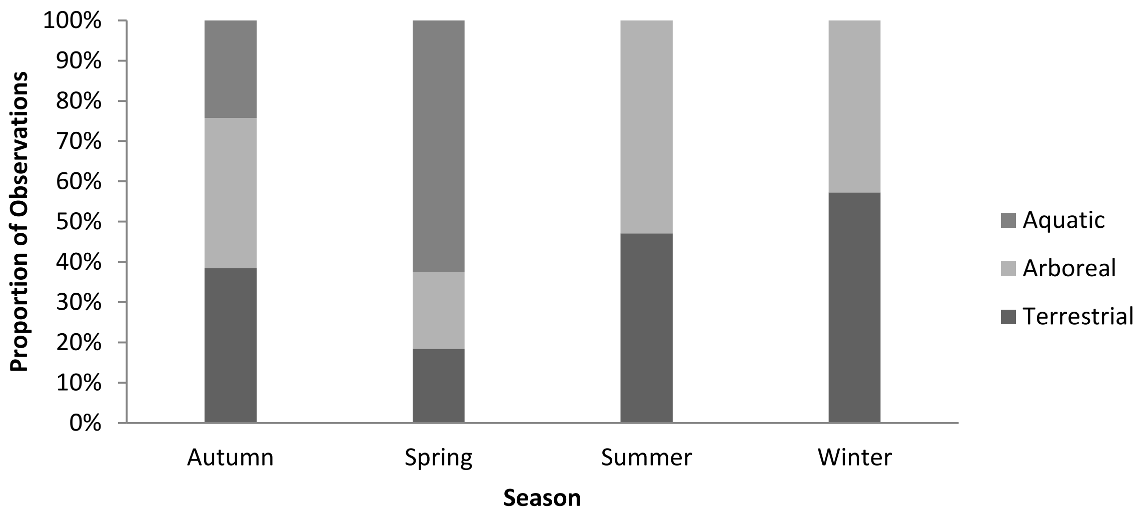


Figure 2.6 Seasonal variation in nocturnal perch sites used by *L. rheocola*

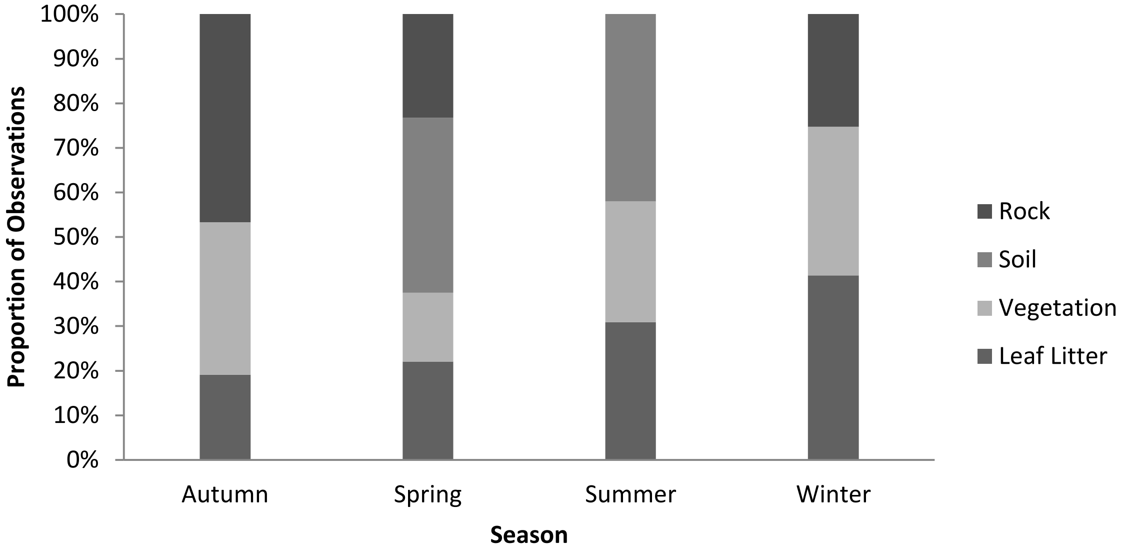


Figure 2.7 Season variation in nocturnal microhabitats selected by *L. rheocola*

Table 2.5 Proportion of most commonly use flora families by *L. rheocola*

Substrate	Diurnally	Substrate	Nocturnally
Arecaceae	0.148	Arecaceae	0.221
Pandanaceae	0.142	Pandanaceae	0.174
Cyatheaceae	0.056	Cyatheaceae	0.080
Sapindaceae	0.041	Philydraceae	0.070
Lauraceae	0.029	Sapindaceae	0.058
Elaeocarpaceae	0.026	Elaeocarpaceae	0.030

Other substrates used include Araliaceae, Amaranthaceae, Blechnaceae, Celastraceae, Euphorbiaceae, Meliaceae, Meliaceae, Monimiaceae, Moraceae, Myristicaceae, Myrtaceae, Phyllanthaceae, Poaceae, Proteaceae, Rosaceae, Rubiaceae, Rutaceae, Smilacaceae, Thymelaeaceae, Zingiberaceae.

Discussion

The behaviour of lowland male *L. rheocola* was superficially similar to that of other *Bd*-susceptible stream-dwelling hylids and whilst most behaviours were probably not detrimental to *Bd*, some behaviours might have been (Hodgkison 1998; Rowley and Alford 2007, Puschendorf 2009). At low elevations, uninfected male *L. rheocola* exhibited seasonal changes in behaviour that coincided with changes in environmental conditions, potentially influencing the likelihood of *Bd* infection and survival. Environmental temperatures at low elevation rise above those tolerable by *Bd* in summer, and are thought to be the dominant driving force in the survival of lowland amphibian populations (Berger et al. 2004). High environmental temperatures promote an increase in the proportion of body temperatures above 25°C (about 20% of the time in summer), and, apparently, a corresponding seasonal decline in *Bd* prevalence (Rowley and Alford 2013; Sapsford et al. 2013). However, during the cooler periods of the year *L. rheocola* do not usually achieve higher body temperatures, as environmental temperatures within lowland rainforest rarely exceed 25°C. When higher body temperatures (above 25°C) were not achieved (for example, in autumn and winter) *Bd* prevalence and intensity increased and could pose a significant threat to lowland amphibian populations (Berger et al. 2004; Piotrowski et al. 2004; Retallick et al.2004; Woodhams and Alford 2005; Puschendorf 2009). Body temperatures were highest in individuals that basked diurnally, a behaviour that negatively influences *Bd* and occurs in many *Bd*-susceptible amphibians, e.g., the spotted treefrog (*Litoria spenceri*), southern mountain yellow-legged frog (*Rana muscosa*) and western toad (*Bufo boreas*) (Lillywhite et al. 1973; Carey 1978; Brattstrom 1979; Bradford 1984; Gillespie and Hollis 1996; Navas 1996; Blaustein and Bancroft 2007; Richards-Zawacki 2009). In the lowland *L. rheocola* I studied, there was evidence of the influence of cool winter temperatures on disease because in winter I found dead and dying individuals (Berger et al. 2004).

Nocturnal movements did not vary seasonally, whereas diurnal movements did. Diurnal movements were largest in summer, particularly distances moved from the stream, perch height, and movements from nocturnal perches to diurnal retreat sites. The shortest movements occurred during winter when temperatures where significantly lower and less suited to amphibian movement. The lack of seasonal variation in nocturnal movement may have been due to the abundance of nocturnal perches available and site fidelity displayed by males. Diurnal movements did vary seasonally, which may have occurred because frogs have specific requirements for diurnal retreats that allow for maintenance of hydrobalance, and avoidance of temperature extremes.

Male *Litoria rheocola* moved very short distances from the stream compared to other stream-dwelling hylids (see Gillespie and Hollis 1996; Hodgkison 1998; Hodgkison and Hero 2001; 2002; Rowley and Alford 2007a; Puschendorf 2009). By remaining close to the stream, individuals may be increasing their probability of infection (see Chapter 3).

I detected no seasonal variation in habitat use, and I did not quantify whether habitats were used in relation to their availability. Use of particular microhabitats may influence infection probability; through regulation of body temperature and avoidance of wet substrates, individuals can influence the probability of acquiring, maintaining and loosing infection (Trenham 2001; Doan 2004; Gillespie et al. 2004; Roznik 2013). Given that I found no significant variation in habitat use among seasons, it is unlikely that microhabitat selection acts as a seasonal disease resistance mechanism (Lemckert and Brassil 2000; Watson et al. 2003) in *L. rheocola*.

Markedly different patterns of behaviour between males and females are common in stream-associated amphibians (Hodgkison 1998; Hodgkison and Hero 2002; Goldingay and Newell 2005). I detected few females in this study, suggesting a major sex bias in the behaviour, habitat use and potentially thermal temperature profiles between sexes. The implications of differences between male and female behaviour in the maintenance of *Bd* in lowland amphibian populations is unknown, but is potentially significant. Females were perched at a greater distance from the stream than males, which also occurred in *L. nannotis* and *L. lesueuri* (complex) (Rowley and Alford 2007; pers. obs.) and may cause a sexual bias in infection prevalence. All females detected in close proximity to the stream (<1 m) in my study were gravid, suggesting females may only visit the stream to reproduce, minimising contact with conspecifics and potentially *Bd*.

Conclusion

The behaviour of lowland populations of *Litoria rheocola* varies seasonally. Microhabitat use and nocturnal movement did not vary seasonally, and are unlikely to seasonally influence *Bd* prevalence in lowland populations of *L. rheocola*. However, diurnal movements and body temperatures did vary seasonally, and may positively influence the probability of survival of *L. rheocola* by influencing contact with *Bd* and increasing temperatures above those tolerable by *Bd*. My study cannot reveal whether there have been behavioural changes in response to *Bd* infection or selection by *Bd* on this population, but behaviours beneficial to host survival and detrimental to *Bd* should be considered significant disease resistance mechanisms and considered in examinations of the amphibian-chytrid relationship. As *Bd* continues to threaten amphibians worldwide, determining how a species persists in the presence of the pathogen may provide insights as to how to best conserve other susceptible species, both locally and internationally.

CHAPTER 3: FROGS INFECTED BY THE AMPHIBIAN CHYTRID FUNGUS (*BATRACHOCHYTRIUM DENDROBATIDIS*) BEHAVE DIFFERENTLY FROM UNINFECTED FROGS

Abstract

Behavioral changes in individuals infected by pathogens can have important epidemiological implications, strongly influencing the spread and dynamics of disease. Here I examined behavioral differences between frogs (*Litoria rheocola*) that were either uninfected or infected by the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*). I tracked these frogs using harmonic radar during winter, when they are most at risk of infection. Infected frogs displayed difference in movement patterns and habitat use to uninfected frogs but no relationship was found between infection status and environmental conditions in diurnal retreat sites. Individuals that used perch and retreat sites closer to the steam, and spent more time in arboreal vegetation diurnally were most likely to be infected. Therefore, individual behaviour may not only influence individual infection and survival, but that of entire amphibian populations.

Introduction

Infectious diseases are an important factor regulating wildlife populations. Diseases may cause changes in host physiology and behaviour, which can lead to altered movement patterns, microhabitat use, foraging efficiency, reproductive output, and predator avoidance (Mcnair and Timmons 1977; Cheeseman et al. 1981; Curtis 1987; Oi and Pereira 1993; Ramsey and Cowan 2003; Farnsworth et al. 2006; Jansen et al. 2007). Changes in host behaviour due to infection are an influential factor affecting pathogen spread among individuals, subpopulations and metapopulations (Samson et al. 1988, cited in Hajek and St. Leger 1994, p. 301; Pie et al. 2004).

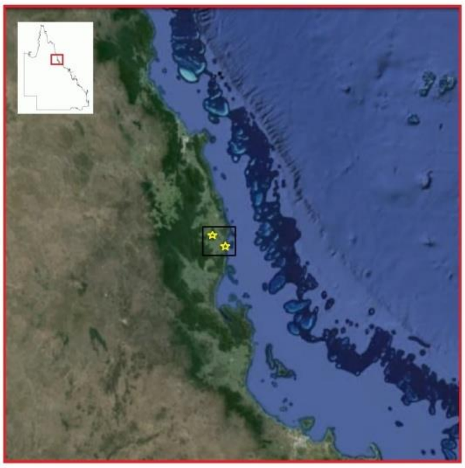
Amphibians, the world's most threatened vertebrate class, are a group in which infectious disease is playing a significant role in species decline (Laurance et al. 1996; Berger et al. 1998). The proximal cause of many declines and extinctions is chytridiomycosis, a disease caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) (Berger et al. 1998; Rachowicz et al. 2006). Infection by *Bd* does not always cause the death of a host; survival is possible, even in highly susceptible species (e.g., the armoured mistfrog, *Litoria lorica*) (Puschendorf 2009).

Environmental conditions, host susceptibility, and infection intensity influence the survival of amphibians infected by *Bd*, and have been studied under both laboratory and natural conditions. On the other hand, behavioural differences between amphibians infected and uninfected by *Bd* have rarely been investigated in the field (Williams and Hero 1998; Puschendorf 2009; Stevenson et al. 2013). Potentially, behavioural differences between infected and uninfected frogs, may differ in many ways, for example in terms of microhabitat selection, inter- and intra-specific contact rates, movement patterns, temperature regulation, and activity patterns, any of which have the potential to influence host survival. Here I aim to compare the behaviours of uninfected common mistfrogs (*Litoria rheocola*) with those infected with *Bd*. Specifically; I examine movement, microhabitat use and body temperatures of *L. rheocola*.

Methods

Study sites

This study was conducted between 26 July and 04 August 2010, during the coolest months of the year, which is the period during which *Litoria rheocola* are most susceptible to *Bd* infection, and mortality (Sapsford et al. 2013). Five sites at three streams were surveyed. The sites were in isolated pockets of tropical lowland rainforest (complex mesophyll vine forest) in the Wet Tropics bioregion of North Queensland, Australia, near the towns of Tully and Mission Beach. Two of the three rainforest streams flowed into Mena Creek; Mena 1 (17°39’03.01”S, 145°59’23.94”E), and Mena 2 (17°39’11.97’S, 145°59’06.96”E). The third stream, Stoney Creek (17° 55' 17.9" S, 146° 04' 07.2" E), flowed from Lacey Creek, 8 km south of Mission Beach (Figure 3.1). Each stream flowed between 0-90 m above sea level (ASL) and did not connect to any mountainous streams (> 300 m elevation). As the study sites were not contiguous with higher elevation areas, and not connected by shared watercourses, the dynamics of the host-pathogen system at lower elevations were not influenced by pathogen dynamics at higher elevations. Each site was defined by a 400-m transect that was established along each stream. Anthropogenic disturbance in the area consisted of access roads for 132kV/275kV transmission lines (Mena Creek) and the Tully-Mission Beach Road. Streams were narrow (1 – 6 m wide), shallow (0.05 – 1.40 m deep) and typically lit with dappled sunlight. However, the width and depth of the streams varied considerably with rainfall. All three streams had permanent water flow through riffles, runs and pools, with rocks of various grain sizes from pebbles to boulders, with a sandy streambed. The dominant understory flora was diverse, and included plants in the family Arecaceae, Pandanaceae, Cyatheaceae, and Philydraceae.



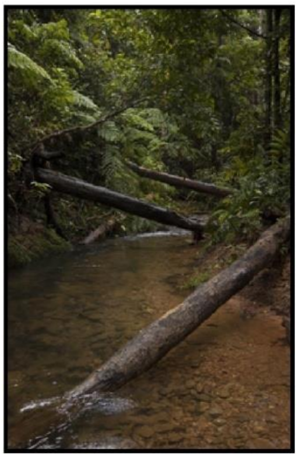


Figure 3.1 Location of Mena Creek and Mission Beach study sites indicated by yellow star, with habitat of Mena Creek adjacent

Study species

Litoria rheocola is a small (males: 23 – 39mm, females: 27-39) pale brown to brown, stream-dwelling frog endemic to the Wet Tropics (Williams and Hero 1998, 2001; Hoskins and Hero 2008) (Figure 3.2). An obligate stream breeder, male *L. rheocola* are common within streams year-round, whereas females and juveniles are observed less often (McDonald and Alford 1999; pers. obs.). Gravid females and calling males have been recorded year-round from streamside vegetation and rocks, suggesting breeding occurs in most months (Hodgkison and Hero 2001). *Litoria rheocola* is listed as Endangered after suffering significant declines during 1989-1994 from chytridiomycosis (Richards et al. 1993, Northern Queensland Threatened Frogs Recovery Team 2001; IUCN 2011).

Harmonic diodes

I used harmonic direction-finding diodes to track frogs. These tracking devices were constructed using a surface-mounted diode (SOT-323 Surface-mount zero bias Schottky detector diode) with a 13 cm, 0.5 mm leader wire as an antenna, glued to the diode with a conductive silver epoxy (Gourret et al. 2011). The end of each antenna was given a silicone-coated colour code to allow visual identification of individuals. Diodes were coated in silicone and attached to 1-mm diameter silicone tubing, through the middle of which cotton thread was inserted, and tied around the frog's inguinal region (Figure 3.2). The cotton thread was intended to decay, which would allow eventual detachment of the tracking device if the tracked individual was not recovered. The silicone tubing and cotton thread were attached so that the device would not slip off, and then cut to the size of the frog's inguinal region. The total mass of the tracking device (approximately 0.15 g) never exceeded 8% of the frog's total mass, restricting tracking to adults over 2 g. Hence, the movement of juveniles, sub-adults and small adults could not be investigated (Lemckert and Brassil 2000).

Figure 3.2 Common mistfrog (*Litoria rheocola*) (Left), and common mistfrog (*Litoria rheocola*) with tracking device (Right).

Capture and processing

Typically, frogs were captured by inverting a clip-lock bag over my hand and placing it directly over the frog. A new clip-lock bag was used for each individual, to prevent disease transmission between frogs and cross-contamination of samples, using the protocols recommended by Mendez et al. (2008) and Phillott et al. (2010). Once captured, snout-urostyle length (SUL) was measured to the nearest 0.1 mm using plastic Vernier callipers, and mass was recorded with a 10 g Pesola^{TM} spring balance prior to the attachment of a tracking device. Only male frogs over 2 g in mass were tracked, to conform to ethical mass requirements for tracking devices.

Batrachochytrium dendrobatidis assessment

To determine *Bd* infection status and intensity, frogs were swabbed using sterile cotton medical swabs (Tubed Dry Swabs, Medical Wire and Equipment, Corsham, Wiltshire U.K.). This detection method has the highest sensitivity (Hyatt et al. 2007). Frogs were swabbed on the front feet, hind feet, inner thighs, the ventral surface and pelvic patches. These surfaces were swabbed because they are most consistently infected by *Bd* (Berger et al. 1998; 1999; Williams et al. 2002; Woodhams and Alford 2005). Swabs were frozen and sent to the Center for Integrated Biotechnology at Washington State University, Pullman, WA, USA. Each swab was tested in a triplicate using real-time quantitative PCR (qPCR) assays for the presence of *Bd* zoospores (Boyle et al. 2004). Once processing and attachment of tracking devices was complete, all adult male *L. rheocola* were released at their points of capture.

Tracking

Frogs were tracked using a harmonic direction finder (RECCO R5 transmitter-receiver Recco Rescue Systems, Lidingö, Sweden). The hand-held direction finder acted as both a transmitter and receiver, creating a sound when it received a reflected signal from a diode (Rowley and Alford 2007). To locate frogs, I slowly walked transects, rotating the receiver in all directions. Once a signal was detected, the direction finder could be focused by decreasing the gain and homing in on the tracking device. The receiver's ability to detect tracking devices declines with distance, and signal strength is reduced by vegetation that absorbs the radio frequencies transmitted (Sizun 2005). Individuals were relocated daily, once at night (18:00-04:00) and once in the day (08:00-17:00). Upon relocation of a frog, I recorded ecological data including, the frog's position in the environment, behaviour, microhabitat and body temperature, as well as movement data. Data recorded on relocated tracked frogs did not involve handling the frogs, and was conducted with minimal disturbance.

Movement

Upon relocation of a tracked frog, flagging tape was used to mark the exact location of each individual for accurate measurements of consecutive relocations. Distances moved were recorded as direct linear measurements between successive locations using a 50 m Komelon^{TM} fibreglass tape measure; so minimum distances were recorded; actual distances moved would be the same or greater (Secor 1994; Webb and Shine 1997; Doak 2005). Movements recorded included distances moved perpendicular to the stream, parallel to the stream, changes in perch height, horizontal distances moved, and distances from rocks, all of which were measured between consecutive locations. Only when an individual was repeatedly located between successive tracking sessions (i.e., day-night and night-day) was movement data recorded. Movements were only recorded when an individual had moved from their location during the previous survey. Nocturnal movements were the movement from a nocturnal perches to a diurnal retreat site, whilst diurnal movements were movement from a diurnal retreat to a nocturnal perch.

Environmental conditions

Environmental conditions including air and water temperatures were recorded to the nearest 0.5°C using Livingstone™ mercury glass thermometers; humidity was recorded with a Brannan™ whirling hygrometer. These variables were assessed diurnally and nocturnally, at the start (0 m mark) of each transect, prior to each tracking event.

To estimate temperature and desiccation rates experienced by *L. rheocola*, I used 25 Plastidip™-coated Thermochron iButton™ data loggers that were embedded into 25 'permeable' frog models, following the methods of Rowley and Alford (2010). These models were made of 3% agar, were permeable to water loss, and accurately estimate the body temperatures of *L. rheocola* (Roznik 2013; Roznik and Alford 2014). Models were positioned in all diurnal microhabitats in which infected and uninfected *L. rheocola* were observed. Models were left for 48 hours and weighed at 24-hour intervals, allowing me to determine the influence, if any, of differences in temperatures and water loss experienced by infected and uninfected *L. rheocola*.

Habitat use

Microhabitat characteristics were recorded upon relocation of any tracked individual. Substrates were recorded in seven broad categories: vegetation, leaf litter, log, rock, under rock, soil and submerged. The distance of individuals from particular substrates (rock, leaf litter) was recorded. The position of the frog was categorised as arboreal, terrestrial or aquatic. Additionally, the section of stream to which the individual was most closely associated (riffle, run, or pool) was recorded. Riffles were distinguished as sections of a stream with shallow, fast, broken water. Runs were defined as slower-moving, unbroken, deeper water, whilst pools were characterised by increased water depth and extremely slow-moving water.

Analysis

I examined the relationship between individual infection status and behaviour, which I separated into three distinct categories: microhabitat use, movement, and selection of temperature and desiccation regimes. For each category of behaviour, I initially prepared a set of candidate generalised linear mixed-effect models, based on a combination of variables relevant to each category. Infection status was coded as a binomial response variable, so I used models with a binomial family and a logit link function. Site was included as a random effect in each model to control for any effects specific to particular sites. To avoid overfitting models, I did not include interactions between variables. For each of the three behavioural categories, my set of candidate models included all possible models with combinations of up to four variables. Variables were selected based on data availability and suitability for comparisons, (i.e., they were not highly correlated with each other) in each category. Median and maximum values for each individual for each variable were included in the models. Individuals that were not observed at least three times were removed from the dataset. For each model, I calculated Akaike's Information Criterion with an adjustment for finite sample size (AICc), and used these values to determine the strength of evidence for each model relative to the candidate set of models, using the criteria of Burnham and Anderson (2002). If more than one model was deemed to best support the model (ΔAICc <2) then these models were averaged to obtain a final model. All statistical analyses were performed in program R, version 2.15.2 (R Core Team 2012) using the lme4 (Bates et al. 2012) and MuMIn (Barton 2013) packages.

The following groups of fixed effects were included in the initial model sets:

Microhabitat: proportion of observations on wet substrates (during the day), proportion of observations on wet substrates (at night), proportion of time in terrestrial microhabitats (at night), proportion of time in arboreal vegetation (during the day), and proportion of time in leaf litter (during the day).

Movement: Maximum distances moved along the stream (during the day), median distances moved along the stream (during the day), median distances moved along the stream (at night), maximum distance moved perpendicular to the stream (at night), maximum horizontal distance (during the day), median perch height (at night), median distance moved away from stream (during the day), and median distance to rock (at night).

Temperature and desiccation: Proportion of temperatures below 20°C, proportion of temperatures above 25°C, mean desiccation rate after 24 hours, and mean desiccation rate after 48 hours. The temperature thresholds selected coincide with temperatures categories at which *Bd* in North Queensland grows fastest (15-25°C) and those at which it grows slowest (>25°C) (Stevenson et al. 2013), however, due to the low number of temperatures below 15°C at my study site, I used values below 20°C.

Results

I tracked a total of 41 adult male *L. rheocola* for up to 10 days. Twenty-two individuals were infected, and 19 individuals were uninfected; infection prevalence was 54%. Infection intensity was low; although the average zoospore count was high (4396 zoospores per infected individual), only four of the 19 infected individuals had individual mean zoospore counts above 202 zoospores (range 1-65202 zoospores). At my study sites in winter, I found that the probability of infection was related to the microhabitat use (Table 3.1) and movements of *L. rheocola* in winter (Figure 3.3, and Figure 3.5), but not the choice of temperature and desiccation regimes (Table 3.4).

Microhabitat

Thirty-two microhabitat models were created within the AICc table. The models with a weight of approximately 5% and above are shown in Table 3.1. Three microhabitat models were strongly supported by my data with ΔAICc < 2 (maximum Nagelkerke R^2 = 81.5%). These models were averaged to create a final model, which was significantly different from a null model containing the intercept and random effect of site (χ^2 = 19.392, df = 2, P < 0.001). This final model suggested that the more time individuals spent in arboreal vegetation during the day, the more likely they were to be infected, and that this effect was modified by spending time in leaf litter, such that spending time in leaf litter during the day decreased the probability of infection (Table 3.1 and Figure 3.3). The final averaged model was significantly different from a null model, which contained only the intercept and random effect of site (χ^2 = 19.392, df = 2, P < 0.001).

Table 3.1 Generalised linear mixed-effects models of microhabitat variables.

Candidate Models			
Model effects	AICc	ΔAICc	Weight
Proportion of day in vegetation	24.25755	0	0.302898
Proportion of day in vegetation + proportion of day in leaf litter	25.7118	1.454281	0.146387
Proportion of day in leaf litter	25.94702	1.689471	0.130146
Proportion of day in vegetation + Proportion of day on wet substrates	27.61985	3.362300	0.056387
Proportion of day in vegetation + proportion of night on wet substrates	27.79992	3.542363	0.051532
Proportion of day in vegetation + Proportion of night in terrestrial microhabitats	27.8423	3.58474	0.050452
Proportion of day in leaf litter + Proportion of night on wet substrates	27.88040	3.622847	0.049500

Final averaged model	
Model effect	**Estimate**
Intercept	-4.886
Proportion of day in leaf litter	-4.058
Proportion of day in vegetation	10.675

Highlighted rows represent those models that are best supported by the data (ΔAICc < 2).

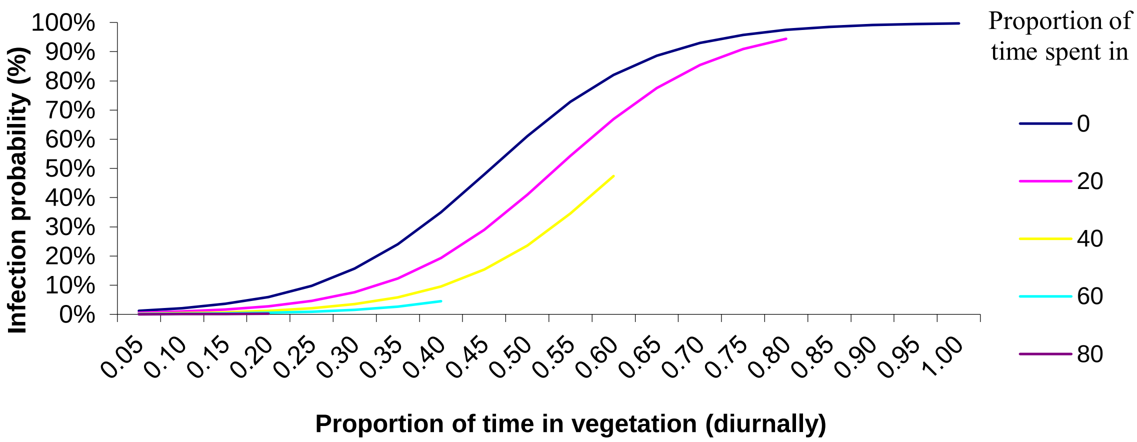


Figure 3.3 Probability of infection as a function of the time spent in arboreal vegetation, and time spent in leaf litter, based on the results of the final averaged microhabitat generalised linear mixed-effects models.

Movement

For movement parameters, fifty-seven models were created. Very few, however, accounted for a substantial proportion of the Akaike weight in the model set. The models with an Akaike weight of approximately 4% and above are shown in. Only one model had a ΔAICc < 3; this model also had by far the greatest weight (44.3%, R^2 = 96.6%). This model indicated that as individuals moved perpendicular to the steam both during the night and the day, their probability of infection decreased (Table 3.2 and Figure 3.5. The model containing these two fixed effects was significantly different from a null model containing only the intercept and random effects of sites (χ^2 = 41.332, df = 2, P < 0.001). The majority of movements recorded were, on average, larger in uninfected frogs than infected frogs; however, infected frogs moved further than uninfected frogs in approximately one third of recorded movement variables. Movements in which infected frogs moved further than uninfected frogs are shown in (Figure 3.4).

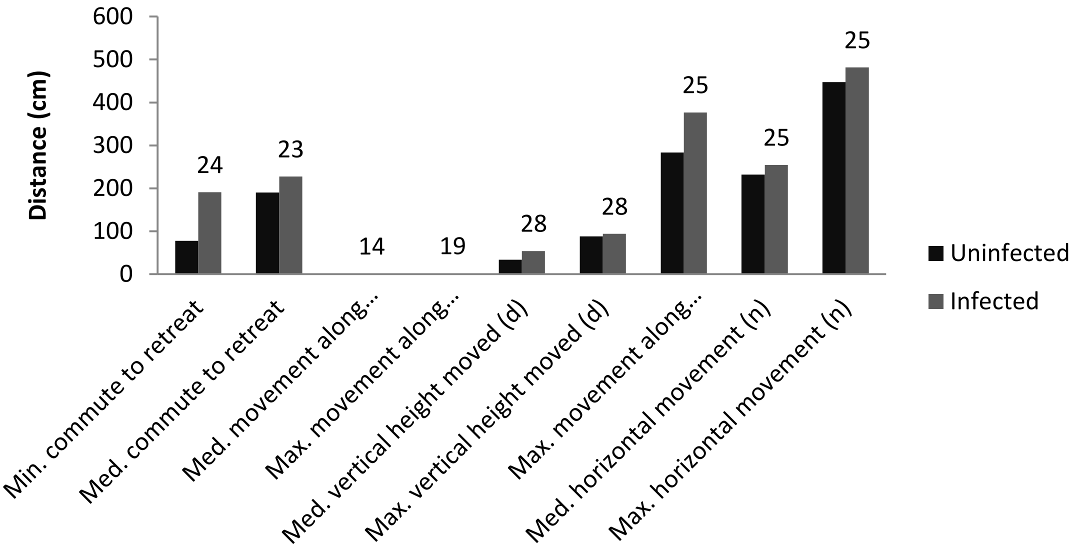


Figure 3.4 Variables examined where infected frogs averaged larger movements than uninfected frogs. Min = Minimum, Med = Median, Max = Maximum, (d) = diurnal, (n) = nocturnal. Labelled values represent sample size (n).

Table 3.2 Generalised linear mixed-effects models of movement variables

Model effects	AICc	ΔAICc	Weight	R^2
Median distance moved perpendicular to stream (diurnally) + Maximum distance moved perpendicular to stream (nocturnally)	26.46435	0	0.443284	0.966316
Median distance moved perpendicular to stream (diurnally) + Median perch height (nocturnally)	30.08258	3.618233	0.072609	0.929633
Median distance moved perpendicular to stream (diurnally) + Maximum horizontal distance (diurnally) + Maximum distance moved perpendicular to stream (nocturnally)	30.08570	3.62135	0.072496	0.971500
Median distance moved perpendicular to stream (diurnally) + Maximum distance moved perpendicular to stream (nocturnally) + Median perch height (nocturnally)	30.51866	4.054312	0.058384	0.969991
Median distance moved perpendicular to stream (diurnally) + Maximum distance moved perpendicular to stream (nocturnally) + Median distance to rock (nocturnally)	31.21521	4.750857	0.041214	0.967462

Highlighted rows represent those models that are best supported by the data (ΔAICc < 2).

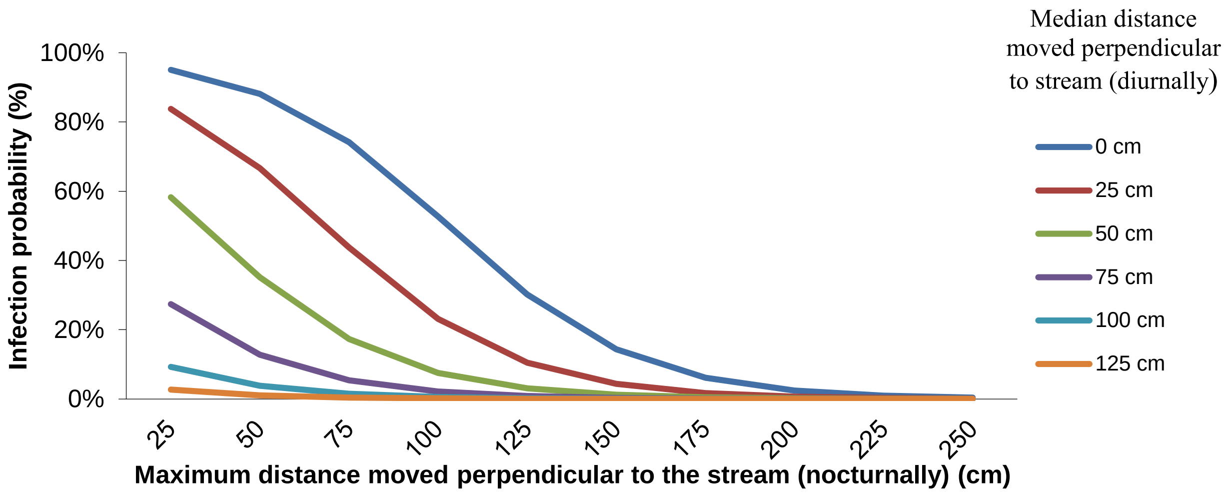


Figure 3.5 Probability of infection as a function of the maximum and median vertical distances moved, based on the generalised linear mixed-effects model best supported by my data

Microenvironment use

Ambient temperatures during the study period remained low and stable (Table 3.3). Environmental temperatures were mostly within the temperature range considered optimal for *Bd* growth and reproduction (15-25°C; Stevenson et al. 2013), and temperatures remained too low to be detrimental to the reproduction and survival of *B. dendrobatidis* (< 28°C; Stevenson et al. 2013). Body temperatures estimated through models showed that uninfected frogs selected microhabitats that did not get as cool as those used by infected individuals (Figure 3.6).

Table 3.3 Environmental conditions experienced by *Litoria rheocola* during winter

Temperature	Minimum (°C)	Average (°C)	Maximum (°C)
Air	14.00	21.10	26.50
Water	19.00	21.67	22.50
Substrate	14.00	21.22	22.70
Retreat Sites	14.00	21.70	26.00
Activity Sites	15.00	19.50	26.50
Uninfected Body Temperature	14.90	20.86	27.06
Infected Body Temperature	14.10	20.55	24.28

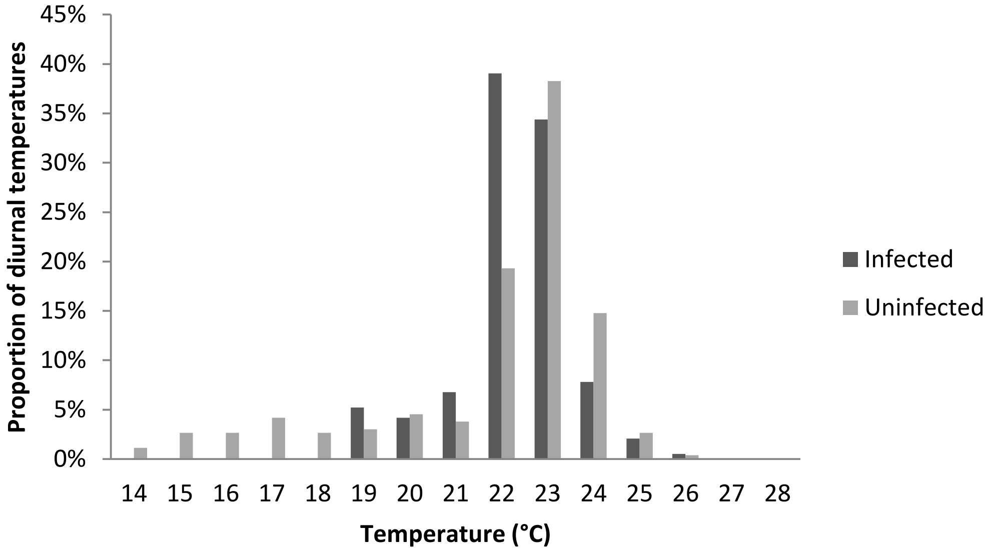


Figure 3.6 Diurnal body temperatures (08:00-17:00 hr) of infected and uninfected *Litoria rheocola,* as estimated by combining temperatures of all physical models placed in the diurnal microhabitats used by frogs.

For temperature and moisture microenvironment selection, sixteen models were created; models with a weight of approximately 3% and above are shown in Table 3.4. There was no evidence that infection status was related to the choice of microhabitat temperatures or desiccation rates of frogs. The intercept-only (null) model had the lowest ΔAICc value, (Table 3.4). Therefore, infection status did not strongly influence choice of temperature and desiccation conditions ($\chi^2 = 6.3174$, df = 4, $P < 0.1767$), so I did not examine any further models.

Table 3.4 Generalised linear mixed-effects models for temperature and desiccation

Model effects	AICc	ΔAICc	Weight	R^2
Null model	21.24829	0	0.453274	0
Proportion of temperatures below 20°C	23.21283	1.964543	0.169733	0.153907
Relative water loss in 24 hour period	24.40043	3.152142	0.093731	0.033714
Relative water loss in 48 hour period	24.51666	3.26837	0.088439	0.02135
Proportion of temperatures greater than 25°C	24.69474	3.446455	0.080904	0.002191
Proportion of temperatures below 20°C + Relative water loss in 48 hour period	26.65353	5.405244	0.030383	0.237288

Highlighted row represents models, where ΔAICc < 2

Discussion

I found that more than half of the *L. rheocola* I assessed in the Wet Tropics lowlands in winter were infected by *Bd*, and that there was a relationship between individual infection status and behaviour. Individuals that used perch and retreat sites closer to the steam, and spent more time in arboreal vegetation diurnally were more likely to be infected. I found no relationship between infection status and environmental conditions in diurnal retreat sites; however, other studies have shown that thermal and hydric conditions can be related to *Bd* infection status and intensity (Sapsford et al. 2013; Rowley and Alford 2013; Roznik 2013; Chapter 2).

Frogs that spent more time above the ground had higher probabilities of infection, compared to those that spent more time in leaf litter. This may have occurred because infected frogs preferred arboreal microhabitats, or because arboreal retreat sites maintained more suitable conditions for the survival of *Bd*. Using dry leaf litter may decrease *Bd* infection risk, because *Bd* growth and survival are reduced in dry environments (Johnson et al. 2013). My findings differ from those of Roznik (2013), who observed that infected *L. rheocola* and the green-eyed treefrog (*L. serrata*), were more likely to use rocks and decaying wood than uninfected frogs, and less likely to use vegetation. However, in Roznik's (2013) study, rocks and decaying wood were typically more humid than vegetation at those sites. Thus, in both studies, infected frogs used more humid sites more often than uninfected frogs.

Movement patterns of infected and uninfected individuals were broadly similar. However, uninfected individuals moved further perpendicular to the stream, whilst infected individuals showed limited movement perpendicular to the stream, as was also reported by Roznik (2013). Infected individuals may remain close to streams because of the impact of their infections, for example because they have lower energy levels, or electrolyte imbalances. Remaining in close proximity to the stream minimizes the diversity of microhabitats and variation in environmental conditions experienced by an individual. Moving away from the stream changes the availability and types of microhabitat and environmental conditions to which individuals are subjected. Access to drier conditions away from the stream may cause variation in the probability of infection. Uninfected individuals typically made larger movements than infected individuals, potentially because they were healthier; however, infected individuals made larger movements in approximately one third of all recorded movement variables. In particular, infected individuals moved further to reach retreat sites, perhaps suggesting they were seeking locations conducive to a reduction in infection. Alternatively, infected individuals may have had to forage across a broader area to increase energy intake to supply immune functions (Weber and Stilianakis 2007), which may have taken them further from nocturnal perches. Allocation of time to different tasks by infected individuals, for example moving along the stream versus perpendicular to the stream or foraging versus calling (Green 1990), may produce differences in individual behaviour similar to those I observed.

The restricted temperature range to which individuals were subjected during winter in my study (14-26.5°C) limited the potential for individuals to increase their body temperatures above 25°C. Temperature above 25°C can be fatal to *Bd* (Stevenson et al. 2013). Many studies have documented the importance of temperature in the survival of *Bd*-infected amphibians, and the benefits of increased body temperatures that can occur through utilisation of warmer microclimates (Richards-Zawacki 2009; Rowley and Alford 2013, Roznik 2013). However, in these studies individuals could increase their body temperatures above 25°C, whilst during my study individuals virtually could not. As temperatures were not available within the higher range, individuals must have relied on factors other than temperature such as innate immune defences, desiccation (Roznik 2013) or behaviour to influence survival during winter, factors rarely considered in studies of *Bd* infection.

It is unclear whether the differences in behaviours of *Bd*-infected and uninfected individuals are caused by infected individuals trying to recover from infections, uninfected individuals trying to avoid infection, or individuals exhibiting behaviours irrespective of *Bd*, which predisposes them to infection or recovery, or behavioural changes directly caused by the pathogen (Moore 2002). The short sampling period and low infection intensities in infected individuals in our study may have caused us to overlook some subtle behavioural differences between infected and uninfected individuals, and a greater range of behavioural differences may be detectable during warmer environmental conditions in other seasons (Richards-Zawacki 2009; Rowley and Alford 2013). I was, however, able to detect a range of differences in the behaviour of infected and uninfected individuals, which were correlated with their probability of infection.

Conclusion

Temperature is considered a major driving force influencing *Bd* infection probability in amphibians; however, other factors including movements and microhabitat use may also be related to infection probability. When temperatures do not exceed 25°C (e.g., during winter at my study site), and are not high enough to be detrimental to *Bd*, amphibian hosts could potentially behaviourally influence their probability of infection. As individual behaviours influence infection probability, it, in turn, influences population infection levels, and, potentially population persistence. To date, the behavioural differences between *Bd*-infected and uninfected amphibians have been underrepresented in research on *Bd*-susceptible amphibians. The range of behavioural differences between *Bd*-infected and uninfected individuals is likely to vary between species, sexes and possibly age classes, as is the significance of the different behaviours. Understanding the significance of these behavioural differences in the survival of *Bd*-susceptible amphibians requires further research.

CHAPTER 4: POTENTIAL RESERVOIRS AND VECTORS OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN QUEENSLAND RAINFOREST STREAMS

Abstract

The presence of *Batrachochytrium dendrobatidis* (*Bd*) on a non-amphibian host challenges our current understanding of the epidemiology of this disease, not only altering what is known of the pathogen's ability to survive, reproduce and potentially disperse, but also pathogen transmission between hosts. An extremely broad range of amphibians are potentially capable of sustaining *Bd*, but a potential diversity of non-amphibian hosts having been overlooked due to the presumed specificity of *Bd* on amphibians. *Batrachochytrium dendrobatidis* infects keratinised tissue in all amphibian life history stages, suggesting that any fauna with external keratin are potentially capable of sustaining *Bd*. I assessed a variety of potential non-amphibian hosts for the presence of *Bd* in the field in lowland rainforest of the Wet Tropics. Eight families of stream-associated invertebrates and two new frog species tested positive to *Bd*, whereas no reptiles tested positive. Survival and reproduction of *Bd* on stream-associated invertebrates could potentially influence amphibian declines, and aid in the large scale movements of the pathogen.

Introduction

Pathogens that cause extinction of susceptible hosts require a reservoir host to facilitate the pathogen's survival (deCastro and Bolker 2005; Lips et al. 2006). Reservoir hosts enable the maintenance of pathogen populations when susceptible hosts are in low densities or not present (Haydon et al. 2002; Davidson et al. 2003; Blaustein et al. 2005) and can facilitate spread (Cullen and Owens 2002; Haydon et al. 2002; Davidson et al. 2003). The dynamics of host-pathogen systems with multiple hosts are not always clear, yet reservoir hosts are a main factor contributing to the extinction of susceptible species, reinforcing the need to determine reservoir hosts and their influence host population dynamics.

Chytridiomycosis caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) has been implicated in the decline and extinctions of amphibians worldwide, (Berger et al. 1998; Stuart et al. 2004; Collins and Crump 2009). Until recently *Bd* was thought to occur exclusively on amphibians (Piotrowski et al. 2004) using the keratin in amphibian skin for nutrients, however, the exclusivity of *Bd* has recently been refuted (Voyles et al. 2011; Kilburn et al. 2011; McMahon et al. 2013). *Batrachochytrium dendrobatidis* has spread globally infecting a diversity of amphibians in a range of habitats including remote pristine areas, implying the presence of a non-amphibian reservoir host that not only tolerates *Bd* infection but aids in its dispersal (Mitchell et al. 2008).

The potential for *Bd* to have naturally occurring, non-amphibian hosts has long been discussed, even though some amphibians can act as reservoir hosts (Davidson et al. 2003; Piotrowski et al. 2004; Rachowicz and Vredenburg 2004; Blaustein et al. 2005; Woodhams and Alford 2005; Alford 2010). Laboratory studies indicate *Bd* can survive on a diverse range of substrates (e.g., sand, leaf litter, rocks, invertebrate exoskeletons, autoclaved snakeskin, cleaned epidermal keratin, algae and potentially biofilm (S. Cashins, pers. Comm.; Johnson and Speare 2003; Piotrowski et al. 2004; Lips et al. 2006; Kirshtein et al. 2007; Walker et al. 2007; Carty 2009 in amphibiaweb; Cossel and Lindquist 2009). However, the fact that *Bd* can survive on these substrates in laboratories does not directly imply the same will occur in nature (McCallum 2005). Field studies have detected *Bd* in natural water samples, on crayfish (*Procambarus alleni*), reptiles, birds, and ground debris (sticks) (Longcore et al. 1999; Johnson and Speare 2003; Woodhams and Alford 2005; Di Rosa et al. 2007; Mitchell et al. 2008; Kilburn et al. 2011; Garmyn et al. 2012; McMahon et al. 2013).

Lowland rainforest pockets isolated from the main body of the Wet Tropics rainforest provide an ideal location for investigating potential non-amphibian reservoir hosts for *Bd*. These regions do not share watercourses with high elevation mountains and are subjected to environmental conditions (e.g., temperature and humidity) that are less buffered and not always within the range in which *Bd* can survive. *Batrachochytrium dendrobatidis* prevalence in susceptible adult amphibian hosts (*Litoria rheocola*) in these rainforest patches declines during the summer, sometimes to undetectable levels (Sapsford et al. 2013). This suggests that *Bd* does not infect amphibians at all times, and yet persists in populations. In addition, these lowland rainforests provide an opportunity to investigate *Bd* in an environment where temperatures and seasonal precipitation are, at times, outside of the optimum environmental regimes of *Bd*. Lowland rainforests support a diverse assemblage of non-amphibian vertebrates, invertebrates and abiotic substrates that have the potential to act as a reservoir host or vector of *Bd*, or both. The aim of this study was to determine whether there were potential non-amphibian reservoir hosts that may sustain *Bd*.

Methods

I sampled five times between the 18 May 2009 and the 05 April 2011, sampling at least once per season. Five sites at three streams were surveyed, in isolated pockets of tropical lowland rainforest (complex mesophyll vine forest) near Tully and Mission Beach in the Wet Tropics bioregion of North Queensland, Australia (Figure 4.1). Two of the three rainforest streams flowed into Mena Creek (Mena 1 [17°39’03.01”S, 145°59’23.94”E], Mena 2 [17°39’11.97’S, 145°59’06.96”E]). The third stream, Stoney Creek (17° 55' 17.9" S, 146° 04' 07.2" E), flowed from Lacey Creek, 8 km south of Mission Beach. Each stream is between 0-90 m above sea level (ASL) and did not connect to any mountainous streams at > 300 m elevation. Anthropogenic disturbance in the area consisted of access roads for 132kV/275kV transmission lines (Mena Creek) and the Tully-Mission Beach Road. Streams were narrow (1 – 6 m wide), shallow (0.05 – 1.40 m deep) and typically lit with dappled sunlight; however, width and depth of these streams varied considerably with rainfall. All three streams had permanent water flow through riffles, runs and pools, with rocks of various grain sizes from pebbles to boulders, with a sandy stream-bed. The dominant understory flora was diverse, and included plants in the family Arecaceae, Pandanaceae, Cyatheaceae, and Philydraceae.

Figure 4.1 Location of Mena Creek and Mission Beach study sites indicated by yellow star, with habitat of Mena Creek adjacent

Batrachochytrium dendrobatidis sampling on stream-associated invertebrates

Invertebrates were sampled throughout the streams in riffles, pools and runs by dip netting using an Aqua One, 25.4 cm coarse-mesh fish net, during the day (09:00 -16:00). Rocks were lifted from the stream and invertebrates attached to the underside of the rocks collected with a gloved hand. Aquatic invertebrates were separated into individual 2-ml vials with high grade ethanol, and identified to family (Gooderham and Tysrlin 2002). Non-aquatic invertebrates with high water contact, e.g., adult dragonflies, were opportunistically captured. Each invertebrate was swabbed for *Bd* individually, on all external body parts.

Batrachochytrium dendrobatidis sampling on vertebrates

Swabbing methods varied among vertebrate group, ensuring that surfaces swabbed were those most consistently or most likely to be infected by *Bd* (Berger et al. 1998; 1999; Williams et al. 2002; Woodhams and Alford 2005). Frogs were swabbed on the front feet, hind feet, inner thighs, the ventral surface and pelvic patches. Reptiles, including eastern water dragons (*Intellagama lesueurii*), brown tree snakes (*Boiga irregularis*), and common tree snakes (*Dendrelaphis punctulata*) were swabbed on the feet (if present), and ventral surfaces in a similar fashion to amphibians. Turtles, including Johnstone River snapping turtle (*Elseya stirlingi*) and saw-shelled turtle (*Wollumbinia latisternum*) were swabbed with two swabs, one used for the skin of the feet, hands and neck, the second used for the plastron and carapace.

Batrachochytrium dendrobatidis assessment

To quantify the presence of *Bd*, both vertebrates and invertebrates were swabbed using sterile cotton medical swabs (Tubed Dry Swabs, Medical Wire and Equipment, Corsham, Wiltshire U.K.), the detection method with the highest sensitivity (Hyatt et al. 2007). All swabbed individuals were held with a new pair of latex gloves to avoid cross-contamination of samples, using the protocols recommended by Mendez et al. (2008) and Phillott et al. (2010). Swabs were frozen and sent to the Center for Integrated Biotechnology at Washington State University, Pullman, WA, USA. Each swab was tested in a triplicate using real-time quantitative PCR (q-PCR) assays for the presence of *Bd* (Boyle et al. 2004).

Results

During August and September 2010, fifteen stream-associated invertebrates from three streams tested positive to *Bd*. A positive result was returned in eight of the sixteen stream-associated invertebrate families tested (Table 4.1). All individuals that tested positive were aquatic species or aquatic life stages of terrestrial species, no terrestrial life stages tested positive. Members of the Baetidae (mayfly) tested positive at all three streams, whilst members of the Psephenidae (water-penny), Calamoceratidae (caddisfly), Diphlebiidae (damselfly) and Gerridae (water striders) tested positive at two streams. No members of the Ecnomidae (caddisflies), Eustheniidae (stoneflies), Gomphidae (dragonflies), Hydropsychidae (caddisflies), Leptophlebiidae (mayflies) or Telephlebiidae (dragonflies) tested positive to *Bd*. The overall prevalence of *Bd* among all stream-associated invertebrates sampled from all sites was can be seen in Table 4.1.

Table 4.1 *Batrachochytrium dendrobatidis* infection in stream-associated invertebrates

Family	Swabbed	Infected	Mean zoospores per individual	Maximum mean zoospores count	Mean standard deviation (+/-)	Prevalence
Atyidae	6	2	91.60	180.89	14.66	0.30
Baetidae	21	3	29.70	70.31	2.42	0.14
Calamoceratidae	3	1	2333.61	2333.61	367.08	0.33
Diphlebiidae	30	2	33.06	42.51	3.35	0.07
Ecnomidae	1	0	-	-	-	-
Eustheniidae	2	0	-	-	-	-
Gerridae	3	2	171.87	340.40	15.32	0.67
Gomphidae	2	0	-	-	-	-
Gyrinidae	3	1	3.15	3.15	0.36	0.33
Hydropsychidae	3	0	-	-	-	-
Leptophlebiidae	21	0	-	-	-	-
Psephenidae	13	3	57.60	132.65	6.31	0.23
Telephlebiidae	2	0	-	-	-	-
Tipulidae	1	0	-	-	-	-
Unknown Family	10	1	153.50	153.50	22.20	0.10

*No Australian stream-associated invertebrates have previously been found to carry *Bd*.

Fifty-one amphibians and eight tadpoles from three streams tested positive to *Bd*. A positive result was returned in six of the eleven amphibian species tested (Table 4.2). Four of the species that tested positive are well known to become infected with *Bd*, whilst two species *Litoria bicolour* and *Hylarana daemeli* have not previously tested positive to *Bd*. The significance of this finding is discussed in chapter 5.

Table 4.2 *Batrachochytrium dendrobatidis* infection in amphibians

Amphibian species	Number swabbed	Number infected	Mean zoospores per individual	Maximum mean zoospores count	Mean standard deviation (+/-)	Prevalence
Cophixalus ornatus	2	0	-	-	-	-
Limnodynastes peroni	1	0	-	-	-	-
Litoria bicolour	5	1	12.99	12.99	2.74	0.20
Litoria dayi	29	2	150.40	288.96	7.33	0.06
Litoria gracilenta	3	0	-	-	-	-
Litoria jungguy	21	2	52.28	52.28	7.29	0.08
Litoria rheocola	312	37	2671.18	65202.00	299.58	0.11
Litoria rheocola (Tadpoles)	105	8	132.94	874.08	8.13	0.07
Litoria serrata	77	8	776.11	7430.64	67.94	0.10
Litoria xanthomera	2	0	-	-	-	-
Egg mass *Litoria sp*.	1	0	-	-	-	-
Hylarana daemeli	12	1	52.28	52.28	7.29	0.08
Rhinella marina	4	0	-	-	-	-

A diversity of reptiles inhabited these streams, however, none of the five commonly occurring reptile species tested positive for *Bd*, although sample sizes are very low (Table 4.3).

Table 4.3 ***Batrachochytrium dendrobatidis*** **infection in reptiles**

Reptile species	Number swabbed	Number infected
Boiga irregularis	2	0
Dendrelaphis punctulata	1	0
Elseya stirlingi	4	0
Intellagama lesueurii	4	0
Wollumbinia latisternum	3	0

*No Australian reptiles have been reported to carry *Bd*.

Discussion

Batrachochytrium dendrobatidis can occur on invertebrates in Australia, and likely elsewhere (Kilburn et al. 2011; McMahon et al. 2013). Stream-associated invertebrates have keratinised surfaces (more specifically, prekeratin in the basal cells of the epidermis) and inhabit microclimates suited for the growth and reproduction of *Bd* (Berger et al. 1999; Voyles et al. 2011). Further, most members of the Chytridiomycota family parasitise invertebrates and decompose chitin; *Bd* may do likewise (Powell 1993; Longcore et al. 1999). The detection of *Bd* on stream-associated invertebrates does not confirm that *Bd* infects or reproduces on stream-associated invertebrates (Haydon et al. 2002); it does however, suggest a potential non-amphibian reservoir host, and mechanisms for localised and large-scale dispersal of the pathogen.

Positive samples were collected at the time of year when mean zoospore counts were highest in common mistfrogs (*Litoria rheocola*) (Sapsford et al. 2013). Of the eight families of stream-associated invertebrates that tested positive to *Bd*, the highest mean zoospore counts (Calamoceratidae) were similar to mean zoospore counts of susceptible co-occurring amphibians (e.g., *L. rheocola*). This suggests that *Bd* may be growing on stream-associated invertebrates. Infection levels may be higher in resistant species (reservoir hosts), than in susceptible species (McCallum and Dobson 1995; Woodhams and Alford 2005), as occurred, for example, in the relatively *Bd* resistant jungguy frogs (*L. jungguy*) and declining Eungella day frogs (*Taudactylus eungellensis*) at Eungella (McCallum and Dobson 1995). Whether or not such a relationship exists between stream-associated invertebrates and amphibians is not known. The prevalence of *Bd* on stream-associated invertebrates is poorly understood and is likely to vary among families, genera, and species.

Batrachochytrium dendrobatidis can grow on the keratinised epidermis of reptiles in vitro, and has been detected on reptiles in nature (Piotrowski et al. 2004; Kilburn et al. 2011). *Batrachochytrium dendrobatidis* has not been detected on an Australian reptile, yet many are ecologically similar to amphibians, particularly in relation to microhabitat use, water contact, and temperature regulation. Thus, some reptiles may be capable of acting as a reservoir host (e.g., turtles, aquatic lizards, and snakes) (Kilburn et al. 2011). Given the small sample of reptiles tested in this and other studies (e.g., Phillott et al. 2009), I cannot conclude that reptiles do not carry *Bd* in these systems.

Other vertebrates (e.g., fish) may be capable of hosting *Bd*, yet have seldom been examined. The diet of many aquatic vertebrates, such as eels, include the aquatic invertebrates that tested positive to *Bd* in this study, providing the potential for direct contact and ingestion of *Bd* (Kilburn et al. 2011). The significance of this should not be dismissed, as many fish can become infected by viruses that infect amphibians (Laurance et al. 1996; Kiesecker et al. 2001; Daszak et al. 2003).

Dispersal of *Bd* over large distances (e.g., along Australia's east coast) is not well understood. Various people have suggested that cross-continental dispersal could have been caused by human transport of amphibians for food (Mazzoni et al. 2003; Schloegel et al. 2009; Alford 2010), research (Reed et al. 2000; Skerratt et al. 2007), pets (Fisher and Garner 2007; Une et al. 2008; Alford 2010), zoos, or by accident (Obendorf 2005). Whilst there is evidence of human induced movement (Pauza and Driessen 2008), it is not considered the sole method of dispersal, and non-amphibian vectors have been suggested (Morehouse et al. 2003; Collins and Crump 2009; Kilpatrick et al. 2009; Vredenburg et al. 2010). The speed at which *Bd* spread in Australia, sometimes across expanses of unsuitable environmental conditions, suggests that amphibians are not the only vector (McCallum 2005; Vredenburg et al. 2010). Localised movements of *Bd* between regions may have been caused by non-amphibian fauna, such as reptiles, birds, fish, or invertebrates (Laurance et al. 1996; Johnson and Speare 2005; McCallum 2005; Woodhams and Alford 2005; Vredenburg et al. 2010; Garmyn et al. 2012).

Many faunal groups could act as vectors for *Bd*; stream-associated invertebrates appear particularly suitable as they are keratinised, abundant, widespread and could transfer *Bd* to amphibians (McMahon et al. 2013). Occurring globally, many stream-associated invertebrate families have pan-tropical distributions, including families that tested positive to *Bd* in this study (Longcore et al. 1999; Daszak et al. 2003).

Flying invertebrates have the potential to carry *Bd* on their external cuticle or in water on their bodies. This could account for the movement of *Bd* within and between streams, water bodies and potentially over long distances. Transmission of the pathogen from invertebrate to amphibian could occur through direct contact, which commonly occurs in mosquitoes (*Uranotaenia lateralis*), sandflies (Culicoides), mayflies (Baetidae), blackflies (Simuliidae), and subcutaneous parasites (*Batrachomyia* spp) or consumption of invertebrates such as hemipterans, dipterans, decapods, trichopteran and coleopterans (Williams and Felt-mate 1992 in Laurance et al. 1996; CSIRO 1991 in Laurance et al. 1996; Hodgkison and Hero 2003; pers. obs). The transportation of *Bd* has the potential to be highly significant to susceptible amphibians, particularly if the pathogen is moved into uninfected areas. Transportation of *Bd* could not only introduce *Bd* into new areas, but also introduce different strains of

Bd into already sensitive areas. It is plausible that stream-associated invertebrates, acting as vectors of *Bd*, could have contributed to the devastating declines of amphibian populations.

Conclusion

Whether *Bd* is capable of surviving and reproducing in nature without amphibians (e.g., on other biotic substrates) is unclear, yet relevant to our understanding of amphibian declines worldwide. I determined that various stream-associated invertebrates can carry *Bd*, though aspects of the relationship between *Bd* and stream-associated invertebrates remain virtually unknown, including whether or not *Bd* can survive on, infect, or reproduce, on invertebrates. Although *Bd* was detected on stream-associated invertebrates, it is still necessary to determine whether or not these invertebrates are acting as a reservoir host or vector. Future works targeting *Bd* and non-amphibian hosts have the potential to detail relationship and host-pathogen dynamics of what was considered an amphibian specific pathogen.

CHAPTER 5: DETECTION OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN THE AUSTRALIAN WOOD FROG (*HYLARANA DAEMELI*) AND NORTHERN DWARF TREEFROG (*LITORIA BICOLOR*)

Abstract

The severe declines experienced by a range of Australian amphibians, and the importance of reservoir hosts in these declines, suggests that testing all amphibian species for the presence of *Bd,* especially in areas that have suffered declines, should be undertaken. However, species that have not exhibited noticeable declines have frequently been overlooked during surveys of *Batrachochytrium dendrobatidis* (*Bd*). Surveys of amphibians along lowland rainforest streams detected *Bd* on two amphibians not previously known to carry the pathogen, the Australian wood frog (*Hylarana daemeli*) and northern dwarf treefrog (*Litoria bicolor*). Infection levels in both species were low (12-52 mean zoospore count) compared with susceptible species. The presence of *Bd* on these species increases not only the number of amphibians known to become infected by *Bd,* but the number of potential reservoirs that may aid in the decline and extinction of susceptible species.

Introduction

Batrachochytrium dendrobatidis (*Bd*) occurs in at least 61 species of Australian frogs (Murray et al. 2010; Speare et al. 2005). *Batrachochytrium dendrobatidis* may infect species with or without causing decline, and is suspected to cause the extinction of some host species (Berger et al. 1998; Lips et al. 2005; Skerratt et al. 2006; Alford 2010). Knowing the range of species that carry *Batrachochytrium dendrobatidis* increases our understanding of the geographic range of *Bd*, potential hosts, and the conditions under which *Bd* can survive, especially at the periphery of its distribution.

Northern dwarf treefrog (*Litoria bicolor*)

Litoria bicolor is a small hylid that occurs in creeks, swamps, flooded grasslands and along rainforest edges in northern Australia, through Cape York Peninsula, the Wet Tropics, across the Top End and the Kimberley region of Western Australia (Tyler and Knight 2009). *Litoria bicolor* occurs over a wide elevation gradient up to at least 1000 m ASL; although most of the species range occurs at lower elevations. *Batrachochytrium dendrobatidis* is present through much of the geographic range of *L. bicolor*, and has been detected in co-occurring and related species (see Murray et al. 2010). *Litoria bicolor* (Figure 5.1) has not suffered *Bd* implicated declines, and unlike the closely related, morphologically and ecologically similar *L. fallax* and *L. olongburensis* (Vanderduys 2013), has not tested positive to *Bd* (Murray et al. 2010; Speare et al. 2005; Clay Simpkins, pers. Comm.). Tests conducted by Keith McDonald at Endeavour Valley, Cooktown, Queensland on at least five *L. bicolor* were negative for *Bd* (Murray et al. 2010).

Australian wood frog (*Hylarana daemeli*)

Hylarana daemeli (Figure 5.1) is unique as it is the only member of the Ranidae family in Australia (Vanderduys 2013). *Hylarana daemeli* ranges across three broad bioregions, the Wet Tropics, Cape York Peninsula and the Arnhem Coast Corner (Williams and Hero 1998; Anstis 2013). In the Wet Tropics it inhabits coastal lowlands and foothills below 600 m ASL. It occurs in rainforest and wet sclerophyll forests associated with streams (Hoskins and Hero 2008). A semi-aquatic species, *H. daemeli* is likely to have been at risk of infection by *Bd* in the Wet Tropics since *Bd* appeared there in approximately 1989 (Laurence et al. 1996). However, *H. daemeli*, like certain other amphibians (striped marsh frog [*Limnodynastes peronii*], ornate burrowing frog [*Platyplectrum ornatum*], and Stoney Creek frog [*Litoria lesueuri*]) has ecological traits that appear to reduce the probability of population decline despite the presence of infection (Williams and Hero 1998). All 68 swabs collected by Keith McDonald and Alistair Freeman from *H. daemeli* in the McIlwraith Range tested negative for *Bd* (Skerratt et al. 2005; Murray et al. 2010) and Speare et al. (2005a).

Lowland amphibian populations often have lower infection intensity and prevalence than do upland populations, and although *L. bicolor* can occur up to at least 1000 m ASL, *H. daemeli* does not occur at elevations >600 m, where significant declines and the highest infection intensities have been recorded (Kriger and Hero 2008). Although not strictly rainforest species, *L. bicolor* and *H. daemeli* do occur in rainforest habitats in which some amphibian species can have high rates of infection (Woodhams and Alford 2005). Surveys targeting *Bd* in Australia have typically excluded these species, focussing on declining species or those that commonly co-occur with declining species (e.g., *Litoria jungguy*).

Figure 5.1 Northern dwarf treefrog (*Litoria Bicolor*) (Left), and Australian wood frog (*Hylarana daemeli*) (Right)

Methods

Study sites

As part of two separate projects, surveys for *Bd* were undertaken at four locations, Mena 1 (17°39'03.01"S, 145°59'23.94"E), and Mena 2 (17°39'00.00"S, 145°59'10.76'E) near Mena Creek, Stoney Creek, (17° 55' 17.9"S, 146° 04' 07.2"E) near Mission Beach, and Ella Bay (17°27'31.99"S, 146°03'20.21"E) (Alford 2010a) near Innisfail (Figure 5.2). Both projects involved swabbing amphibians to detect *Bd* and results from these surveys have been combined. At Ella Bay a two night *Bd* survey was undertaken on the 26 August 2009 and 27 August 2009 by two people. Surveys at Mena Creek and Stoney Creek were undertaken between 07 July 2010 and 13 February 2011. I swabbed a range of species but specifically targeted species highly susceptible to *Bd* infection (e.g., *Litoria rheocola*). A total of 538 individual frogs of 13 species were swabbed for the pathogen during surveys (Table 5.1).

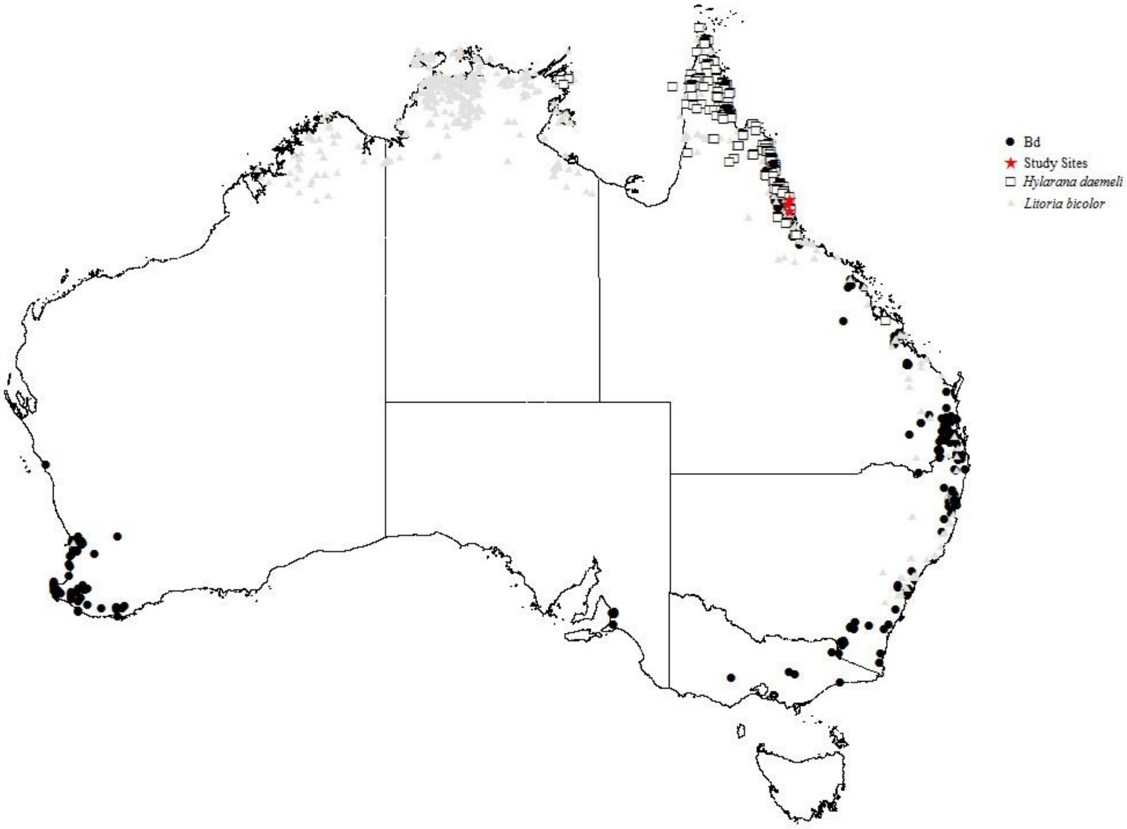


Figure 5.2 Known occurrences of *Hylarana daemeli*, *Litoria bicolor* and *Batrachochytrium dendrobatidis*. Study sites for at which *Hylarana daemeli* and *Litoria bicolor* were tested for *Batrachochytrium dendrobatidis*. Data collected from Atlas of Living Australia, and Murray et al. (2010)

Batrachochytrium dendrobatidis assessment

To quantify infection, frogs were swabbed using sterile cotton medical swabs (Tubed Dry Swabs, Medical Wire and Equipment, Corsham, Wiltshire U.K.), the detection method with the highest sensitivity (Hyatt et al. 2007). Frogs were swabbed on the front feet, hind feet, inner thighs, the ventral surface and pelvic patches. These surfaces were swabbed as they are most consistently infected by *Bd* (Berger et al. 1998; 1999; Williams et al. 2002; Woodhams and Alford 2005). All individuals swabbed were held with a new pair of latex gloves to avoid cross contamination of samples (Mendez et al. 2008; Phillott et al. 2010). Swabs were frozen and sent to the Center for Integrated Biotechnology at

Washington State University, Pullman, WA, USA. Each swab was tested in triplicate using real-time quantitative PCR (q-PCR) assays for the presence of *Bd* (Boyle et al. 2004).

Results

Of the five *L. bicolor* swabbed only a single individual tested positive from Mena 1 (Table 5.1). Of the 14 swabs collected from *H. daemeli* during surveys, two tested positive to *Bd*, one from Mena 1 and one from Ella Bay (Table 5.2). The quantitative PCR analysis for each sample returned positive results for the presence of *Bd* in all three replicate subsamples. Five other species tested positive to *Bd* (*Litoria dayi*, *L. rheocola*, *L. serrata*, *L. jungguy* (complex), and *Limnodynastes peronii*) (Table 5.1). With the exception of *L. peronii* these species regularly test positive to *Bd* in the Wet Tropics and in some species there has been population declines (McCallum and Dobson 1995; Laurence et al. 1996).

Table 5.1 Species and numbers of frogs swabbed for *Bd* between the 07 July 2010 and 13 February 2011, and the outcomes

	Mena 1		Mena 2		Stoney Creek		Ella Bay	
Species	Swabbed	Infected	Swabbed	Infected	Swabbed	Infected	Swabbed	Infected
Litoria bicolor	3	1	2	0	0	0	0	0
Litoria dayi	23	2	6	0	0	0	0	0
Litoria gracilenta	1	0	1	0	1	0	0	0
Litoria infrafrenata	9	0	6	0	1	0	0	0
Litoria rheocola	180	24	110	11	32	5	4	1
Litoria rubella	0	0	0	0	0	0	14	0
Litoria serrata	18	1	33	2	29	4	2	0
Litoria xanthomera	2	0	0	0	0	0	0	0
Litoria jungguy (complex)	11	0	5	0	5	1	7	1
Limnodynastes peronii	0	0	1	1	0	0	0	0
Rhinella marinus	0	0	2	0	2	0	12	0
Hylarana daemeli	4	1	4	0	4	0	2	1
Cophixalus ornatus	0	0	1	0	0	0	1	0

Table 5.2 Results of swabs taken from infected *H. daemeli* and *L. bicolor* from Mena Creek and Ella Bay

Date	Species	Season	Site	Infection status	Mean zoospore count	StdDev
27/08/2009	*H. daemeli*	Winter	Ella Bay	Infected	-	-
1/08/2010	*H. daemeli*	Winter	Mena 1	Infected	52.283	7.292
7/07/2010	*L. bicolor*	Winter	Mean 1	Infected	12.994	2.735

Discussion

Both *L. bicolor* and *H. daemeli* have been swabbed for *Bd* (see Skerratt et al. 2005; 2010; Speare et al. 2005a; Murray et al. 2010) in areas north of the northernmost regions from which *Bd* is recorded in Australia on *Bd* on Cape York Peninsula north of Big Tableland (15.7°S, 145. 2°E) (Speare et al. 2005). I swabbed these two species in the Wet Tropics bioregion in areas that contain *Bd*, and found an overall prevalence of *Bd* of 14% in *H. daemeli*, and 20% in *L. bicolour*. There is no evidence that either of these species declined or were negatively influenced by *Bd* in the past. However, neither of the two species displays the traits of declining species, which include low fecundity, breeding in flowing streams and inhabiting high elevation rainforest habitats (Williams and Hero 1998). These species could serve as reservoir hosts in the locations where they occur.

Considering the phylogenetic relationship of *L. bicolor* and *H. daemeli* to susceptible and declining species both of these species should be considered for more thorough testing. Over the past 20 years many species of *Litoria* and *Hylarana* have tested positive to *Bd* (e.g., big-eyed frog [*H. grandocula*], variable backed frog [*H. similis*], Schlegel's frog [*H. chalconota*], Luzon frog [*H. luzonensis*], bronzed frog [*H. temporalis*]), many of which have suffered declines, including armoured mistfrog (*Litoria lorica*), Australian lace-lid (*L. dayi*), common mistfrog (*L. rheocola*), waterfall frog (*L. nannotis*) and mountain mistfrog (*L. nykalensis*) which is presumed extinct (Richards et al. 1993; Savage et al. 2011; Swei 2011; Lowe et al. 2012).

There is a high degree of overlap in the distribution of *Bd*, *L. bicolor* and *H. Daemeli* in the Wet Tropics, yet the distribution of both *L. bicolor* and *H. daemeli* extend well outside the known range of *Bd* (Figure 5.2). This implies that if *Bd* expanded its geographic range these species have the potential to act as reservoir hosts, retaining and transmitting infection in these areas.

Amphibians at all rainforest stream sites are potential reservoirs of *Bd*. Although no microhylidae have ever tested positive to *Bd*, any amphibian found within the rainforest stream environment could potentially host *Bd* (Murray et al. 2010). Susceptible species, including those that have suffered declines at high elevation, such as *Litoria rheocola, L. serrata,* and *L. dayi* maintained high infection intensity and prevalence at all sites (Puschendorf 2009; Roznik 2013; Sapsford et al. 2013).

Conclusion

The detection of *Bd* in additional amphibian species will continue. Testing species that are not currently at risk of decline is important, because although *Bd* does not pose a risk to all amphibian species; it does pose a risk to some species with which non-declining species are closely associated. The role of reservoir hosts in species decline and extinction is well established; knowing which species could act as reservoir hosts should, therefore, be a priority.

CHAPTER 6: GENERAL DISCUSSION AND FUTURE DIRECTIONS

Batrachochytrium dendrobatidis (*Bd*) continues to be a serious threat to amphibians globally; a diversity of species are at serious risk of extinction. The relationship between amphibian species and *Bd* varies, and in many cases is unclear, with some populations showing declines, whilst others continue to thrive (McDonald and Alford 1999). Features aiding in the persistence of amphibian species are becoming apparent; with ecology and individual behaviour playing important, yet insufficiently studied roles. In the Wet Tropics of north eastern Australia, considerable research has focussed on species that have suffered declines (Puschendorf, 2009; Sapsford et al. 2013; Roznik 2013; Woodhams & Alford 2005), providing insights into a diversity of aspects of the amphibian-*Bd* system. To increase our understanding of this relationship I described seasonal changes in the ecology of *Litoria rheocola*, and examined behavioural differences in *Bd*-infected and uninfected *L. rheocola*. I also examined a number of potential amphibian and non-amphibian reservoirs and vectors. I examined species not previously recorded with *Bd,* and non-amphibian hosts, which are areas of research that may change our current conceptions about the presumed specificity of *Bd* and amphibians, and provide an explanation for the method of spread of the pathogen.

Seasonal variation in amphibian behaviour

All amphibians are subjected to seasonal fluctuations in environmental conditions, altering their behaviour accordingly. Describing these behaviours is easy in some cases (e.g., burrowing during the dry season in desert frogs), whilst in other cases behavioural differences are less obvious, especially when suitable environmental conditions occur year-round (e.g., within tropical rainforest). Our understanding of seasonal behavioural changes in amphibian species varies dramatically among species, and descriptions of seasonal behavioural changes are not available for many species. Examinations of seasonal behaviour in *L. rheocola* revealed broad similarities with other Australian stream-dwelling hylids. Seasonal differences were detected in body temperatures, which were higher in the summer months, and lower in winter, and movement patterns, which differed significantly diurnally, but not nocturnally. Throughout the year, *L. rheocola* remained closer to streams than has been recorded in any other Australian stream-associated hylid. *Batrachochytrium dendrobatidis* infection levels became undetectable during the summer months; a pattern that has not been recorded within populations of *Bd* susceptible species. The disappearance of the pathogen from the *L. rheocola* population indicates the presence of a reservoir, and suggests that isolated patches of lowland rainforest provide conditions in which populations of *L. rheocola* can persist with endemic *Bd* infection. With further research, other ecological factors such as habitat requirements, diet, mating, breeding sites and the significance of infection on behaviour can become more apparent.

Behaviour and *Batrachochytrium dendrobatidis*

Higher environmental and body temperatures typically influence *Bd* negatively. The temperatures of the tropical lowland rainforest were, at times, detrimental to the survival and reproduction of *Bd*, apparently tipping the balance of the amphibian-chytrid fungus relationship in favour of the host for extended periods of the year (Sapsford et al. 2013). However, during the cooler months, lower temperatures provided *Bd* with suitable conditions for growth and reproduction, increasing infection prevalence and intensity. As such, the cooler (autumn and winter) periods pose a risk to *Bd*-susceptible amphibians. During periods when *Bd* is a threat to individual health, an individuals' behaviour may be critical in avoiding, managing and surviving infection. Examination of behavioural differences between infected and uninfected *L. rheocola* under natural conditions provided information on how behaviour may act as a disease resistance mechanism. In the case of *L. rheocola*, the probability of *Bd* infection is influenced by individual selection of microhabitat and movement patterns and this is likely to be similar in other amphibian species. Uninfected *L. rheocola* sat further from the stream, and used different microhabitats from infected *L. rheocola*, and although not statistically significant, uninfected *L. rheocola* moved significantly further and sat higher in vegetation during this and other studies (Roznik, 2013). Observations of behavioural differences between infected and uninfected amphibians such as these, add to the broader understanding of factors that aid in the natural persistence of *Bd* infected amphibian populations. The significance of behavioural differences between infected and uninfected individuals suggests such differences warrant further investigation. Given the relatively recent threat of *Bd* (<25 years) to many amphibian species, variations in behaviour may be evidence of functional adaptations to the negative effects of the pathogen. There are, of course, other possibilities, for example that the behavioural differences are caused by infection, but further research is required to determine the significance, cause and how widespread these differences in behaviour may be.

Batrachochytrium dendrobatidis reservoirs and vectors

The detection of *Bd* DNA on stream-associated invertebrates has not previously occurred in Australia. Detection of *Bd* on non-amphibian hosts is becoming increasingly common as more research into non-amphibian hosts is undertaken, providing further evidence that amphibians are not the only reservoirs of the amphibian chytrid fungus. Although sample sizes within this study were low, a range of stream-associated invertebrate families tested positive for *Bd*, providing the potential to alter our current understanding of *Bd* transmission methods. Recent works (McMahon et al. 2013) confirmed transmission of *Bd* from a non-amphibian host to an amphibian under laboratory conditions, emphasising the need to study non-amphibian reservoirs in natural systems. Stream-associated invertebrates and other non-amphibian reservoirs may also act as vectors of the pathogen, potentially explaining both the large- and fine-scale spread of *Bd* across its now expansive range. Determining which faunal groups are capable of carrying, spreading and transmitting *Bd* requires additional research, across all *Bd* inhabited countries.

To the future

Amphibians around the world continue to survive, despite the presence of *Bd*. How they do it appears to be species specific, and reliant on numerous factors. The tropical lowlands of Australia's Wet Tropic bioregion are a stronghold for many amphibian species that suffered significant declines in the same region at higher elevations. These and other extant amphibian populations provide the opportunity to investigate the amphibian-*Bd* relationship from a diversity of viewpoints, including behaviour, disease resistance, and host-pathogen interactions under naturally fluctuating conditions in the presence of a potentially large range of non-amphibian reservoirs. *Batrachochytrium dendrobatidis* research examining non-amphibian hosts has the potential to dramatically change our understanding of *Bd* in natural systems.

Studying *Bd* and extant amphibian populations under field conditions can provide a wealth of information that could aid in the conservation of declining species, particularly as other threatening processes become more important, e.g., climate change and other chytrid funguses (e.g., *Batrachochytrium salamandrivorans*). For many species, *Bd*-caused extinction has already occurred and for others it is likely. To minimise the likelihood of extinction, we must gain information from naturally surviving *Bd*-susceptible species, particularly species that have naturally recovered despite the presence of *Bd* (e.g. *Litora serrata*). This requires field research into aspects of amphibian ecology and factors that aid in survival, such as naturally occurring antimicrobial bacteria to provide information on how species are surviving and more importantly recovering in the presence of *Bd*. Captive management and breeding are currently considered a last resort for many *Bd*-susceptible amphibians (e.g., southern corroboree frog [*Pseudophryne corroboree*], Kroombit tinker frog [*Taudactylus pleione*], and Baw Baw frog [*Philoria frosti*]), however, the natural resilience of many species also provides other options such as translocations that may allow species to recover under natural conditions. A greater understanding of many aspects of the amphibian-chytrid fungus relationship is required to reduce further loss of wild amphibian populations.

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