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Interactions between amphibian skin sloughing and a cutaneous fungal disease:

infection progression, immune defence, and phylogenetic patterns

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Abstract

Worldwide, there has been an unprecedented rise in emerging infectious diseases of wildlife, and this has contributed to a widespread biodiversity crisis. Amphibian populations, in particular, are threatened by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), which in post-metamorphic animals only infects the skin, and causes the potentially lethal disease chytridiomycosis. Amphibians regularly slough their skin, and in doing so remove many skin-associated microbes. Thus, skin sloughing may play an important role in the pathogenesis of chytridiomycosis. To investigate this association, the influence of Bd infection on amphibian skin sloughing, and the role of sloughing in regulating infection, was examined. Furthermore, to better understand the variation in skin sloughing rates across species and ecological groups, and make inferences about the role of this process in susceptibility to this fungal disease, amphibian skin structure and function was investigated within a phylogenetic context.

To determine the relationship between skin sloughing and disease progression (chapter 2), adult green tree frogs (*Litoria caerulea*) were exposed to an Australian Bd strain, and sloughing rates and infection load were monitored on a naturalistic cycling temperature regime (15 - 23˚C). Sloughing rates were determined by filming frogs and infection intensity was monitored before and after sloughing with conventional swabbing and quantitative PCR. Sloughing rate was found to increase with Bd infection load in infected frogs, but sloughing itself did not affect Bd load on the ventral skin surface. Although a faster sloughing rate might be considered advantageous for Bd-infected animals, it does not appear to curb the progression of disease in susceptible species. In fact, sloughing may actually contribute to the loss of physiological homeostasis seen in terminally ill frogs by further inhibiting water and electrolyte transport across the skin.

In some species less susceptible to chytridiomycosis, it has been demonstrated that Bd growth remains epibiotic, without penetrating the underlying epidermal layers. Therefore, sloughing may more effectively remove Bd zoospores in less susceptible species (chapter 3). To test this hypothesis, five Australian frog species, *Lit. caerulea*, *Platyplectrum ornatum, Lechriodus fletcheri, Limnodynastes peronii,* and *Lim. tasmaniensis,* were exposed to an Australian Bd strain, and their sloughing rates and infection loads monitored over time. Utilising an improved methodology to remove any artefacts from the swabbing itself, sloughing was found to reduce Bd load on the ventral skin surface, in all five species, despite wide ranging variation in susceptibility to Bd infection and subsequent disease. In less susceptible species, sloughing reduced Bd load up to 100%, leading to infection clearance. However, the drop in Bd load was only temporary in susceptible species, potentially due to the invasive growth of Bd in skin layers underlying the

stratum corneum in these species. If less susceptible species are able to clear themselves of Bd infection via the routine process of skin shedding, amphibian sloughing may be a more important immune defence than previously thought. This work has implications for understanding the pattern of Bd growth on individual hosts, as well as population-level dynamics.

Finally, the relationship between susceptibility to chytridiomycosis and skin structure and function between species was investigated within a phylogenetic context (chapter 4). The sloughing rates of 21 frog species from around the globe were measured, including Australia (9), Central and South America (11), and Southeast Asia (1). In addition to measuring sloughing rates, epidermal thickness and the number of replacement layers in preserved specimen of seventeen of these species were also measured. Utilising a phylogenetic linear mixed model framework, the association of these skin turnover traits with the evidence for Bd-driven declines was assessed, based on information from the IUCN Red List, published papers, grey literature, and personal communications. It was determined that sloughing rate demonstrates high phylogenetic signal, but was not associated with the evidence of Bd-driven declines, or other skin characteristics, within this subset of species. This is the first comparison of sloughing rate across a wide range of amphibian species, and creates the first database of amphibian sloughing behaviour. Given the strong phylogenetic signal observed in sloughing rate, approximate sloughing rates of related species may be predicted based on phylogenetic position, and may help to explain differences in the severity of infection in genera with relatively slow skin turnover rates (e.g. *Atelopus*). A clear understanding of epidermal turnover in amphibian genera particularly affected by Bd may help focus conservation mitigation efforts.

Despite the restriction of Bd to the post-metamorphic amphibian epidermis, the role of skin sloughing as an immune defence mechanism has so far been overlooked. This work investigating the physiology of amphibian skin and the host-pathogen relationship at the site of pathogen colonisation brings us closer to understanding the factors driving observed variation in intra- and interspecific susceptibility across a wide range of amphibian hosts. This understanding can help improve species-specific predictions of host extinction risk in natural populations.

Declaration by author

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Publications during candidature

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Michel E. B. Ohmer (Candidate)	Concept and design (60%), data collection (100%), data analysis (90%), writing and editing (80%)
Rebecca L. Cramp	Concept and design (20%), writing and editing (10%)
Craig R. White	Data analysis (10%), writing and editing (5%)
Craig E. Franklin	Concept and design (20%), writing and editing (5%)

Contributions by others to the thesis

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-*Travels with Charley*, John Steinbeck

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List of Abbreviations

Bd – *Batrachochytrium dendrobatidis*

BSA – bovine serum albumin

DAPI – 4',6-diamidino-2-phenylindole

DNA – deoxyribonucleic acid

GG – granular gland

IMI – intermoult interval

IUCN – International Union for Conservation of Nature

MG – mucous gland

ML – maximum likelihood

PCNA – proliferating cell nuclear antigen

PCR – polymerase chain reaction

PLMM – phylogenetic linear mixed model

PVC – polvvinyl chloride

REML – restricted maximum likelihood estimation

SB – *stratum basale*

SC – *stratum corneum*

SG – *stratum granulosum*

SVL – snout-vent length

ZE – zoospore equivalents

CHAPTER 1

General Introduction

Amphibian declines and emerging infectious diseases

We are in the age of the Anthropocene (Steffen et al. 2011), in which human-induced environmental change and degradation has led to what many believe is the start of the sixth great mass extinction (Wake & Vredenburg 2008; Barnosky et al. 2011). With this continued loss of biodiversity, understanding the threats affecting species and communities and devising effective mitigation strategies are of utmost importance. An understanding of the physiological mechanisms underlying responses of organisms to new and ongoing threats, such as climate change, loss of habitat, and emerging pathogens, and an integration of physiology and behaviour (Cooke et al. 2014), can greatly increase the effectiveness of such strategies (Blaustein et al. 2012; Cooke et al. 2013).

The recent rise in emerging infectious diseases of wildlife is one such threat to biodiversity (Daszak et al. 2000, Smith et al. 2009b) that has been linked to increased anthropogenic environmental change and degradation (Daszak et al. 2000; Aguirre & Tabor 2008). While declines and extinctions in amphibians are a component of the biodiversity loss occurring globally (Houlahan et al. 2000; Collins & Storfer 2003), amphibians have received special attention for the enigmatic and catastrophic nature of many declines, particularly in the last three decades (Stuart et al. 2004; Hof et al. 2011). In fact, amphibians are the most threatened group of vertebrates (Stuart et al. 2004), experiencing extinction rates that are four-orders greater than background estimates (Alroy 2015). A major contributor to these declines was determined following the discovery and description of the fungal pathogen *Batrachochytrium dendrobatidis* (hereafter Bd) in 1998 and 1999 (Berger et al. 1998; Longcore et al. 1999), and its salamander-specific sister-species *B. salamandrivorans* in 2013 (Martel et al. 2013; Martel et al. 2014), which cause the insidious disease chytridiomycosis in their amphibian hosts (Skerratt et al. 2007).

As a generalist pathogen with a broad host range within the class Amphibia, the spread of Bd has been, and continues to be, implicated in amphibian declines and extinctions in Australia (Berger et al. 2004; Woodhams & Alford 2005; Schloegel et al. 2006; Murray et al. 2009), North (Vredenburg et al. 2010), Central and South America (Berger et al. 1998; Lips et al. 2006; Crawford et al. 2010), and Europe (Bosch et al. 2001), and has now been recorded in over 500 amphibian species globally (Kilpatrick et al. 2010a; http://www.bd-maps.net/surveillance/; Olson et

al. 2013). Among this wide range of amphibian hosts, susceptibility to Bd infection and subsequent disease occurs along a continuum and ranges from resistance or tolerance to uncontrolled infection and mortality (Beldomenico & Begon 2010;Woodhams et al. 2011). In addition, this variation in susceptibility can be seen both intra and inter-specifically (Woodhams & Alford 2005; Tobler & Schmidt 2010), as evidenced by differing rates of decline between populations (Kriger et al. 2007; Van Sluys & Hero 2009; Bradley et al. 2015) and in sympatric species (Alford & Richards 1999; Stuart et al. 2004; Lips et al. 2006). While there have been many advances in understanding the ecology and epidemiology of this disease, research is still hindered by a lack of basic biological understanding regarding the Bd-host relationship (Voyles et al. 2011) at the site of pathogen colonisation: the skin.

This chapter first describes the unique role of the skin in amphibian physiology, an overview the pathogen, Bd, and the relationship between Bd and its amphibian hosts. Then, the drivers of variation in susceptibility to this generalist fungal pathogen are expanded upon, and the potential role of skin sloughing, given what we currently know about this elusive amphibian behaviour and its physiological importance, is introduced. Finally, a framework is provided for the research in this thesis and its significance, and an overview of the structure of the subsequent chapters.

Amphibian skin structure and function

The skin of vertebrates serves as a protective boundary between the organism and its external environment, including any parasites and pathogens that may occur in that environment. For amphibians, the skin is a site of constant flux through which water, energy, osmolytes, and respiratory gases are exchanged (Boutilier et al. 1992). Considered more permeable than the skin of other vertebrates, the amphibian integument plays a vital role in maintaining physiological homeostasis, although many species have adapted to depart from strict homeostasis when necessary (Boutilier et al. 1992; Voyles et al. 2010). Generally, the amphibian epidermis contains 5-7 layers of epithelial and specialised cells: 1-2 superficial layers of flattened keratinised cells (*stratum corneum*), 3-4 intermediate layers (*stratum granulosum*), and a basal layer (*stratum germinativum/ basale*) from which all epidermal cells originate and differentiate (Farquhar & Palade 1965; Guo et al. 2003; Figure 1.1A).

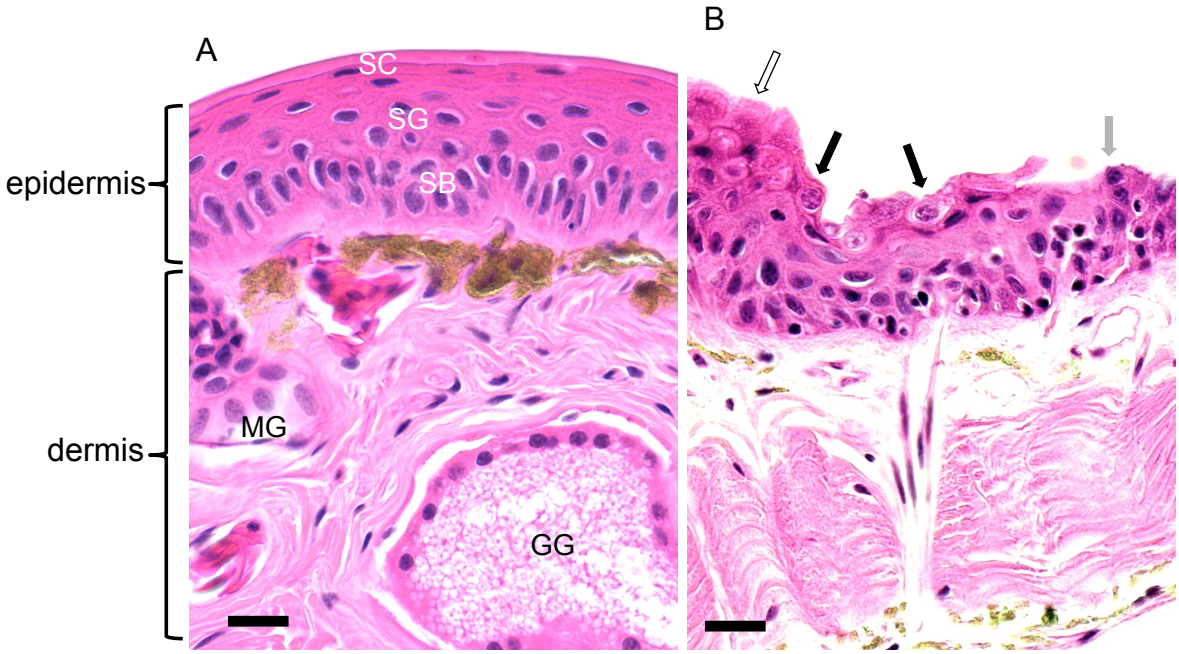

Figure 1.1 Epidermal and dermal layers of *Litoria caerulea* skin: (A) Healthy skin and (B) skin infected with *Batrachochytrium dendrobatidis* (Bd). Skin sectioned at 5 µm and stained with haematoxylin and eosin. SC = *stratum corneum*, SG = *stratum granulosum*, SB = *stratum basale*, MG= mucous gland, GG = granular gland, black arrows = Bd zoosporangia growing intracellularly, grey arrow = erosion, white arrow = hyperkeratosis. Scale bar = 25 µm.

In order to keep the skin in optimal condition, epidermal turnover occurs in amphibians on a regular basis as the old *stratum corneum* is replaced with a newly keratinised skin layer (Barker Jørgensen 1988). This epidermal turnover is called sloughing, or moulting, and time between sloughing events is termed the intermoult interval. When the *stratum corneum* begins to separate from the underlying epidermal layers, mucus is secreted into the resulting subcorneal space in order to aid in the process, and the skin is removed behaviourally with the assistance of complicated whole body, limb, and mouth movements (Ling 1972; Budtz & Larsen 1975; Barker Jørgensen 1988; Figure 1.2A-B). Unlike mammalian vertebrates, the slough is usually shed in its entirety, and then consumed, perhaps for the partial recycling of raw materials for future skin layers (Ling 1972; Weldon et al. 1993; Duellman & Trueb 1994). While the mechanism is not fully understood, sloughing is hormonally controlled via the thyroid, pituitary, and adrenal glands (Jørgensen & Larsen 1960, 1964; Barker Jørgensen et al. 1965; Larsen 1976; Barker Jørgensen 1988), and follows an autonomous rhythm that is species-specific and may be influenced by age, size, and breeding timing (Jørgensen & Larsen 1961; Larsen 1976). Intermoult interval is also highly

dependent on ambient temperature, with moulting frequency increasing at higher temperatures (Stefano & Donoso 1964; Cramp et al. 2014). Given the regularity of the sloughing process and its importance for epidermal functioning, it may play an important role in immune defence in the face of invading cutaneous pathogens.

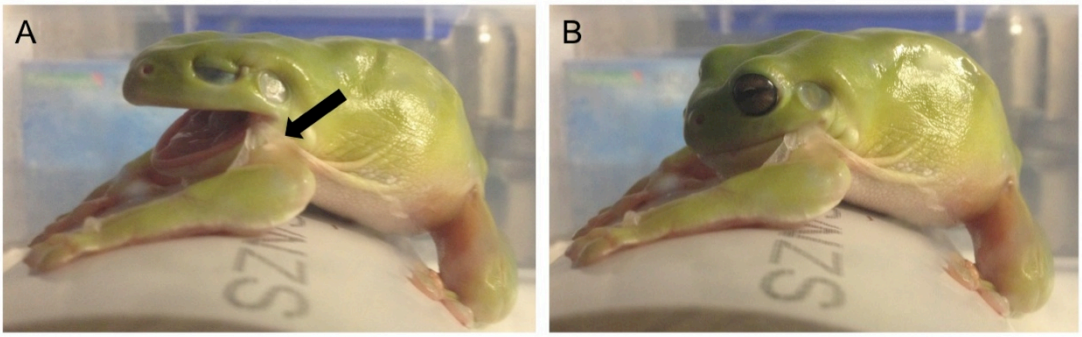

Figure 1.2 Adult *Litoria caerulea* exhibiting classic sloughing behaviour, in which the back is arched, and the mouth opens (A) and closes (B) with the rhythmic movement of the limbs to pull the shed skin towards the corners of the mouth (see black arrow), where it is ingested. Photo: M.E.B. Ohmer

The amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Bd)

Bd infection is restricted to the keratin and prekeratin-containing cells in the superficial layers of the amphibian's skin, and the keratin-containing mouthparts of larvae (Marantelli et al. 2004; Berger et al. 2005a). As one of only two members of the genus *Batrachochytrium,* the ability of Bd (and recently described *B. salamandrivorans*) to parasitise vertebrate hosts is completely unique within the phylum Chytridiomycota (Berger et al. 1998; Pessier et al. 1999). Bd has a two-part lifecycle consisting of a motile flagellated zoospore, and a stationary thallus that develops into a reproductive zoosporangium (Pessier et al. 1999; Piotrowski et al. 2004; Berger et al. 2005a). Under optimal conditions, zoospores are motile for up to 24 hours before they encyst on a suitable substrate, which involves retracting the flagellum after attachment, and developing a chitin wall (Berger et al. 2005a; Greenspan et al. 2012). A germination tube then forms that penetrates cell membranes of the host, usually through more than one cell layer (Greenspan et al. 2012; Van Rooij et al. 2012). It is through this germ tube that cellular contents of the zoospore body are transferred to the host cell, where a zoosporangium then develops (Berger et al. 2005a). Zoosporangia are cleaved into daughter zoospores, and these then leave the zoosporangium through one or more

discharge papilla that are directed toward either the external body surface or intercellular spaces within the skin of the host (Berger et al. 2005a; Figure 1.3). However, in some hosts such as the tolerant species *Xenopus laevis*, growth of Bd appears to remain epibiotic, and does not penetrate past the *stratum corneum*, which is routinely shed (Van Rooij et al. 2012).

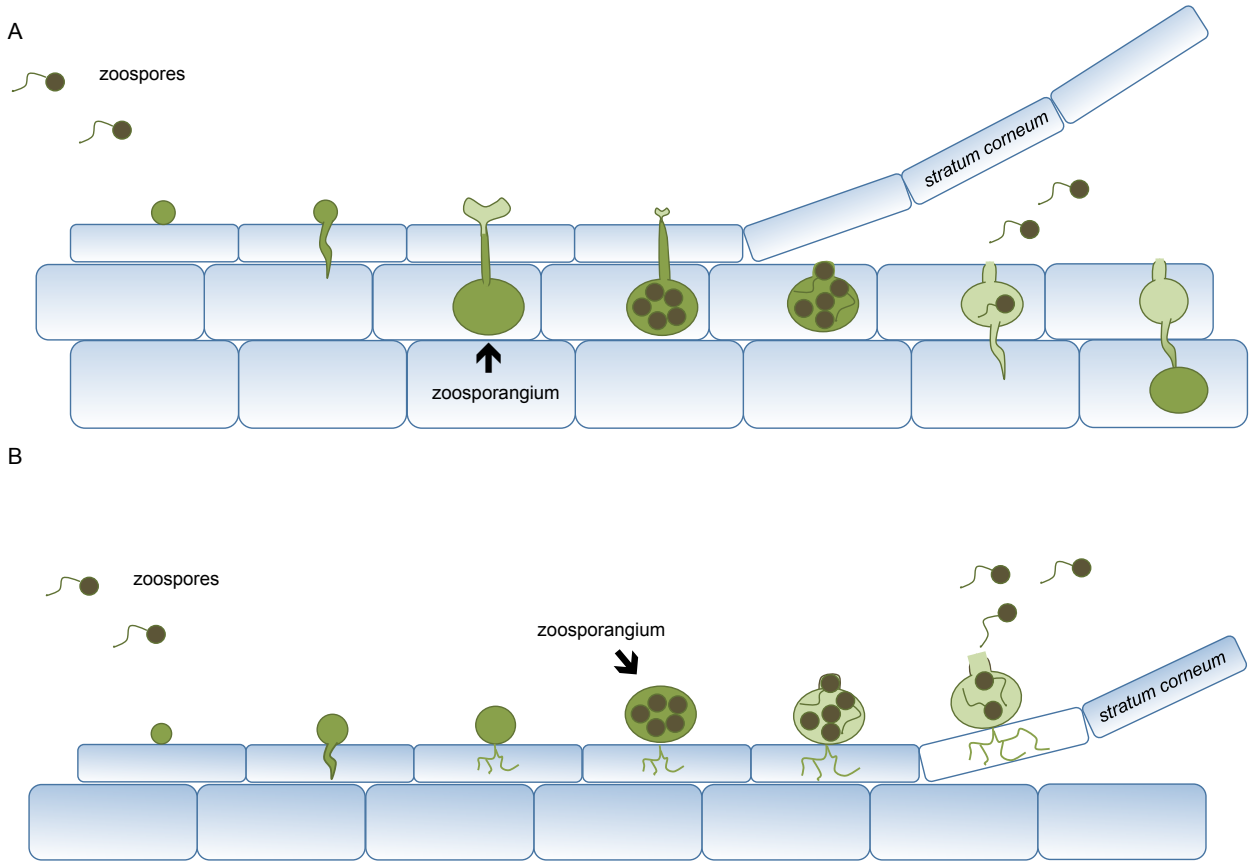

Figure 1.3 Schematic of the growth of *Batrachochytrium dendrobatidis* (Bd) in the amphibian host. (A) A zoospore encysts on the epidermal surface, and then produces a germ tube that penetrates into the *stratum corneum*, or through one or more cell layers. Cellular contents of the zoospore are transferred into the host cell, forming a zoosporangium. Depending on the intermoult interval of the host, the *stratum corneum* is eventually shed, and zoospores are released through a discharge papilla into the environment. Zoosporangia may also produce further germ tubes to infect deeper cell layers. (B) Alternative growth pattern of Bd on amphibian skin, in which zoosporangia grow epibiotically and are shed with the *stratum corneum* (adapted from Greenspan et al. 2012 and Van Rooij et al. 2015). Figure not to scale.

Like all organisms, Bd grows best in certain environments, and is very sensitive to high temperatures and drying. In culture, Bd can grow at temperatures as low as 4˚C, but grows optimally between 17-25˚C. With prolonged exposure (8+ h) to 30˚C, half of zoospores die, and reproduction ceases (Piotrowski et al. 2004). However, optimal growth in the amphibian host is likely dependent on the thermal performance curves (Rohr et al. 2013), and varying responses to temperature variability (Raffel et al. 2013), of both the parasite and its host. Furthermore, virulence of Bd can vary with the strain (Berger et al. 2005b; Farrer et al. 2011), and has been linked to that strain's growth rate, production of zoospores, and zoosporangia size in culture (Piovia-Scott et al. 2014; Berger et al. 2016), and strain phenotype, specifically zoosporangia size, can predict Bd prevalence in a population (Lambertini et al. 2016).

Chytridiomycosis in the amphibian host

Bd infection causes regions of skin thickening (hyperkeratosis), thinning (hypokeratosis), and cell proliferation (hyperplasia) within the *stratum corneum* and *stratum granulosum* of the amphibian epidermis (Berger et al. 1998; Pessier et al. 1999; Berger et al. 2005a). Invasion of host epidermal cells and development of zoosporangia results in reorganisation, vacuolisation and finally dissolution of the cell (Berger et al. 2005a; Figure 1.4). In severely infected individuals, the normal sloughing mechanism appears to be disrupted; some regions of the skin experience hyperkeratosis and an increase in epidermal cell turnover leading to an accumulation of up to four keratinised layers before sloughing, while other regions experience skin thinning, likely because the sloughing rate is faster than epidermal cell turnover and keratinization (Berger et al. 2005a; Figure 1.1B). Bd appears to be well suited to living in amphibian skin, because the timing of zoospore development follows the maturation of the epidermal skin layers, with the final stages occurring when infected cells are most superficial (Berger et al. 2005a; Voyles et al. 2011). This timing often results in self-reinfection of the parasitised host, which is necessary for Bd to reach lethal infection intensities (Ohmer 2011; Briggs et al. 2010). When re-infection rates are greater than the loss of zoosporangia from the frog's skin, exponential Bd growth occurs, leading to severe clinical signs and mortality in infected hosts (Briggs et al. 2010; Vredenburg et al. 2010). Clinical signs of Bd infection manifest in what appears to be excessive or irregular skin sloughing, anorexia or lack of appetite, lethargy, abnormal posture, cutaneous eurythema, dorsal cutaneous discolouration, and finally, loss of righting reflex (Nichols et al. 2001; Voyles et al. 2009).

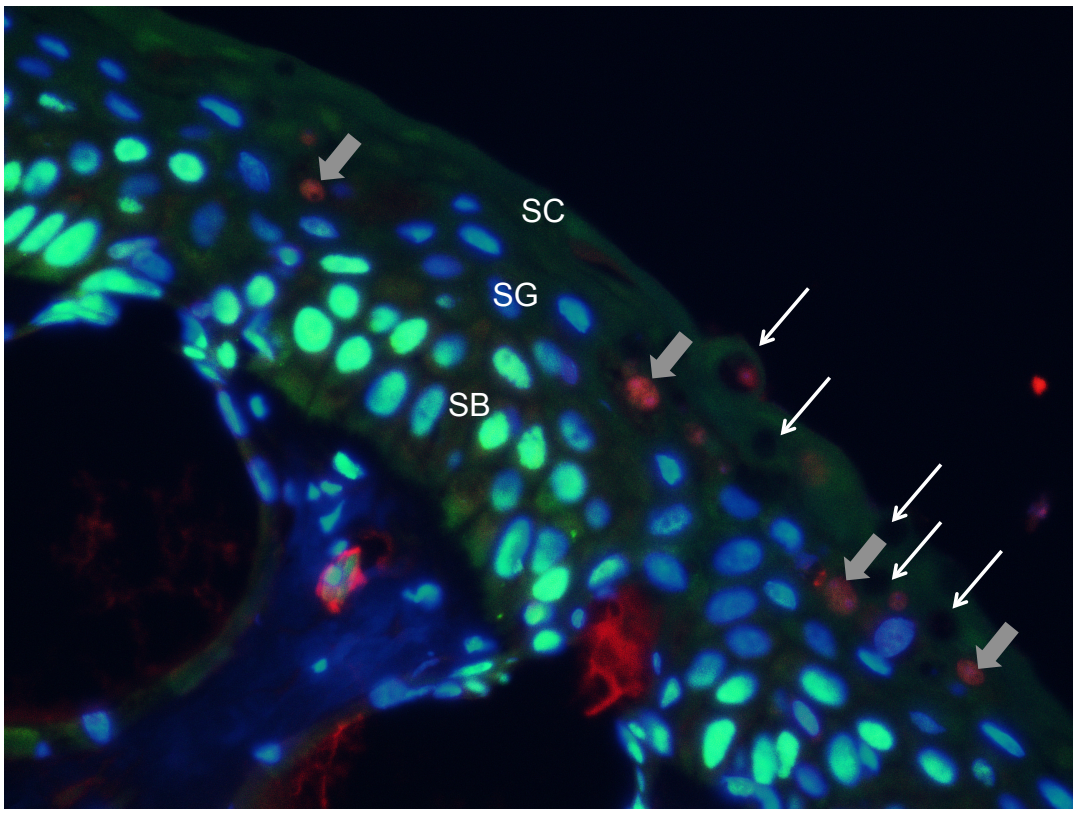

Figure 1.4 Epidermis of *Litoria caerulea* heavily infected with *Batrachochytrium dendrobatidis* (Bd), stained with immunofluorescently-labelled antibodies. Anti-caspase-3 antibody (red) indicates cellular death (apoptosis), anti-proliferating cell nuclear antigen (anti-PCNA) antibody (green) indicates cellular proliferation, and DAPI counterstain (blue) identifies nuclei. White narrow arrows = empty zoosporangia, thick grey arrows = cells infected with Bd, demonstrating cell death (red), SC = *stratum corneum*, SG = *stratum granulosum*, SB = *stratum basale*

How a fungal pathogen restricted to the superficial epidermis can cause mass mortality in its amphibian hosts has been the subject of great debate (Berger et al. 2005a). While the ultimate cause of mortality in severely infected frogs is still unknown, the proximate cause is thought to be a systematic loss of electrolytes, particularly Na^+, K^+, and Cl^-, resulting in reduced cardiac electrical activity (Voyles et al. 2009; Campbell et al. 2012). While Bd infection is thought to result in reduced Na^+ uptake via the skin, as of yet the mechanism behind K^+ wasting is unknown (Campbell et al. 2012). However, Rosenblum et al (2012) found evidence that ion imbalances are caused by indirect leakage of ions from the severely damaged epidermis, as demonstrated by a downregulation of skin integrity gene classes in the skin. Furthermore, increased haematocrit levels concomitant with a decrease in mass indicate dehydration in infected frogs in the wild (Voyles et al. 2012). If the process of skin sloughing is a physiologically vulnerable period for an amphibian, it may be that

this routine process of skin turnover could play a role in disease progression and pathophysiology. Interestingly, the role of sloughing and potentially an increase in sloughing rate during disease development has yet to be investigated directly.

Variation in susceptibility to chytridiomycosis

Variation in susceptibility to Bd is the combined result of the 'epidemiological triad': host and pathogen ecology/biology, and environmental cofactors (Fisher et al. 2009b). Both the host and the pathogen have ecological, biological, and physiological constraints and conditions in which they perform optimally, and different co-factors, such as climate and seasonality (Rohr & Raffel 2010a), population size and community structure (Searle et al. 2011a; Becker et al. 2014a), host behaviour (Richards-Zawacki 2010), and innate and acquired immunity (Ramsey et al. 2010), McMahon et al. 2014), may influence the host-pathogen relationship further (Daszak et al. 2003; James et al. 2015). Furthermore, not all strains of Bd may be equally virulent or resilient (Fisher et al. 2009a; Lambertini et al. 2016), and not all amphibian hosts are suitable or equal targets for infection (Searle et al. 2011b; Gahl et al. 2012; Ohmer et al. 2013; James et al. 2015).

While some amphibian species are considered generally very susceptible to mortality from chytridiomycosis, such as *Atelopus zeteki* (and likely many other species of this genus) in Central and South America (La Marca et al. 2005; Bustamonte et al. 2010; Becker et al. 2015; Ellison et al. 2015), *Rana muscosa* and *Rana sierrae* in North America (Briggs et al. 2010; Rosenblum et al. 2012), and Corroboree frog species (*Pseudophryne corroboree* and *P. pengilleyi*) in Australia (Hunter et al. 2010; Brannelly et al. 2015), others appear to be resistant to infection, given they can reduce or eliminate Bd infection loads. There is little definitive evidence of species demonstrating widespread resistance to chytridiomycosis, but some species appear to demonstrate greater resistance than most, including the notorious invader *Lithobates catesbeianus* (Gervasi et al. 2013; Eskew et al. 2015), and threatened species in the genus *Leiopelma* (Bishop et al. 2009; Ohmer et al. 2013), amongst others (Woodhams et al. 2007a; Gahl et al. 2012; Searle et al. 2011b). Furthermore, additional species are thought to be largely tolerant to infection, most notably the common laboratory species *Xenopus laevis* from Africa (Ramsey et al. 2010;Van Rooij et al. 2012), and *Pseudacris regilla* in California (Reeder et al. 2012). Tolerance is an alternative strategy for hosts to respond to a pathogen, in which the fitness effects of infection are reduced or controlled despite high pathogen burdens (Medzhitov et al. 2012, Råberg 2014).

Ecology and life history traits have been proposed to influence the survivorship of wild amphibians exposed to Bd, with species having small range sizes, restriction to high elevations, low clutch sizes, and a dependence on water having a greater risk of Bd-related declines (Kriger & Hero

2007; Bielby et al. 2008). Of course, a range of responses to infection occurs within species as well, with differences in susceptibility arising between individuals and populations. This may be driven by body condition (Beldomenico & Begon 2010; Ramsey et al. 2010), age or life stage (Longo & Burrowes 2010), and the capacity of the host to thermally acclimate relative to the pathogen (Raffel et al. 2013), or behaviourally thermoregulate (Richards-Zawacki 2010), in given environmental conditions. In addition, body size is thought to influence susceptibility within species, with smaller animals succumbing to Bd infection faster (Carey et al. 2006; Ohmer et al. 2013). This has been proposed to be the result of a surface-area dependent threshold in Bd infection intensity, with smaller frogs having a lower threshold and thus reaching that threshold faster (Carey et al. 2006). Finally, variation in immune defences, some of which have a genetic origin (i.e. immunogenetics, Savage & Zamudio 2011; Ellison et al. 2015), and their efficacy in fighting off Bd infection likely play a large role in the observed variation in susceptibility observed between species and populations (Richmond et al. 2009; Rosenblum et al. 2012).

Facets of the amphibian immune system

Amphibians have comprehensive immune systems with innate and acquired components (Carey et al. 1999; Richmond et al. 2009). In addition to the physical barrier of the epidermis, the mucus on the surface of the amphibian's skin is the first line of defence against an invading pathogen such as Bd (Rollins-Smith et al. 2011). This mucus contains secreted anti-microbial peptides (AMPs) produced by granular glands in the epidermis (Rollins-Smith et al. 2011), lyzozymes (Zhao et al. 2006), and antibodies that have been shown to bind to Bd zoospores in certain species (Ramsey et al. 2010). AMP activity against Bd zoospores in the laboratory has been shown to correlate with susceptibility to Bd infection in some species (Woodhams et al. 2007a), but not others (Rollins-Smith et al. 2006), indicating that AMPs may act differently on the amphibian host than in culture (Voyles et al. 2011). Skin mucus may also contain metabolic products produced by symbiotic bacteria, which have been shown to be species specific even among sympatric species (McKenzie et al. 2011), and variable in their efficacy to prevent Bd infection (Harris et al. 2009; Becker et al. 2011; Flechas et al. 2012b). Collectively, these skin defence components of the mucus have been termed the 'mucosome' (Woodhams et al. 2014). When measuring entire mucosome function against Bd, which includes both AMPs and bacterial symbionts, Woodhams et al (2014) found that higher Bd inhibition predicted lower infection risk in wild populations of the same species. Thus, a holistic view of innate immune defences found on the skin of amphibians may be helpful for understanding potential susceptibility to Bd.

To date, very few studies have demonstrated an acquired immune response to Bd in amphibians. The common laboratory species *Xenopus laevis*, which appears to be tolerant of Bd

infection, does produce Bd-binding antibodies in response to repeated Bd exposure (Ramsey et al. 2010), but the susceptible species *Rana muscosa*, *R. sierrae*, and *Silurana tropicalis*, do not demonstrate a robust immune response with Bd infection (Rosenblum et al. 2009; Rosenblum et al. 2012). It has been demonstrated that Bd may produce factors that reduce or evade the normal cell-mediated immune response (Rosenblum et al. 2008; Rollins-Smith et al. 2011), and recently Bd zoospores and supernatant were found to inhibit splenic lymphocyte functioning and proliferation, and increase apoptosis *in vitro* (Fites et al. 2013). However, certain amphibian species can acquire resistance in the form of reduced infection burden and mortality after repeated exposures to the pathogen (McMahon et al. 2014).

Finally, it has been suggested that variation in skin sloughing frequency may play a role in the observed inter and intraspecific variation in susceptibility to disease (Voyles et al. 2011; Meyer et al. 2012; Cramp et al. 2014). Given that heat therapy is known to clear infection in some species (Woodhams et al. 2003), and sloughing rate increases with temperature (Cramp et al. 2014; Meyer et al. 2012), higher temperatures may help individuals rid themselves of disease-causing organisms by increasing moult frequency (Berger et al. 2004). In addition, sloughing rate may increase in response to pathogen invasion. Thus, sloughing may play a role in amphibian immune defence.

Skin sloughing: both immune defence and sign of disease?

Recent work indicates that periodic skin sloughing results in up to a 100% short-term reduction of epicutaneous flora and fauna in amphibians (Meyer et al. 2012; Cramp et al. 2014). Thus, sloughing periodicity may play an important role in regulating the growth of Bd, and subsequent pathogenicity of the fungus in the host. Conversely, sloughing may also make an amphibian more susceptible to disease, by way of removing beneficial symbiotic bacteria, potentially interacting with the mucosal defences of the mucosome, and disrupting physiological homeostasis. Regardless, understanding innate variation in sloughing frequency across amphibian species may help to explain variation in susceptibility to disease.

First, however, we need to understand the sloughing-Bd relationship on an individual level. The routine mechanical process of skin turnover via sloughing might interact with a cutaneous pathogen, such as Bd, differently depending on the nature of Bd growth in the skin, the environmental context, and the physiological state of the amphibian. Anecdotal reports indicate an increase in the amount of sloughed skin associated with Bd infected hosts (either on the individual, or within enclosures; Berger et al. 1998; Lips 1999; Davidson et al. 2003; Bovero et al. 2008; Padgett-Flohr 2008; Becker & Harris 2010; Carver et al. 2010). This has been attributed to an increase in sloughing rate, and has been hypothesised to be beneficial if it removes unwanted

cutaneous pathogens (Davidson et al. 2003; Berger et al. 2004; Becker & Harris 2010; Greenspan et al. 2012). In fact, the initial establishment of Bd could be dependent on the timing of exposure to Bd with respect to the sloughing cycle. Exposure to Bd immediately before a sloughing event could potentially remove the pathogen entirely, or delay reinfection and an increase in infection intensity. This has been demonstrated in *Daphnia magna*, in which moulting within 12 h of exposure to the parasitic bacterium *Pasteuria ramosa* significantly reduced infection (Duneau & Ebert 2012). However, if sloughing does not occur soon after exposure, encysted Bd zoospores may have more time to produce germination tubes to penetrate into deeper epidermal cell layers, beneath the *stratum corneum* (Greenspan et al. 2012; Van Rooij et al. 2012).

The number of intermediate and replacement cell layers in an amphibian's skin may dictate the level of 'moulting plasticity' a particular species can endure (Greenspan et al. 2012). Those with more replacement cell layers, e.g. the American bullfrog (*Lithobates catesbeianus),* may be able to endure continual sloughing without losing cutaneous function (Greenspan et al. 2012). Furthermore, species-specific aspects of amphibian skin structure, or immune defences, may limit the growth of Bd intracellularly. For example, in skin explants of the Australian green tree frog (*Litoria caerulea*), Bd was able to penetrate the outer skin layers within hours of encysting on the skin surface, resulting in exclusively intracellular growth (Van Rooij et al. 2012). However, in skin explants of the African clawed frog (*Xenopus laevis*), a species tolerant of Bd infection, Bd infection remained epibiotic, and did not penetrate into deeper cell layers (Van Rooij et al. 2012). Whether this alternative growth pattern occurs in the whole host has yet to be determined (Van Rooij et al. 2015). In addition, premature keratinisation of infected replacement layers may aid the removal of encysted zoospores and zoosporangia during the sloughing process in some species (Greenspan et al. 2012).

Skin sloughing may also contribute to the pathophysiology of chytridiomycosis, given the temporary increase in skin permeability to water and electrolytes before, during and after the sloughing event, which may lead to a net sodium loss in an aquatic environment (Jørgensen 1949; Voyles et al. 2011). In a dry environment, sloughing may result in higher rates of evaporative water loss, and potentially dehydration (Appendix A). Given there is evidence of amphibians avoiding Bd-infected water (McMahon et al. 2014), and behaviourally thermoregulating when infected with Bd (Richards-Zawacki 2010), dehydration may be more likely to be observed in the heterogeneous environment experienced by wild amphibians than in a captive laboratory situation (Voyles et al. 2012). Furthermore, skin sloughing may be more or less effective at removing encysting Bd zoospores depending on the environment in which the host is sloughing. Aquatic and semi-aquatic species may be more likely to encounter zoospores in the environment than terrestrial species, and

sloughing location could influence pathogen colonisation success. Sloughing may also benefit the pathogen by revealing newly keratinised epidermis free of cutaneous symbiotic bacteria. It has been demonstrated that some symbiotic microbes on the skin of amphibians can prevent morbidity associated with Bd infection (Harris et al. 2009; Becker & Harris 2010; Becker et al. 2015), thus, sloughing may actually increase susceptibility of the epidermis to invading pathogens.

Thesis research framework

This research aims to provide a solid foundation for understanding the role of amphibian skin sloughing in immune defence and disease progression, specifically in the face of the generalist fungal pathogen, Bd. It has been asserted that chytridiomycosis is responsible for the greatest loss of amphibian biodiversity in recorded history (Skerratt et al. 2007), and has resulted in both population extirpations and species extinctions in Australia, and worldwide (see Fisher et al. 2009b). With over 32% of amphibian species threatened with extinction, amphibians are now considered one of the most endangered classes of vertebrates on the planet (IUCN 2008). Understanding what drives innate variation in susceptibility to infection with Bd, and disease development, can greatly advance conservation efforts. In order to fully understand an entirely cutaneous pathogen, such as Bd, and how it interacts with the amphibian host, the ubiquitous mechanism of skin sloughing cannot be ignored. While previous work has suggested that sloughing might remove zoosporangia embedded in the superficial *stratum corneum* and regulate infection loads, as indicated by highly variable infection intensities over time in severely diseased individuals (Berger et al. 2005a; Olsen et al. 2004), no one has investigated the mechanism further.

Despite anecdotal reports that Bd infection results in increased sloughing in amphibians (Davidson et al. 2003), there is little evidence to support this claim directly. This is largely because it is unclear whether sloughing rate actually increases, or if ingestion of sloughed skin decreases, or if sloughed skin is being removed in many small pieces, rather than as a whole, making it seem more frequent (Meyer et al. 2012). Relying on counting sloughed skin pieces within enclosures has led to this confusion (Davidson et al. 2003; Padgett-Flohr 2008). By developing a novel skin marking technique combined with the use of closed-circuit surveillance cameras, the exact timing of the sloughing event itself could be pinpointed, rather than using indirect evidence, such as the counting of shed sloughs. Using this technique, the intraspecific variation in sloughing frequency in healthy animals, as well as how sloughing changes with disease progression in infected animals, could be examined. With an exact understanding of when frogs are sloughing and the timing of the sloughing cycle, questions could be addressed that have largely eluded researchers thus far.

Furthermore, by combining visual monitoring of sloughing frequency and behaviour with quantitative genetic testing (qPCR) for Bd before and after sloughing occurs, the effect of a sloughing event on individual infection load can be investigated. Since Bd is largely restricted to the outer skin layers that are shed periodically, the capacity to reduce fungal burdens (possibly entirely) may explain some of the temporal variability in Bd infection intensity that has been reported (Berger et al. 2004; Ohmer et al. 2013). It might also explain why some species, potentially those with a higher intrinsic rate of sloughing, might be able to 'self cure' following an exposure event. Furthermore, the nature of Bd growth may vary depending on the amphibian host; thus, skin sloughing could be more effective at reducing fungal load in hosts in which Bd grows epibiotically (Van Rooij et al. 2012).

Sloughing frequency can vary widely among amphibian species, ranging from every day to every other week (see Ling 1972) and may play an important role in determining whether or not Bd exposure results in infection. Many studies have attempted to infer the extinction risk of amphibians based on their macroecology (Cooper et al. 2008; Hero et al. 2005), or a combination of ecology, life history and environmental context (Sodhi et al. 2008; Bielby et al. 2008). Some studies have gone a step further, and integrated documentation of Bd infection in the wild or the pathogen's predicted worldwide distribution into decline risk (Bielby et al. 2008; Rödder et al. 2008; Sodhi et al. 2008). Importantly, these studies have incorporated phylogenetic relatedness into their comparative analyses, given that species are not statistically independent units that share common evolutionary histories (Felsenstein 1985). Given our scarce understanding of the variation in sloughing rate across amphibian species, the first step required is measuring sloughing rates over a global subset of amphibian species.

By comparing sloughing rates across a range of frog species with different susceptibilities to chytridiomycosis and evidence of Bd-driven declines on a global scale, inferences can be made about the effects of intermoult interval on disease susceptibility within a phylogenetic comparative context. Furthermore, sloughing rate, combined with measures of variation in epidermal thickness between species using museum specimens and histological techniques, can indicate whether the number of replacement epidermal cell layers dictates the amount of moulting plasticity a species can endure (Greenspan et al. 2012). Those species that experience high susceptibility to Bd in the laboratory should have fewer replacement epidermal cells layers, while those that can tolerate or resist Bd infection would benefit by having more replacement cell layers, in order to slough more often without physiological harm.

Thus far, no studies have focussed on skin structure and function with respect to observed variation in susceptibility to Bd. Given that Bd infects a wide range of amphibian hosts, there are

likely to be notable differences in skin structure between species. My approach to this question is novel in that it utilises amphibians already in captivity in zoos and breeding centres, largely as a result of disease susceptibility in the wild, to measure sloughing rates across a range of representative taxa. There is little baseline information on sloughing rate in amphibian species, thus the collection of this data has formed the first database on sloughing behaviour.

Aims of research and structure of thesis

The overall aim of this thesis is to increase our knowledge of the host-pathogen relationship, particularly with respect to the poorly understood phenomenon of skin sloughing in amphibians, and its possible role in innate host defence. This work helps clarify the relationship between sloughing and Bd infection progression, which until now had remained elusive (Voyles et al. 2011), at the individual, species, and global scale. A greater understanding of the factors driving observed variation in intra- and interspecific susceptibility can better inform management of chytridiomycosis in wild populations, particularly by helping to make predictions about species declines in newly affected areas.

This thesis is comprised of three experimental chapters (chapters 2-4), which are written as independent manuscripts comprising an abstract, and an introduction, methods, results, and a discussion. Chapter 2 is published in *Functional Ecology* (Ohmer et al. 2015), and investigates the relationship between Bd infection intensity and sloughing rate in the model species *Litoria caerulea.* Chapter 3 then expands on this understanding by comparing the efficacy of sloughing in reducing Bd load in five Southeast Queensland amphibian species with varying levels of susceptibility to chytridiomycosis. Next, in chapter 4, sloughing rates and epidermal thicknesses were measured across over twenty frog species to enable a phylogenetic comparison of sloughing rate and susceptibility to Bd on a global scale within the Anura.

The final chapter of this thesis discusses the implications of this work in the context of amphibian conservation, and expands upon future research directions.

All experiments were approved by the University of Queensland Animal Ethics Committee (Approval Number: SBS/452/12/URG), and animals were collected under Scientific Purposes Permit WISP12218412.

CHAPTER 2

Skin sloughing rate increases with chytrid fungus infection load in a susceptible amphibian

Abstract

Amphibian chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), is responsible for the greatest disease-driven loss of vertebrate biodiversity in recorded history. Understanding drivers of host susceptibility to this cutaneous disease is hindered by gaps in our knowledge of the host-pathogen relationship. One such overlooked aspect of susceptibility is variation in skin maintenance processes, particularly skin turnover via routine sloughing. It has been suggested that sloughing plays a role in immune defence, by removing skin-associated microbes. Thus, skin sloughing may play an important role in the pathogenesis of chytridiomycosis. To determine the relationship between skin sloughing and disease progression, we exposed adult Australian green tree frogs (*Litoria caerulea*) to a local Bd strain, and monitored sloughing rates and individual infection load on a naturalistic cycling temperature regime (15 - 23˚C). We determined sloughing rates in real time by using an array of infrared video cameras to film frog behaviour, and monitored infection load before and after sloughing by swabbing and analysis with quantitative PCR. We found that sloughing rate increased with Bd infection load in infected frogs, but sloughing itself did not affect Bd load on the ventral skin surface. Furthermore, Bd infection did not affect the duration of characteristic sloughing behaviour and sloughing retained rhythmicity even at high infection loads. Although an increased sloughing rate might be considered advantageous for Bd-infected animals, it does not appear to curb the progression of disease and may actually contribute to the loss of physiological homeostasis seen in terminally ill frogs by further inhibiting water and electrolyte transport across the skin. By measuring sloughing rates directly for the first time, our results shed light on how Bd interacts with the physiological processes of the skin, and indicate that variation in skin sloughing frequency may play a role in the observed inter- and intraspecific variation in susceptibility to disease.

Introduction

While fungal pathogens have long been considered a threat to global plant species, particularly in agriculture, devastating effects of pathogenic fungi on animals have only recently been documented (Jones et al. 2008; Fisher et al. 2012). One pervasive example is *Batrachochytrium dendrobatidis* (Bd), a skin-invading fungal pathogen of amphibians that has

driven more vertebrate biodiversity loss than any other pathogen in recorded history (Stuart et al. 2004; Skerratt et al. 2007). Bd has been reported to have infected over 500 species worldwide (Kilpatrick et al. 2010b; http://www.bd-maps.net/surveillance/; Olson et al. 2013), but not all amphibian species are equally susceptible, and some populations persist with disease, while others decline (Alford & Richards 1999; Woodhams & Alford 2005; Lips et al. 2006; Kriger et al. 2007; Van Sluys & Hero 2009; Briggs et al. 2010; Tobler & Schmidt 2010; Ohmer et al. 2013). Bd only infects the superficial layers of an amphibian's skin, and yet exponential growth of the fungus can lead to disruption of cutaneous functioning and eventually mortality (Voyles et al. 2009). While there have been many advances in understanding the ecology and epidemiology of this disease, research is still hindered by a lack of basic biological understanding of the host-pathogen relationship (Voyles et al. 2011). Thus, in order to ultimately understand variation in susceptibility across species, we need to better understand the processes governing amphibian skin function in the presence of disease.

Amphibian skin is a dynamic organ that functions in osmoregulation, ion and acid-base balance, and gas exchange (Boutilier et al. 1992). Considered more permeable than the skin of most vertebrates, the amphibian integument plays a vital role in maintaining physiological homeostasis (Boutilier et al. 1992; Voyles et al. 2010). To keep skin functioning and in optimal condition, the thin outer layer of keratinised skin cells, or *stratum corneum*, is regularly shed and replaced in a process called sloughing, the last step in epidermal turnover (Barker Jørgensen 1988). Healthy frogs slough regularly, anywhere from every day to every other week, and sloughing rate varies across species and is positively correlated with temperature (Stefano & Donoso 1964; Castanho & de Luca 2001; Meyer et al. 2012; Cramp et al. 2014). Recent work indicates that periodic skin sloughing results in up to a 100% short-term reduction of cutaneous flora and fauna (Meyer et al. 2012; Cramp et al. 2014). Thus, sloughing periodicity may play an important role in regulating the establishment and growth of Bd on the skin, and subsequent pathogenicity.

The routine process of sloughing might be beneficial or detrimental in the face of a cutaneous pathogen, such as Bd. First, given that heat therapy is known to clear Bd infection in some species (Woodhams et al. 2003), and sloughing rate increases with temperature (Meyer et al. 2012; Cramp et al. 2014), higher temperatures may help individuals rid themselves of disease by increasing moult frequency (Berger et al. 2004). But while sloughing has been hypothesised to be beneficial if it removes unwanted cutaneous pathogens (Davidson et al. 2003; Berger et al. 2004; Becker & Harris 2010; Greenspan et al. 2012; Meyer et al. 2012), if may also be harmful if excessive sloughing contributes to water and electrolyte imbalance (Jørgensen 1949; Voyles et al. 2011). In addition, sloughing may further disease progression by revealing newly keratinised

epidermis that is free of cutaneous symbiotic bacteria. It has been demonstrated that some symbiotic microbes on the skin of amphibians can prevent morbidity associated with Bd infection (Harris et al. 2009; Becker & Harris 2010), thus sloughing may actually increase susceptibility to disease.

Secondly, if sloughing effectively eliminates the microbial community on the skin of an amphibian, infection outcome may be dependent on the timing of exposure with respect to the sloughing cycle. Exposure immediately before a sloughing event could prevent immediate establishment of the pathogen, delaying reinfection and an increase in infection load, and possibly remove the pathogen entirely (Duneau & Ebert 2012). Conversely, exposure directly after a sloughing event may render an individual more vulnerable, given that zoospores would have more time to encyst in the deeper epidermal cell layers before the next sloughing event occurs (Greenspan et al. 2012; Van Rooij et al. 2012).

Finally, exposure to and subsequent infection with Bd may directly interrupt or impair the sloughing mechanism. This is consistent with many observations of 'abnormal sloughing' in infected amphibians, including sloughing frequently and in small pieces (Davidson et al. 2003). However, sloughing in small pieces at the point of pathogen invasion may also be an innate immune response (Dahl 1993), and thus extremely important in ridding amphibians of localised infections in the early stages of pathogen invasion.

While abnormal sloughing is a symptom of chytridiomycosis in terminally ill animals (Berger et al. 1998; Longcore et al. 1999), there is still speculation about the relationship between disease progression and changes in skin sloughing before exponential pathogen growth on the skin occurs. Anecdotal reports indicate an increase in the amount of sloughed skin associated with Bd infected hosts (either on the individual, or within enclosures; Berger et al. 1998; Davidson et al. 2003; Bovero et al. 2008; Padgett-Flohr 2008; Becker & Harris 2010; Carver et al. 2010). This has been attributed to an increase in sloughing rate, but there is little evidence to support this claim directly. It is unclear whether sloughing rate actually increases following Bd infection, or if ingestion of sloughed skin decreases, or if sloughing is occurring in many small pieces, rather than as a whole, making it seem more frequent (Meyer et al. 2012). This confusion arises because of a reliance on observational data obtained by counting sloughed skin pieces within enclosures (Davidson et al. 2003; Padgett-Flohr 2008); such an approach cannot discriminate among the possible causes of an increase in the amount of sloughed skin associated with Bd infected hosts.

Figure 2.1 An adult Australian green tree frog (*Litoria caerulea*). Photo: M. E. B. Ohmer

In the present study we overcome previous limitations by quantitatively examining the relationship between skin sloughing and Bd infection progression in the susceptible frog species, *Litoria caerulea* (Figure 2.1). By utilising infrared cameras to record behaviours, we were able to accurately measure sloughing rates in infected and healthy amphibians. First, we hypothesised that sloughing rates would increase in Bd infected frogs, possibly indicating an immune response. Second, we hypothesised that exposure timing with respect to the sloughing cycle would influence infection outcome, with frogs exposed just after sloughing more likely to develop infection than those exposed just before sloughing. Finally, because sloughing can reduce cutaneous microbial loads (Meyer et al. 2012), we hypothesised that Bd infection load would be lower immediately following a sloughing event. By measuring sloughing rates directly for the first time, we provide a much clearer picture of how Bd interacts with the physiological processes of the skin, and shed

light on how variation in skin sloughing frequency may play a role in the observed inter- and intraspecific variation in susceptibility to disease (Voyles et al. 2011; Meyer et al. 2012).

Methods

Animal collection and husbandry

This study was conducted with the widespread Australian green tree frog (*Litoria caerulea*). *L. caerulea* may be considered a model species for chytridiomycosis research, given the disease was originally described in this species (Berger et al. 1998; Pessier et al. 1999), and it experiences high infection intensities and mortality as a result of Bd exposure in the laboratory (Berger et al. 2005b; Voyles et al. 2007; Voyles et al. 2009). *L. caerulea* breeds in shallow ephemeral water bodies over the summer months, but adults are usually found in thick vegetation or anthropogenic structures far from water throughout the year. They are widespread in the coastal and arid regions of northern and eastern Australia (Tyler & Knight 2011). Dead and dying *L. caeruela* infected with Bd have been found in the wild, although no population declines have been recorded for this species (Berger et al. 1998; Berger et al. 2004; Commonwealth of Australia 2006).

Adult *L. caerulea* (n = 21, SVL (mm) = 70.1 ± s.d. 5.0, both sexes) were collected from wet roads in non-protected areas of southeast Queensland. Sterile gloves were worn when handling animals, and gloves were changed between frogs to prevent the possibility of disease transmission. Frogs were placed (individually) in moistened plastic bags and transported to The University of Queensland. Upon return, frogs were housed individually in ventilated clear plastic containers (26.2 x 23.7 x 12 cm) on a substrate of paper towels saturated with 100 - 150 ml of aged tap water and PVC pipe for shelter. On a weekly basis, frogs were fed five large crickets and enclosures were cleaned. Lighting and temperature regime replicated natural conditions, with a 12 hour photoperiod and daily temperatures increasing from an overnight minimum of 15˚C, to a daytime maximum of 23˚C (Figure 2.2).

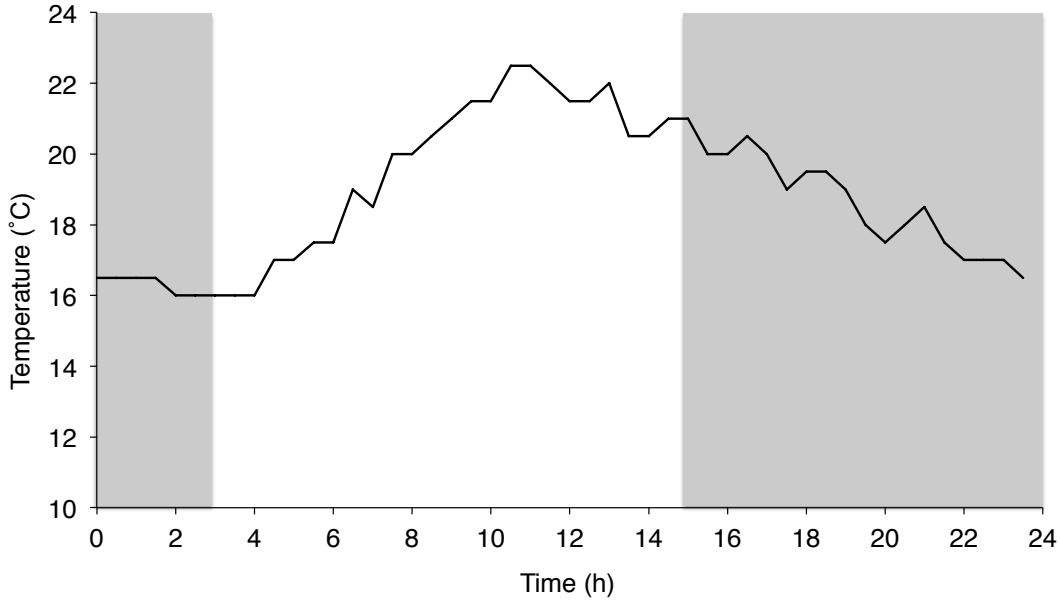

Figure 2.2 Lighting and temperature regime for the housing of experimental animals, *Litoria caerulea,* to mimic natural conditions (15 - 23 ˚C, mean = 18.8 ˚C). Shaded areas indicate when lights were off.

Inoculum preparation and experimental exposure

Bd strain EPS4 isolated by E.P. Symonds (The School of Veterinary Sciences, The University of Queensland) from a *Mixophyes fleayi* tadpole in March 2012, originating from Gap Creek, Main Range National Park, Queensland, Australia, was used for experimental infection. This strain was utilised because it originated from the same region as the study animals. Cultures were maintained at 4˚C until four days before exposure date. The strain was then passaged onto 25 new 1% agar, 0.5% tryptone, 0.5% tryptone-soy plates, to be maintained at 21˚C. After four days, zoospores were harvested by flooding plates with sterile distilled water for 30 min with periodic gentle agitation. Zoospore suspension was then collected, and zoospore concentration calculated using a haemocytometer following Boyle et al. (2004).

Frogs were randomly assigned to Exposed (n =11) and Control (n = 10) groups. Exposed frogs were exposed to a dose of ~250,000 Bd zoospores in 40 ml aged tap water within 300 ml plastic containers (12.5 x 8.3 x 5 cm) for 5 h (Berger et al. 2005b; Ohmer et al. 2013). Control frogs were treated in the same manner, but exposed to aged tap water containing no zoospores. After exposure, frogs were returned to their enclosures.

Sloughing monitoring

We employed a closed-circuit infrared continuous video monitoring system (Generic 16 channel H.264 digital video recorder, model MDR688ZB (AU)-E, 600TVL Weatherproof infrared cameras, model CI20B-65H) to record when frogs sloughed their skin. Sloughing monitoring began two weeks before experimental exposure to determine the baseline sloughing rate for each individual. In addition, frogs were marked with a small amount of non-toxic waterproof ink on their dorsal surface and checked twice daily to record the disappearance of a mark, which would indicate that sloughing had occurred. Once a mark disappeared, it was reapplied. This marking system helped to pinpoint when to review the recorded video to confirm the occurrence of sloughing. The time in hours between sloughing events was termed the intermoult interval (IMI).

Measuring infection load

Given the possibility of infection in natural *L. caerulea* populations, frogs were tested for Bd using quantitative PCR prior to beginning the experiment (Boyle et al. 2004; Hyatt et al. 2007). Infection load was then monitored by swabbing all frogs beginning two weeks after exposure, and then monthly and opportunistically before and after select sloughing events. The swabbing protocol involved firmly running a sterile fine-tipped cotton swab (MW100-100; Medical Wire & Equipment, Wiltshire, England) three times over the frog's ventral surface (including drink patch), sides, thighs, feet, webbing, and toes (Kriger et al. 2006; Retallick & Miera 2007). Swabs were then extracted in 50 µl PrepMan Ultra (Applied Biosystems, Foster City, CA, USA) and analysed in triplicate with quantitative PCR (Boyle et al. 2004; Hyatt et al. 2007), on a Mini Opticon real-time PCR detection system (MJ Mini Cycler, Bio-Rad Laboratories, Inc.) to determine infection load in zoospore equivalents (ZE). A modified 15 µl reaction volume was used for cost efficiency (Garland et al. 2010). Specifically, we followed the protocol of Boyle et al (2004), but with a modified 15 µl reaction volume (7.5 µl TaqMan Universal PCR master mix, 0.54 µl ITS1-3 Chytr primer, 0.54 µl 5.8S Chytr primer, 0.3 µl Bovine serum albumin (BSA), 0.15 µl TaqMan MGB probe, 0.97 µl MilliQ water, and 5 µl diluted (1:10) sample DNA; Ohmer 2011). BSA was added to reduce inhibition (Garland et al. 2010).

In order to measure infection load before and after sloughing occurred, sloughing events were predicted based on their cyclical nature and frogs were swabbed before and after. It was determined that sloughing events were predictable after watching many hours of video footage. Frogs often sloughed at the same time of day, on a consistent cycle. This allowed us to predict sloughing events with some accuracy, and swab frogs before and after a sloughing event occurred. We confirmed that frogs were swabbed before or after a sloughing event by reviewing the video

footage. If a sloughing event did not occur between predicted before and after swabs, those swabs were excluded from analyses. Timing of swabs ranged from 3 min to 55 h before or after the sloughing event, so time between sloughing and swabbing was taken into account in analyses.

Frogs were monitored daily for clinical signs of chytridiomycosis, including lethargy, inappetence, abnormal posture, pieces of sloughed skin not fully removed or visible within enclosure, and loss of righting reflex, as well as discoloured or reddened skin, and weight loss (Nichols et al. 1998; Daszak et al. 1999; Nichols et al. 2001). If a frog demonstrated advanced clinical signs, including slow righting reflex and body mass loss, it was removed from the experiment and treated with 20mg L^{-1} topical chloramphenicol, placed in an incubator at 28˚C for up to 14 days (Young et al. 2012), and given oral 12% Whitaker-Wright solution (242 mMol l^{-1} NaCl, 4.3 mMol l^{-1} $MgSO_47H_20$, 2.85 mMol l^{-1} $CaCl_2$, 2.85 mMol l^{-1} KCl) daily to help correct electrolyte imbalance (Voyles et al. 2009).

Statistical analyses

All analyses were performed in the program R (R Core Team 2013). Change in IMI from pre- to post-exposure among Control, Infected (exposed frogs that tested positive for Bd) and Uninfected (exposed frogs that tested negative for Bd) frogs was compared with a linear mixed effects model (function 'lme', package 'nlme', Pinheiro et al. 2013). IMI is defined as the time in hours between sloughing events; the shorter the IMI, the faster the sloughing rate for an individual frog. Δ*IMI* (IMI - mean pre-exposure IMI) was the response variable, and fixed effects included *Group* (control, infected, or uninfected), *Cycle* (slough cycle number post-exposure*),* and the interaction between these effects. *Frog ID* was included as a random effect to take into account the correlated error from taking multiple measurements on the same individual. All model fitting was performed using Maximum Likelihood (Pinheiro & Bates 2000), and comparisons of nested models were performed with likelihood ratio tests (function 'anova', R base package [51]).

Exposed frogs were later divided into an additional three categories based on infection outcome: Uninfected, Nonclinical, and Clinical. Uninfected frogs never tested positive for Bd, and never demonstrated clinical signs of chytridiomycosis. Nonclinical frogs tested positive for Bd, but never demonstrated clinical signs during the experimental period, whereas Clinical frogs tested positive and demonstrated clinical signs. To compare the change in Bd load in zoospore equivalents (ZE, averaged from triplicate results) over time in the Clinical and Nonclinical groups, we fitted a linear mixed effects model (function 'lme', package 'nlme', Pinheiro et al. 2013) including *Group* (Clinical or Nonclinical), *Days post-exposure*, *Days post-exposure*^{2} (quadratic term), and the random effect *Frog ID*. The response variable, Bd load, was log +1 transformed to normalise the

data. The relationship between Bd load (ZE) over time and IMI in Clinical frogs was also compared with a linear mixed effects model, with *Frog ID* as a random effect.

Infection load (ZE) pre and post sloughing was compared for multiple sloughing events per frog to determine if infection load was reduced following a sloughing event. *Before* and *After* Bd infection loads (ZE) were compared with a linear mixed effects model (function 'lme', package 'nlme', Pinheiro et al. 2013), including *Days post-exposure* and *Time (hours) between swab and sloughing event* as fixed effects, and *Frog ID* as a random effect.

To determine if the time (hours) to first sloughing event post-exposure influenced the *Time to endpoint* (days, log-transformed) for Clinical frogs during the experimental period, a linear regression was performed (function 'lm', base package, R Core Team 2013). The endpoint was removal from the experiment for treatment after the development of advanced clinical signs. A mixed-effects model was also used to assess the relationship between *Mass (g)* or *SVL (mm)* and both Δ*IMI* and *IMI* for all frogs, with *frog ID* as a random effect. Finally, the relationship between frog *Mass* (g) and *Time to endpoint* (days was also assessed with a linear regression (function 'lm', R base package, R Core Team 2013).

Results

Experimental exposure

All frogs were Bd negative prior to the start of the experiment. Control frogs (n = 10) remained healthy and Bd negative for the duration of the experiment. Only two Exposed frogs never tested positive for Bd infection, and are referred to as Uninfected. Of the remaining exposed frogs (n = 9), five developed advanced clinical signs of chytridiomycosis between 62 and 83 days post-exposure, while an additional two did not develop these signs until 133 - 189 days post-exposure. Three Clinical frogs succumbed rapidly to infection even with treatment and died, but four were successfully treated for chytridiomycosis and cleared infection. The remaining two exposed frogs became infected initially, but later cleared that infection, and were considered Nonclinical.

Sloughing behaviour

Observed sloughing behaviour was consistent across individuals and aligns with previously published descriptions (Taylor & Ewer 1956; Larsen 1976; Barker Jørgensen 1988). Characteristically, the physical behaviour of removing the *stratum corneum*, as observed on infrared video recordings, was as follows. Prior to sloughing, frogs became inactive, and breathing

rate noticeably increased. Immediately before the active sloughing phase, individuals assumed a hunched posture, with front limbs extending in front of the head and back arched. During sloughing, an individual frog rhythmically moved the sides of its body, open and closed its mouth, and pushed its arms and legs over its dorsum and towards its mouth. This motion brought the sloughed skin into the corners of the frog's mouth, allowing it to be ingested (see example Video S1 at http://onlinelibrary.wiley.com/doi/10.1111/1365-2435.12370/full). The duration of sloughing lasted from 3-25 minutes, with a median of 7 minutes. Prior to experimental exposure, one individual in the exposed group sloughed for 121 minutes on one occasion, but this was an extreme outlier.

Healthy individuals and clinically diseased individuals demonstrated similar sloughing behaviours. However, animals that were heavily infected with Bd and demonstrating clinical signs of disease displayed difficulty in performing the sloughing action. Movement of the hands and feet across the body often propelled the animal across its enclosure or off of the PVC pipe provided for shelter (see example Video S2 at http://onlinelibrary.wiley.com/doi/10.1111/1365-2435.12370/full). Despite this, the duration of sloughing (min, log-transformed) was not significantly longer in exposed frogs (linear mixed-effect model with *Group* (Control or Exposed) and *Slough number* as fixed effects and *Frog ID* as a random effect: *Group* (Exposed), $\beta = 0.031$, s.e. = 0.061, $p = 0.61$, 95% CI = -0.09 – 0.16), but duration did decrease over time in both groups (*Slough number,* $\beta = -0.007$, s.e. = 0.0025, $p = 0.0049$, 95% CI = -0.01 – -0.0021; Table 2.1). As an indication of sloughing quality, we recorded the number of skin pieces found in enclosures. The number of skin pieces found in exposed frog enclosures was significantly greater than the number of skin pieces found in control frog enclosures (linear mixed-effect model with *Group* (Control or Exposed) and *Week* as fixed effects and *Frog ID* as a random effect: $\beta = 0.65$, s.e. = 0.19, $p = 0.0031$, 95% CI = 0.25 – 1.06; Table 2.2). This indicates that the *stratum corneum* of exposed frogs was no longer being sloughed in one piece, or the action of sloughing was less effective in exposed frogs.

Table 2.1 Statistical results from a linear mixed effects model examining sloughing duration (min, log-transformed) in control *Litoria caerulea* and those exposed to the pathogen *Batrachochytrium dendrobatidis.* Fixed effects were group (Control [n = 10] or Exposed [n = 11]) and Slough number, and Frog ID was included as a random variable to take into account correlated error from repeated measures on the same individual. s.e. = standard error, s.d. =standard deviation, d.f. = degrees of freedom, bold p-values are significant

Fixed effects	**Estimate**	**s.e.**	**t-value**	**d.f.**	***p***	**Confidence intervals**	
						2.5%	**97.5%**
Intercept	2.07	0.053	39.06	446	**<0.0001**	1.97	2.18
Group (Exposed)	0.031	0.061	0.52	21	0.61	-0.09	0.16
Slough number	-0.0070	0.0025	-2.82	446	**0.0049**	-0.01	-0.0021
Random effects	**s.d.**				**Residual**		
Frog	0.12				0.35	0.08	0.18

Table 2.2 Statistical results from a linear mixed effects model examining the number of skin pieces found in enclosures of control *Litoria caerulea* and those exposed to the pathogen *Batrachochytrium dendrobatidis*. Fixed effects were group (Control [n = 10] or Exposed [n = 11]) and week (since starting experiment), and Frog ID was included as a random variable to take into account correlated error from repeated measures on the same individual. s.e. = standard error, s.d. =standard deviation, d.f. = degrees of freedom, bold p-values are significant

Fixed effects	**estimate**	**s.e.**	**t-value**	**d.f.**	***p***	**Confidence intervals**	
						2.5%	**97.5%**
Intercept	0.37	0.21	1.76	330	0.079	-0.04	0.78
Group (Exposed)	0.65	0.19	3.39	19	**0.0031**	0.25	1.06
Week	-0.020	0.011	-1.79	330	0.074	-0.042	0.0018
Random effects	**s.d.**				**Residual**		
Frog	0.30				1.31	0.14	0.65

Change in intermoult interval (IMI)

Infected frogs demonstrated a significant negative change in IMI for successive sloughs post-exposure, indicating an increase in sloughing rate. IMI in infected frogs decreased from a mean of 4.04 days (± 0.43 s.d.) to 3.30 days (± 0.43 s.d.) over the course of the experiment. There were main effects of *Group (Infected)* ($\beta = 7.98$, s.e. = 3.27, $p = 0.025$, 95% CI = 1.16-14.80) and an interaction of *Group (Infected)*Cycle* ($\beta = -1.33$, s.e. = 0.15, $p < 0.00001$, 95% CI = -1.64– -1.04) in the model comparing the change in IMI over time between Control, Infected, and Uninfected frogs (Table 2.3). These results demonstrate that IMI changed over time for the infected

group (Figure 2.3). In comparison, IMI did not significantly change for Control (mean = 3.68 d ± 0.42 s.d.) and Uninfected frogs (mean = 3.99 d ± 0.20 s.d.; Figure 2.3). Of all sloughing events, 70% occurred within four hours of the lights turning off in both Control and Exposed frogs (Figure 2.4).

Table 2.3 Statistical results from a linear mixed effects model examining the change in intermoult interval (ΔIMI, h) in *Litoria caerulea* after exposure to the pathogen *Batrachochytrium dendrobatidis* or sham-exposure. Fixed effects included cycle (number of sloughing cycles post exposure) and group (infected [n = 9], uninfected [n = 2], or control [n = 10]). Frog ID was included as a random variable to take into account correlated error from repeated measures on the same individual. s.e. = standard error, d.f. = degrees of freedom, bold p-values are significant

Fixed effects	**estimate**	**s.e.**	**t-value**	**d.f.**	***p***	**Confidence intervals**	
						2.5%	**97.5%**
Intercept	2.02	2.25	0.90	364	0.37	-2.37	6.42
Group (Infected)	7.98	3.27	2.44	18	**0.025**	1.16	14.80
Group (Uninfected)	0.56	5.57	0.010	18	0.92	-11.05	12.16
Cycle	-0.047	0.11	-0.44	364	0.66	-0.26	0.16
Group (Infected) * Cycle	-1.33	0.15	-8.67	364	**<0.00001**	-1.64	-1.04
Group (Uninfected) * Cycle	0.14	0.28	0.50	364	0.62	-0.41	0.70
Random effects	**s.d.**				**Residual**		
Frog	6.00				7.79	4.31	8.35

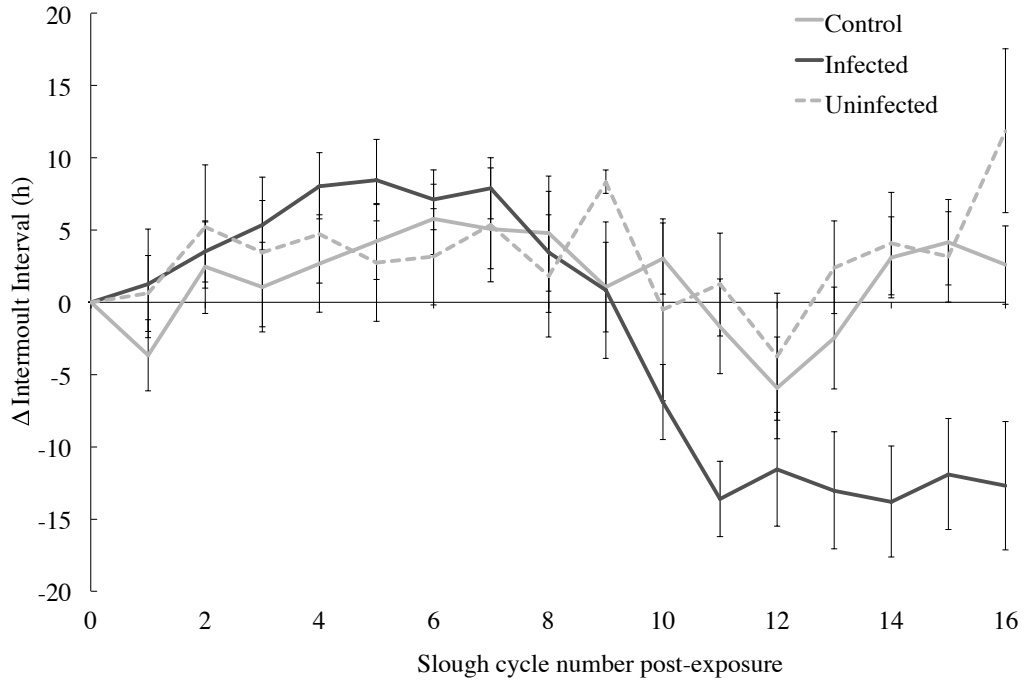

Figure 2.3 Change in intermoult interval (hours) compared to baseline (pre-exposure) intermoult interval for successive sloughing cycles post-exposure in Control (n = 10), Uninfected (n = 2), and Infected (n = 9) *Litoria caerulea* exposed to *Batrachochytrium dendrobatidis* for the first 16 sloughing cycles post exposure. A negative value indicates that intermoult interval has decreased, and consequently sloughing rate has increased. Error bars indicate standard error.

Relationship between infection load and IMI

The change in Bd load (ZE, averaged from before and after swabs at each time point) over the first 75 days post exposure was significantly different in Clinical and Nonclinical frogs (Figure 2.5a), with Bd load increasing over time in Clinical frogs, and peaking then diminishing in Nonclinical frogs. *Days post-exposure* ($\beta = 0.12$, s.e. = 0.04, $p = 0.007$, 95% CI = 0.042-0.20), *Days post-exposure*^{2} ($\beta = -0.0015$, s.e. = 0.00045, $p = 0.002$, 95% CI = -0.0024– -0.00069), and the interaction *Group*Days post-exposure* ($\beta = 0.072$, s.e. = 0.014, $p < 0.00001$, 95% CI = 0.045-0.099), were all significant terms in the linear mixed effects model (Table 2.4). The significant squared term demonstrates that the relationship between Bd load and time was nonlinear, and best defined by a quadratic relationship. Overall, mean Bd load was 36,853 ZE (± 77,926 s.d.) for Clinical frogs and 315 ZE (± 514 s.d.) for Nonclinical frogs.

There was a significant negative relationship between IMI and Bd load (ZE, averaged from Before and After swabs at each time point, and the final swab for late clinical frogs) in the Clinical group ($\beta = -7.05$, s.e. = 0.99, $p < 0.0001$, 95% CI = -9.05– -5.05; Figure 2.5b). A

decrease in IMI results in an increase in sloughing rate, thus sloughing rate increased with increasing Bd load.

Table 2.4 Statistical results from a linear mixed effects model examining the change in infection load (log [Bd load+1] in zoospore equivalents) in *Litoria caerulea* over the first 75 days post-exposure to the pathogen *Batrachochytrium dendrobatidis.* Fixed effects include group (Clinical [n = 7] or Nonclinical [n = 2]), days post exposure and days post exposure^2, and the interaction group*days post exposure. Frog ID was included as a random variable to take into account correlated error from repeated measures on the same individual. s.e. = standard error, s.d. =standard deviation, d.f. = degrees of freedom, bold p-values are significant

Fixed effects	**estimate**	**s.e.**	**t-value**	**d.f.**	***p***	**Confidence intervals**	
						2.5%	**97.5%**
Intercept	-0.09	1.18	-0.076	22	0.94	-2.34	2.16
Group (Clinical)	-1.93	1.10	-1.76	7	0.12	-4.33	0.46
Days post exposure	0.12	0.04	2.95	22	**0.007**	0.042	0.20
Days post exposure^2	-0.0015	0.00045	-3.45	22	**0.002**	-0.0024	-0.00069
Group * Days post exposure	0.072	0.014	5.07	22	**<0.00001**	0.045	0.099
Random:	**s.d.**				**Residual**		
Frog	0.93				0.64	0.55	1.59

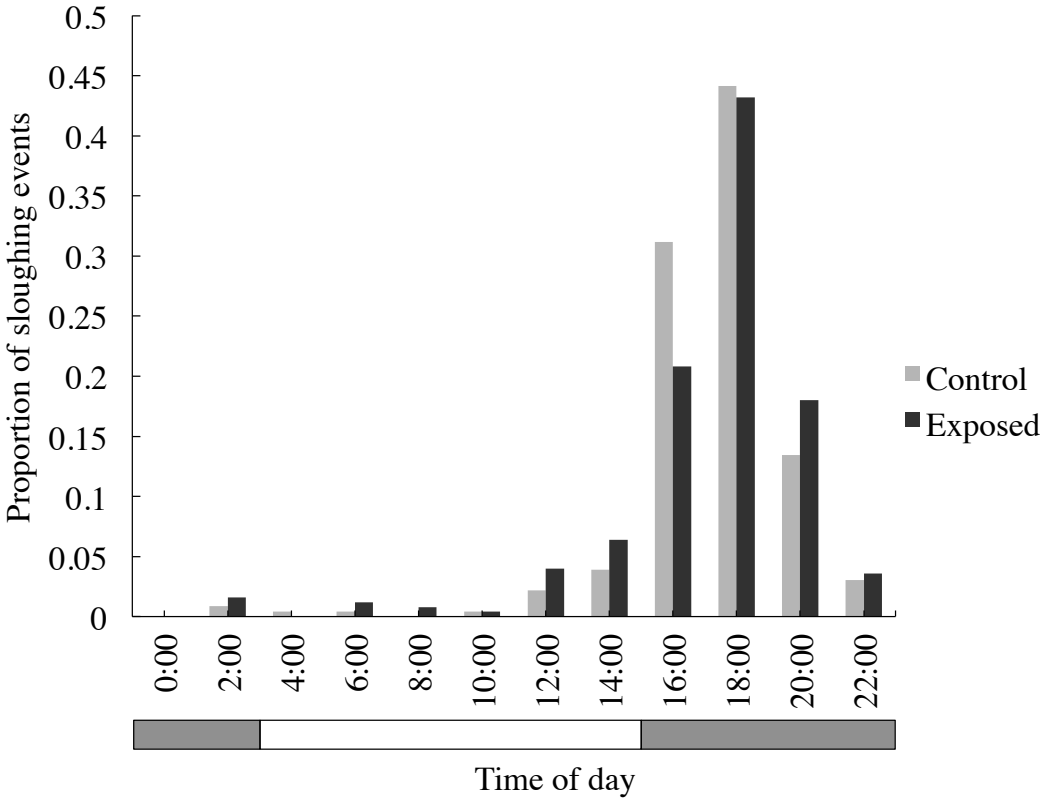

Figure 2.4 The timing of sloughing events in *Litoria caerulea* during a 24 h day, expressed as a proportion of all sloughing events recorded for both Control (n = 10) and Exposed (n = 11) groups. Shaded horizontal bar indicates when lights were off.

Infection load before and after sloughing

Bd load before a sloughing event was not significantly different from Bd load after a sloughing event in Infected frogs (β = 0.011, s.e. = 0.27, p =0.97, 95% CI= -0.52–0.54). Only *Days post-exposure* was a significant main effect of the model comparing ZE from Before and After swabs (β = 0.041, s.e. = 0.0069, p <0.00001, 95% CI= 0.027–0.054), reflecting the general increase in Bd load over time in Infected frogs (Table 2.5).

Table 2.5 Statistical results from a linear mixed effects model examining the effects of sloughing on infection load in *Litoria caerulea* exposed to the pathogen *Batrachochytrium dendrobatidis*. Fixed effects include swab (before or after sloughing event), days post exposure, and time between (a swab and the sloughing event). Frog ID was included as a random variable to take into account correlated error from repeated measures on the same individual. s.e. = standard error, s.d. =standard deviation, d.f. = degrees of freedom, bold p-values are significant

Fixed effects	**estimate**	**s.e.**	**t-value**	**d.f.**	***p***	**Confidence intervals**	
						2.5%	**97.5%**
Intercept	0.37	0.54	0.69	56	0.50	-0.68	1.43
Swab (Before or After)	0.011	0.27	0.042	56	0.97	-0.52	0.54
Time Between	0.014	0.0093	1.52	56	0.13	-0.0039	0.032
Days post exposure	0.041	0.0069	5.93	56	**<0.00001**	0.027	0.054
Random:	**s.d.**				**Residual**		
Frog	1.12				1.08	0.66	1.89

Relationship between exposure timing and infection outcome

Also, *Time to endpoint* (days, log-transformed) was not significantly predicted by the timing of the first sloughing event post-exposure (h; $F(1,5) = 0.17$, $R^2 = -0.16$, $\beta = -0.0034$, s.e. = 0.0082, $p = 0.70$). However, there was a marginally significant positive relationship between *Time to endpoint* (days) and *Mass* (g; Figure 2.6; $F(1,5) = 6.25$, $R^2 = 0.47$, $\beta = 7.68$, s.e. = 3.07, $p = 0.055$), with larger frogs harbouring subclinical infections longer than smaller frogs. Finally, neither frog *Mass* (g) nor *SVL* (mm) was significantly associated with the change in IMI across all groups (Mass: $\beta = 0.51$, s.e. = 0.29, $p = 0.091$, 95% CI = -0.087-1.10; SVL: $\beta = 0.47$, s.e. = 0.30, $p = 0.13$, 95% CI= -0.15-1.10). Overall, frog *SVL* (mm) was significantly associated with IMI ($\beta = 0.68$, s.e. = 0.28, $p = 0.024$, 95% CI = 0.10-1.25), but *Mass* (g) was not ($\beta = 0.55$, s.e. = 0.28, $p = 0.062$, 95% CI = -0.02-1.13).

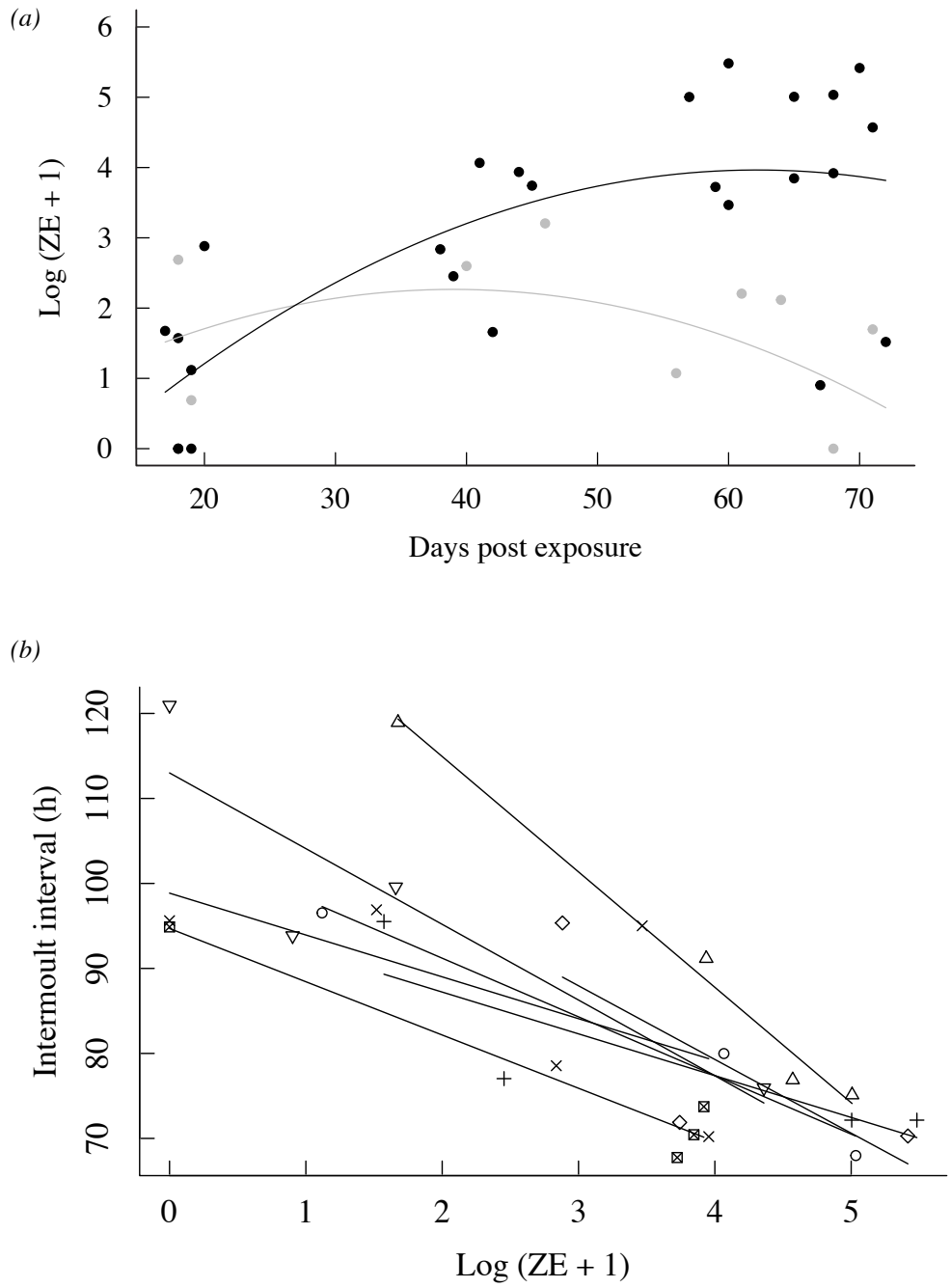

Figure 2.5 (a) Change in Bd load expressed as zoospore equivalents (Log [ZE + 1]) in *Litoria caerulea* exposed to the fungal pathogen *Batrachochytrium dendrobatidis* (Bd) for the first 75 days post exposure. Quadratic curves are fit to each of the two groups: Clinical (black, n = 7), or those demonstrating clinical signs of disease, and Nonclinical (grey, n = 2); (b) The relationship between intermoult interval (h) and Bd load (Log [ZE +1]) in individual *L. caerulea* that demonstrated clinical signs of chytridiomycosis (n = 7).

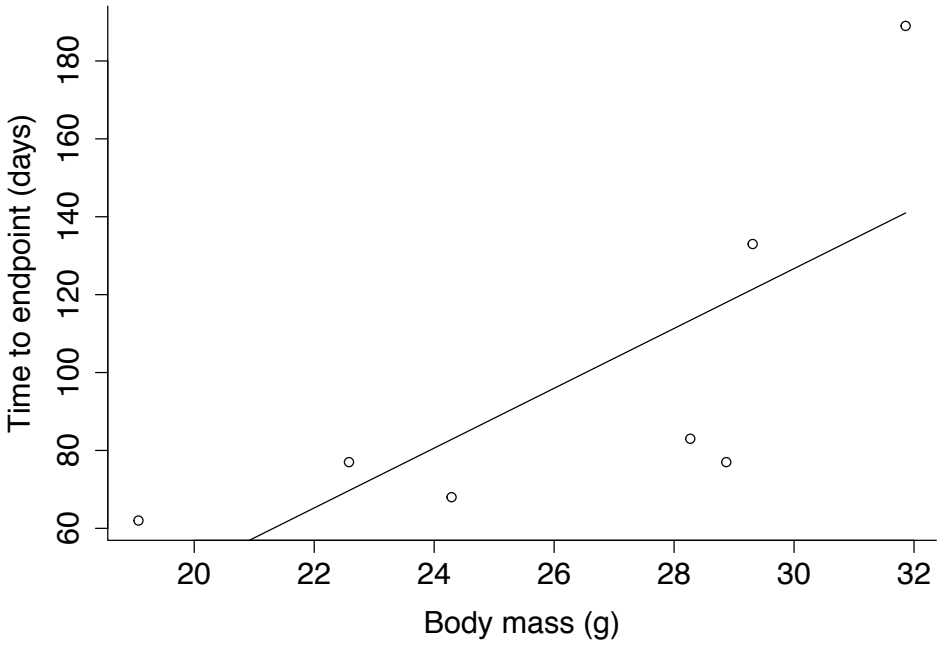

Figure 2.6 The relationship between time to endpoint (days) and body mass (g) of *Litoria caerulea* that became infected with *Batrachochytrium dendrobatidis* and eventually demonstrated clinical signs of disease (n = 7). Endpoint was removal from the experiment for treatment after the development of advanced clinical signs.

Discussion

Understanding the host-pathogen relationship is critical for developing informed disease mitigation strategies (Carey 2005; Blaustein et al. 2012). In the case of amphibians infected with *Batrachochytrium dendrobatidis* (Bd), a fungal pathogen restricted to the superficial epidermal layers, understanding pathogenesis requires comprehension of a process involved in maintaining amphibian skin function: sloughing. To our knowledge, the present study is the first that attempts to measure the effects of disease progression on amphibian sloughing rates. Our results are threefold: First, sloughing rate increases with infection load in exposed frogs, but not until Bd load reaches high levels. However, the rhythmicity of the sloughing cycle is not disrupted, even at high infection loads. Second, there is no effect of exposure timing with respect to the sloughing cycle on infection outcome. And third, the act of sloughing does not appear to significantly reduce infection load on the ventral skin surface. Although a faster sloughing rate might be considered advantageous for Bd-infected animals, it does not curb the progression of disease and may actually contribute to the loss of physiological homeostasis seen in terminally ill frogs (Voyles et al. 2011) by increasing the disruption of water and electrolyte transport across the skin (Jørgensen 1949).

We found that sloughing rate increased with Bd infection load in infected frogs, but sloughing did not lose rhythmicity. This finding is consistent with anecdotal accounts of an increase in sloughing rates in frogs infected with Bd (Berger et al. 1998; Davidson et al. 2003; Bovero et al. 2008; Padgett-Flohr 2008; Becker & Harris 2010; Carver et al. 2010). However, the observed increase in sloughing rate did not occur until fairly high infection loads were reached. In adult *Litoria caerulea*, intermoult interval decreased by 21.2 h (± 4.83 s.d.) on average at a mean Bd load of 7,854 ZE (± 7,769 s.d.) in frogs that developed clinical chytridiomycosis. A recent in vitro study suggests that Bd cell wall constituents might enable the pathogen to evade the immune system of *Xenopus laevis* by inhibiting the production of lymphocytes and inducing their apoptosis (Fites et al. 2013). If sloughing rate is a skin repair mechanism, a delay in sloughing rate increase may be the result of Bd evading clearance until infection load reaches a critical level. In addition, an increase in sloughing may be linked to a higher metabolic rate in the diseased state, which is connected to the stress response (Peterson et al. 2013).

The timing of sloughing appears to be tightly linked with the light-dark cycle on an infradian (greater than 24 h) rhythm, and this is particularly evident during the sudden but limited drop in intermoult interval in Bd infected frogs. This suggests that the plasticity of the sloughing cycle may be constrained by daily light cycles. Previous work indicates that *L. caerulea* in captivity usually slough soon after lights turn off (Cramp et al. 2014). This pattern was also observed in this study, with 70% of sloughing events occurring within four hours of lights out. 'Abnormal' sloughing times, or those that occurred outside of this window, did occur during times of transition from a normal sloughing rate of approximately every four days, to an increased sloughing rate of approximately every three days in infected frogs.

While sloughing rates did increase in infected frogs, the sloughing mechanism was not disrupted, even after infected frogs demonstrated advanced clinical signs of chytridiomycosis. Frogs that were treated after developing clinical signs sloughed every 3.30 days on average up until treatment commenced. However, diseased frogs did shed skin in small pieces, and often did not eat shed skin, which is consistent with previous reports (Berger et al. 1998). Chytridiomycosis appears to disrupt skin functioning (Voyles et al. 2009) and integrity (Berger et al. 2005a), but does not appear to interrupt the rhythmicity of characteristic sloughing behaviour, in which the frog assumes a hunched stance and proceeds to manually remove skin through a series of limb and body movements. This implies that endogenous control of this behaviour is likely not affected by skin deterioration caused by chytridiomycosis.

We did not find that the timing of exposure to Bd within the sloughing cycle influenced infection outcome. Previous work in the crustacean *Daphia magna* indicated that moulting within

12 h after parasite exposure greatly reduced the possibility of infection (Duneau & Ebert 2012). However, Bd demonstrates qualities of both a microparasite, in that it is a small organism with a short generation time in relation to its hosts, and a macroparasite, in that it does not multiply within the host but relies on self-reinfection for individual infection intensities to reach clinical levels (Briggs et al. 2010). In this study, there were numerous opportunities for host reinfection beyond initial exposure, both from within the enclosure and from additional zoospores produced from the infected host. Thus, it may be difficult to predict infection outcome from the timing of initial exposure in the sloughing cycle.

Finally, contrary to predictions, sloughing itself did not affect Bd infection load on the ventral skin surface. This was surprising given that previous work indicates sloughing can reduce cultivable cutaneous bacterial and fungal loads almost entirely (Meyer et al. 2012; Cramp et al. 2014). That being said, Bd encysts on the *stratum corneum*, but can also penetrate this outer layer with a germination tube and develop a zoosporangium in the cell layer immediately below, the *stratum granulosum* (Greenspan et al. 2012; Van Rooij et al. 2012). Thus, although sloughing of the *stratum corneum* may remove zoospores on this outer layer (i.e. those in the initial stages of attachment or being shed from the host), the animal could remain infected because Bd can persist in the underlying skin layer. In addition, the lifecycle of Bd appears to be well adapted to amphibian skin, because the timing of zoosporangium maturation and zoospore release seems to be in sync with the timing of epidermal turnover (Berger et al. 2005a). Thus, sloughing may actually allow a mature zoosporangium to release zoospores external to the host, thereby encouraging self-reinfection of a newly keratinised epidermis that is free of potentially beneficial anti-fungal symbiotic bacteria (Berger et al. 2005a; Meyer et al. 2012). Therefore an increase in Bd load, rather than a sharp drop, might be expected from a skin swab directly after sloughing. Conversely, no change in zoospore numbers from pre- to post-exposure would be expected if frogs were immediately re-infected from their environment. Our results demonstrate no significant difference between before and after swabs, with a little over half (57%) demonstrating a decrease in Bd load after sloughing. Another possibility is that skin swabbing may not be a reliable method for detecting a change in Bd load on or near a sloughing event, given that pieces of the *stratum corneum* containing zoosporangium may be inadvertently removed during swabbing and thereby bias the result of quantification. Further work at the cellular level is required to fully understand the relationship between Bd and sloughing, and the changes in infection load that may occur during this turnover.

This is the first study to demonstrate that Bd infection affects skin turnover rates in a susceptible amphibian. In *L. caerulea* infected with Bd, sloughing rate sustains a limited but

significant increase with disease progression. However, given this increase occurs at high infection loads, and sloughing itself does not appear to reduce the cutaneous Bd burden, it seems unlikely that an increased sloughing rate is a beneficial response. Physiologically, moulting may be a vulnerable period for an amphibian, given a temporary increase in skin permeability to water and electrolytes may occur during and after the sloughing event (Jørgensen 1949). Jørgensen (1949) found that immediately after the *stratum corneum* separated from the underlying epidermis in *Bufo bufo*, water permeability increased 3-4 fold, and salt permeability increased up to 20 fold, resulting in a net loss of sodium. In frogs with clinical chytridiomycosis, plasma sodium levels are markedly decreased, and electrolyte imbalance is a symptom of severe disease (Voyles et al. 2009; Voyles et al. 2012). Thus, an increased sloughing rate may contribute to the imbalance in fluid and electrolyte levels seen in diseased animals (Voyles et al. 2009; Voyles et al. 2012). However, the plasticity of the increased sloughing response may vary across species, particularly in those that demonstrate low susceptibility to chytridiomycosis. In particular, a thicker epidermis as a result of more replacement cell layers might confer such sloughing rate plasticity without physiological harm, which has been hypothesised to be the case in the bullfrog, *Lithobates catesbeianus* (Greenspan et al. 2012). Further work is needed to better understand variation in normal sloughing rates across species and size classes in order to make broader conclusions about the role of sloughing in the pathogenesis of Bd.

CHAPTER 3

Skin sloughing in susceptible and resistant amphibian hosts regulates infection with a fungal pathogen

Abstract

Disease is increasingly recognised as a major threat to wildlife populations. Amphibians worldwide are threatened by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), which in post-metamorphic animals only infects the skin, and causes the potentially lethal disease chytridiomycosis. Amphibians regularly shed their skin in a process called sloughing, and this behaviour has been associated with a reduction in the cultivatable microbes on the skin surface. However, in a previous study, we found no reduction in Bd infection load on the ventral skin surface after sloughing in the susceptible species *Litoria caerulea*. In species less susceptible to chytridiomycosis, it has been demonstrated that Bd growth remains epibiotic, without penetrating the underlying epidermal layers. Therefore, we hypothesised that sloughing would more effectively remove Bd zoospores in less susceptible species. To test this hypothesis, five Australian frog species, *Lit. caerulea*, *Limnodynastes peronii, Limnodynastes tasmaniensis, Platyplectrum ornatum,* and *Lechriodus fletcheri,* were exposed to a local Bd strain, and their sloughing rates and infection loads were monitored over time. Utilising an improved methodology to remove any artefacts from the swabbing itself, we found that sloughing reduced Bd load on the ventral skin surface, in all five species, despite wide ranging variation in susceptibility to Bd infection and subsequent disease and mortality. In less susceptible species, sloughing reduced Bd load up to 100%, leading to infection clearance. The drop in Bd load was only temporary in susceptible species, potentially due to the invasive growth of Bd in skin layers underlying the *stratum corenum* in these species. If less susceptible species are able to clear themselves of Bd infection via the routine process of skin shedding, amphibian sloughing may be a more important immune defence than previously thought. In addition, this finding has implications for understanding the pattern of Bd growth on individual hosts, and how this may translate to population-level effects.

Introduction

Disease is increasingly recognised as a major threat to wildlife populations (Daszak et al. 2000; Smith et al. 2006; Smith et al. 2009a), and "conservation pathogens", or those that contribute greatly to population declines (Willis 2015), can lead to extinctions and biodiversity loss. Amphibian populations are experiencing declines and extinctions on a global scale (Stuart et al. 2004; Chanson et al. 2008), and many of these declines have been attributed to the conservation

pathogen *Batrachochytrium dendrobatidis* (Bd), which infects the keratinised layers of an amphibian's skin (Berger et al. 1998; Skerratt et al. 2007). Given the importance of the amphibian epidermis for a multitude of physiological functions, from cutaneous gas exchange, to water and ion balance (Boutilier et al. 1992), infection with Bd can cause severe disease, known as chytridiomycosis, and mortality (Berger et al. 1998). Bd is a generalist pathogen that infects a wide range of amphibian hosts, and both host and pathogen ecology and physiology, within the context of environmental cofactors such as temperature and precipitation, influence differences in susceptibility within and between species (Fisher et al. 2009b; James et al. 2015). On one end of the spectrum are individuals, populations, or species that demonstrate complete resistance to infection, while on the other end are those that develop high infection loads and succumb rapidly to disease (Searle et al. 2011a; Ohmer et al. 2013; DiRenzo et al. 2014). In the post-metamorphic amphibian host, Bd is entirely restricted to the skin, and skin defences play a central role in preventing disease (Rollins-Smith et al. 2011). Thus, a better understanding of the physiology of amphibian skin will help elucidate how the amphibian-killing fungus interacts with its amphibian hosts, and particularly, how that interaction varies across species.

Amphibian skin is the first line of defence against invading pathogens, and it is constantly renewed to ensure optimal physiological function (Alibardi 2003). This renewal process is termed sloughing or moulting, in which an amphibian sheds the outer skin layer, the *stratum corneum*, in its entirety on a regular basis (Larsen 1976). Sloughing is hormonally controlled (Barker Jørgensen et al. 1965; Barker Jørgensen 1988), and occurs on a regular cycle anywhere from every 24 hours, to every two weeks, depending on the species (Stefano & Donoso 1964; Castanho & de Luca 2001; Cramp et al. 2014; Ohmer et al. 2015). Like most physiological processes in ectotherms, sloughing rate is affected by temperature (Meyer et al. 2012; Cramp et al. 2014). The timing of moulting is also tightly linked with the light-dark cycle, although usually on an infradian (longer than 24 h) rhythm (Cramp et al. 2014; Ohmer et al. 2015). Unlike skin shedding in squamate reptiles, amphibian sloughing occurs frequently, and is difficult to observe, given the comparably short time in which the skin is removed (often 5-20 minutes, Ohmer et al. 2015), and that the shed skin is usually ingested during the process of sloughing, or immediately after. These factors have made this behaviour difficult to study. By employing networked infrared video cameras, sloughing behaviour can be observed as it occurs, and can be used to investigate the role of sloughing in controlling pathogen growth on the skin.

Amphibian skin shedding has been hypothesised to play a role in the regulation of cutaneous microbes, by removing resident populations of bacteria and fungi on a regular basis (Meyer et al. 2012). This regulation has been demonstrated to remove up to 100% of the

cultivatable microbes on the skin surface (Meyer et al. 2012; Cramp et al. 2014), and may help prevent dysbiosis events (Colombo et al. 2015). Previously, we endeavoured to determine the effect of Bd infection on skin sloughing in the Australian green tree frog (*Litoria caerulea*), a species susceptible to Bd infection and chytridiomycosis (Ohmer et al. 2015). While high Bd infection loads were correlated with increased sloughing rates in individual frogs, Bd load on the ventral skin surface did not decrease with sloughing (Ohmer et al. 2015). We hypothesised that sloughing was not effective at removing Bd in the susceptible species *L. caeruela*, and that perhaps an increase in sloughing rate may actually exacerbate the loss of physiological homeostasis seen in terminally ill frogs (Jørgensen 1949; Voyles et al. 2009). However, the growth pattern of Bd in amphibian skin is host-dependent, and an investigation of sloughing across amphibian species may highlight differences in the role of sloughing in pathogen removal.

Bd infects the outer keratinised layers of amphibian epidermis, principally the *stratum coreum* and the *stratum granulosum*, via a swimming flagellated zoospore (Berger et al. 1998; Longcore et al. 1999; Berger et al. 2005a). After encysting on the surface of the *stratum corneum*, zoospores develop into zoosporangia, and produce germ tubes to invade the deeper skin layers and grow intracellularly (Greenspan et al. 2012; Van Rooij et al. 2012). Recently, it has been demonstrated that Bd invokes two very different growth patterns in susceptible versus tolerant hosts. In susceptible species, such as *Litoria caeruela* and *Alytes muletensis*, Bd is found growing almost exclusively intracellularly in the keratinised and partially-keratinised skin layers (Van Rooij et al. 2012). However, in species that demonstrate higher levels of infection tolerance, and eventually infection clearance, such as *Xenopus laevis*, Bd growth is seen as entirely epibiotic, with growth only occurring on the skin surface (Van Rooij et al. 2012). This host-dependent variation in Bd growth pattern may indicate that routine sloughing of the outer *stratum corneum* may be more effective at removing zoospores and zoosporangia in less susceptible amphibian species, in which only epibiotic Bd growth occurs.

We set out to determine the role of amphibian skin sloughing in the regulation of Bd growth in five Australian frog species, in order to compare species with different inherent susceptibility to disease. We hypothesised that unlike in *Litoria caerulea*, a species usually highly susceptible to Bd infection and chytridiomycosis in the laboratory, species that experience low mortality rates and infection clearance would demonstrate a decrease in Bd load on the ventral skin surface after sloughing. In addition, we investigated whether sloughing rates increase in less susceptible frog species after Bd exposure, as they do in *L. caerulea*, and if this occurred at lower Bd loads. We hypothesised that less susceptible species would increase their sloughing rates at lower Bd infection loads, potentially demonstrating an immune response to infection. The amphibian integument varies

widely in structure and function across species. Understanding the role of the ubiquitous and largely understudied process of amphibian skin sloughing, and how it varies across species, may provide clues as to how some amphibians overcome Bd infection. In addition, if sloughing regulates Bd growth on the skin of some frog species, sloughing rate may inform models needed to predict the effects of Bd infection at specific sites and populations (Louca et al. 2014).

Methods

Study species

In order to examine a range of susceptibilities to Bd infection and subsequent disease, we compared sloughing rates and infection loads of five fairly common species of frog from Southeast Queensland: spotted and striped marsh frogs (*Limnodynastes tasmaniensis* and *Lim. peronii*), ornate burrowing frogs (*Platyplectrum ornatum)*, black-soled frogs (*Lechriodus fletcheri*), and green tree frogs (*Litoria caerulea*). Bd infections have been previously recorded in wild populations of all of these species (Berger et al. 2004; Commonwealth of Australia 2006), but there is no definitive evidence of disease-related declines in these species. Previous exposure experiments indicate that *Lit. caerulea* is susceptible to chytridiomycosis in the laboratory (Voyles et al. 2009; Ohmer et al. 2015), while *Lim. tasmaniensis* and *Lim. peronii* demonstrate fairly low susceptibility (Woodhams et al. 2007a; Stockwell et al. 2010). There are no published studies in which *Le. fletcheri* has been exposed to Bd in a laboratory setting.

Animal collection and maintenance

Lim. tasmaniensis, Lim. peronii, *P. ornatum,* and *Le. fletcheri* were collected as spawn in south eastern Queensland, Australia and reared in the laboratory. Egg masses of *Lim. tasmaniensis* and *P. ornatum* were collected from flooded roadsides near Dalby, Queensland while egg masses of *Lim. peronii* were collected from ephemeral pools in St. Lucia, Queensland. Egg masses of *Le. fletcheri* were collected from pools on private land on the Lamington plateau, Canungra, Queensland, with permission. At the time of experimentation, all frogs had reached adult size and were about two years of age, with the exception of *Le. fletcheri*, which had only reached subadult size, and were about 1.5 years old (Table 3.1a). Adult *Lit. caeruela* were collected from wet roadsides in non-protected areas near Fernvale, Queensland. Frogs of a single species originated from one population, eliminating the need to account for population or site differences within a species. In the laboratory, frogs were kept on a cycling temperature regime (15-23˚C) with a 12 h photoperiod (see Ohmer et al. 2015 for detailed temperature cycle). Frogs were housed individually in ventilated clear plastic boxes (*Lit. caerulea:* 262 x 237 x 120 mm, *Le. fletcheri*: 172.5 x 120 x 75 cm, all others: 235 x 170 x 120 mm), with a substrate of paper towels saturated with aged tap

water and a plastic cup or PVC pipe for shelter. Enclosures were cleaned and frogs were fed vitamin and calcium-dusted crickets (5 large or medium, depending on frog size), weekly. All frogs tested negative for Bd infection prior to the start of the experiment.

Table 3.1 (a) Exposure groups and descriptive statistics for five Australian frog species exposed to *Batrachochytrium dendrobatidis* (Bd). All species were exposed to Bd strain EPS4 twice. (b) Details of third experimental exposure to *Batrachochytrium dendrobatidis* (Bd) for *Litoria caerulea* only. Exposure 3 utilised strain *Waste point-Lverreauxii-2013-LB, RW, 2*, and exposure and control groups were re-organised to attain a greater number of infected individuals. *L. caerulea* in the 'new controls' group were previously exposed animals that never became infected, those in the 'new exposed' were previously controls, and two animals ('not re-exposed') were demonstrating signs of chytridiomycosis and not included in the third exposure

(a)

Species	**Control (n)**	**Exposed (n)**	**SVL (mm) mean ± s.d.**	**Life stage**
Lechriodus fletcheri	3	4	26.8 ± 2.2	Subadult
Limnodynastes peronii	3	6	38.6 ± 1.4	Adult
Limnodynastes tasmaniensis	4	7	35.6 ± 3.1	Adult
Litoria caerulea	6	11	83.2 ± 5.7	Adult
Platyplectrum ornatum	4	6	36.5 ± 3.5	Adult

(b)

Group (n)	**Exposure 3 (*L. caerulea* only)**
Control	3
Exposed	5
New control	4
New exposed	3
Not re-exposed	2

Exposure to Bd and infection monitoring

Frogs were exposed to Bd strain EPS4, which was isolated by E.P. Symonds (School of Veterinary Sciences, The University of Queensland) in March 2012 from a *Mixophyes fleayi* tadpole (Gap Creek, Main Range National Park, Queensland, Australia). Bd culture was maintained in flasks at 4˚C until four days before exposure, when it was re-cultured onto 1% agar, 0.25% tryptone, 0.25% tryptone-soy plates for 5-7 days at 21˚C. Once zoospore production peaked, plates were flooded with distilled water for 30 min with periodic gentle agitation. The resulting suspension of zoospores was collected, and zoospore concentration was calculated using a haemocytometer (Boyle et al. 2004).

All frogs were experimentally exposed to Bd at least twice to ensure infection, on 8 April 2015 at a dose rate of ~250,000 zoospores, and two weeks later on 22 April 2015 at a dose rate of ~500,000 zoospores (Table 3.1a). Green tree frogs were exposed a third time, on 20 May 2015, with a different strain of Bd (strain *Waste point-Lverreauxii-2013-LB,RW,2*, isolated by Lee Berger at James Cook University, dose: 500,000 zoospores), in order to attain a greater number of infected individuals (Table 3.1b). Exposure protocol followed that of Ohmer et al (2015), with frogs exposed for five hours in 300 mL plastic containers containing 40 mL of aged tap water (see Table 3.1 for exposure groups). Control frogs were treated in the exact same manner as exposed frogs, but were exposed to aged tap water containing no zoospores.

At two days and seven days following each Bd exposure, all animals were swabbed to assess infection status. Swabbing protocol followed Ohmer et al (2015), with the exception of swabs taken before and after sloughing, which followed a specific protocol to avoid any potential artefacts from the swabbing itself (see *Monitoring infection load before and after sloughing*). Subsequently, animals were swabbed approximately every two weeks, and swabs were analysed with quantitative PCR (qPCR) following Boyle et al (2004) and Hyatt et al (2007). Briefly, swabs were extracted in 50 µL Prepman Ultra (Applied Biosystems, Foster City, CA, USA) and analysed in triplicate with qPCR on a Mini Opticon real-time PCR detection system (MJ Mini Cycler, Bio-Rad Laboratories, Inc.). Infection load was determined by multiplying by 100 to account for dilution and expressed as zoospore equivalents (ZE). A modified 15-µL reaction volume was used (Garland et al. 2010; Ohmer et al. 2015).

Sloughing monitoring

Frogs were recorded continuously with twelve 600TVL Weatherproof infrared Cameras (model EN-CI20B-65H, Eonboom Electronics Limited) at a frame rate of 1.52 frames per second (FPS). Video was recorded on a 16 Channel H.264 Digital Video Recorder (DVR), model MDR688ZB (AU)-E. 600TVL). Sloughing behaviour is unique and easy to recognize on recorded video when played back at 16x normal speed (see Ohmer et al 2015 for example recordings).

Monitoring infection load before and after sloughing

Frog sloughing rates were analysed from recorded video, in order to predict their sloughing rhythm. Individual frogs often slough on an anticipated cycle and at a consistent time of day in the laboratory, allowing for sloughing events to be predicted with some accuracy. Frogs were swabbed as close as possible to this predicted sloughing time, and then swabbed again as soon as possible after sloughing occurred. To achieve this, surveillance cameras were networked, allowing the remote viewing of frog behaviour as it occurred (a methodological update from Ohmer et al. 2015).

To avoid any artefact from swabbing itself, only one side of the ventral surface of each frog (right or left, divided along the sagittal plane) was swabbed before sloughing (randomly chosen), and the opposite side was swabbed after sloughing. When swabbing one side, swabs were run up and down the left or right ventral surface including the drink patch, the thigh, the side of the torso, one fore foot and one hind foot, three times each.

All frogs were monitored daily for clinical signs of disease. If a frog began to demonstrate severe clinical signs, including lethargy, inappetence, abnormal posture (particularly with legs splayed out and head bent over toward substrate), and poor righting reflex, a final swab was taken to determine infection load, and the animal was humanely euthanised in 0.3% neutral buffered MS-222. Clinically infected *Lit. caerulea* were euthanised with an intracoelomic injection of 60 mg kg^{-1} body mass thiopentone sodium (Ilium Thiopentone, Troy Laboratories, NSW, Australia), to allow for additional studies of the skin post-mortem.

Phylogenetic relationships

A phylogenetic tree of all species analysed was obtained from the Open Tree of Life (Hinchliff et al. 2015), accessed via the R package 'rotl' (Michonneau et al. 2015), and Grafen's arbitrary branch lengths were used for tree creation (Grafen 1989; Figure 3.1).

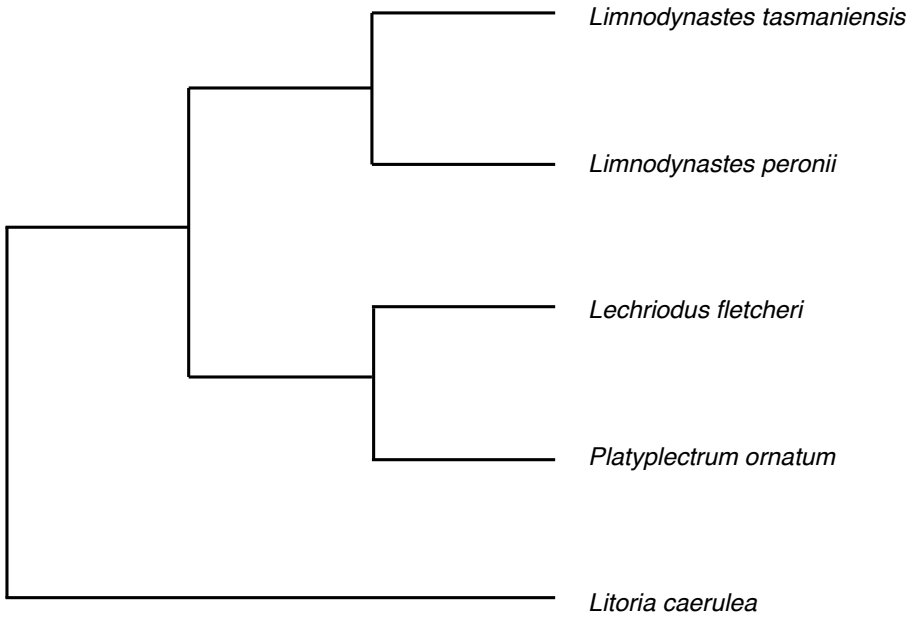

Figure 3.1 Phylogenetic relationships between the species included in this study, displayed with Grafen's arbitrary branch lengths.

Statistical Analyses

All statistical analyses were performed in the program R (R Core Team 2013). Phylogenetic linear mixed models (PLMMs), implemented in ASReml-R, were utilised to account for multiple measurements on the same individuals over time, and phylogenetic non-independence between species (function 'asreml', package 'asreml', Butler et al. 2009). For all PLMMs, a Wald type F-test was used to test for the significance of fixed effects (Kenward & Roger 1997), and the significance of random effects were determined using likelihood ratio tests (Self & Liang 1987, Tobias et al. 2014). Approximate standard errors for the estimate of phylogenetic heritability were calculated using the R pin function (White 2013). Phylogenetic heritability is equivalent to the more widely-used λ (Pagel 1999), and was used as an estimate of phylogenetic signal. Phylogenetic heritability was calculated as the proportion of the variance in the trait, conditioned on the fixed effects (Wilson 2008), which is explained by the relationship among taxa as given by the phylogeny (Housworth et al. 2004). PLMMs were reduced using likelihood ratio tests (Self & Liang 1987, Tobias et al. 2014).

In order to examine the change in infection load (log[ZE+1]) over time in exposed frogs that became infected with Bd, a PLMM was fitted with the interaction between *Species* and *Days post exposure,* and *Days post exposure*^{2}, as the fixed effects, and *Frog ID* nested within *Species* and a phylogenetic variance-covariance matrix constructed from the phylogeny as random effects. Survival curves were compared between species with a log-rank test (function 'survdiff', package 'survival'). The natural log of slough duration (min) was also compared across species, with *Days post exposure* and *Group* as fixed effects, and the same random effects as the previous model.

To test for a change in Bd load (log [zoospore equivalents (ZE)+1]) after sloughing, a PLMM was performed, including individual *Frog ID* nested within *Species, Days post exposure,* and a phylogenetic variance-covariance matrix constructed from the phylogenetic tree as random effects, and *Before or After* (sloughing) as the fixed effect. To examine the role of swabbing timing after sloughing in the observed percent change in Bd load, we used a PLMM to examine the percent change in Bd load (log[ZE+1]) from before to after sloughing, with the same random effects as the previous model, and *Time between slough and swab (min)* as the fixed effect.

The intermoult interval, or IMI, throughout the experiment was compared across species in a PLMM, with *Days post exposure* ('day' indicating the first slough date of consecutive sloughs between which an IMI was calculated) and *Group* (Control, Infected, Uninfected, or Clinical) as fixed effects, and *Frog ID* nested in *Species*, as well as the variance-covariance matrix from the phylogeny as the random effects. *Days post exposure* was used as a proxy for time progression in

the experiment, due to missing values for a few sloughing events in individual frogs (particularly those the burrow, e.g. *P. ornatum*). IMI is the time in hours between sloughing events, with shorter IMIs indicating a faster sloughing rate. To further explore differences in IMI within species, separate linear mixed effects models with *Days post exposure,* and *Group* were performed (function 'lme', library 'nlme', using Maximum Likelihood, Pinheiro & Bates 2000, Pinheiro et al. 2013). With the same random effects as previous PLMMs, the change in IMI with Bd load (log [ZE+1]) was also compared across all species.

Results

Experimental exposure

All control frogs remained Bd negative for the duration of the experimental period. Infection prevalence and mortality rate in exposed frogs varied across species, with *P. ornatum* experiencing the highest mortality rate (100%) and average prevalence (73.3%, s.e. = 6.1%), and *Lim. tasmaniensis* experiencing the lowest mortality rate (0%), and average prevalence (34.7%, s.e. = 9.3%). Survival curves were significantly different between species ($\chi^2 = 27.9$, d.f.=4, $p = 1.29 \times 10^{-5}$; Figure 3.2). Both days post exposure and the interaction between days post exposure and species were significant in the model describing infection load over time, which reflects differences in infection outcome between species (e.g. Bd clearance or mortality; days post exposure: F= 30.31, d.f. = 1,187.4, $p = 6.6 \times 10^{-9}$, days post exposure*species: F= 17.85, d.f. = 4, 201.9, $p = 1.5 \times 10^{-12}$, days post exposure^2: F= 25.0, d.f. = 1,187.9, $p = 4.9 \times 10^{-3}$; Figure 3.3, Table 3.2). In order to compare sloughing duration based on infection outcome, exposed frogs were divided into the following groups: clinical (developed clinical signs of disease), infected (tested positive for Bd without developing clinical signs), and uninfected (never tested positive for Bd).

Sloughing behaviour

In control frogs, *Lit. caerulea* demonstrated the longest IMI on average of 4.02 days (± 0.47 s.d.), while *Lim. peronii* and *Lim. tasmaniensis* demonstrated the shortest IMIs of 2.47 (± 0.53 s.d.) and 2.57 days (± 0.49 s.d.), respectively. *Le. fletcheri* and *P.ornatum* sloughed on average every ~3 days (2.82 ± 0.55 s.d. and 2.96 ± 0.41 s.d.). Sloughing behaviour was similar across species and is in line with previous reports (Ohmer et al. 2015), and the duration of the sloughing behaviour did not vary across group (F= 1.67, d.f. = 3, 42.7, $p = 0.21$), or days post exposure (F= 1.56, d.f. = 1, 604.3, $p = 0.19$), but did vary across species (Table 3.3).

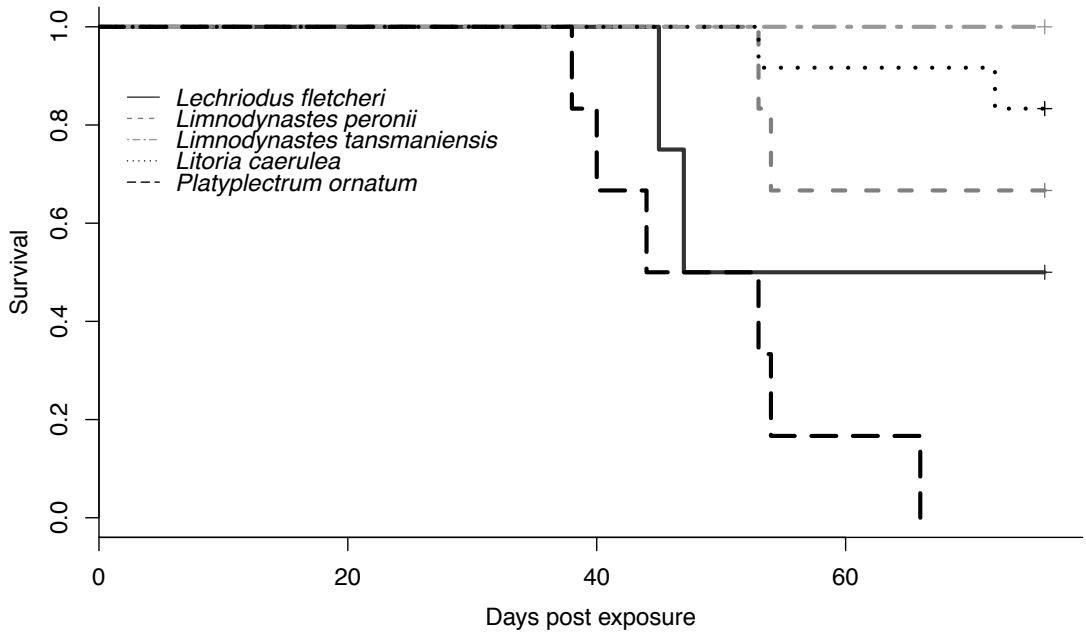

Figure 3.2 Survival curves for five frog species found in Southeast Queensland, Australia, after exposure to *Batrachochytrium dendrobatidis* (Bd).

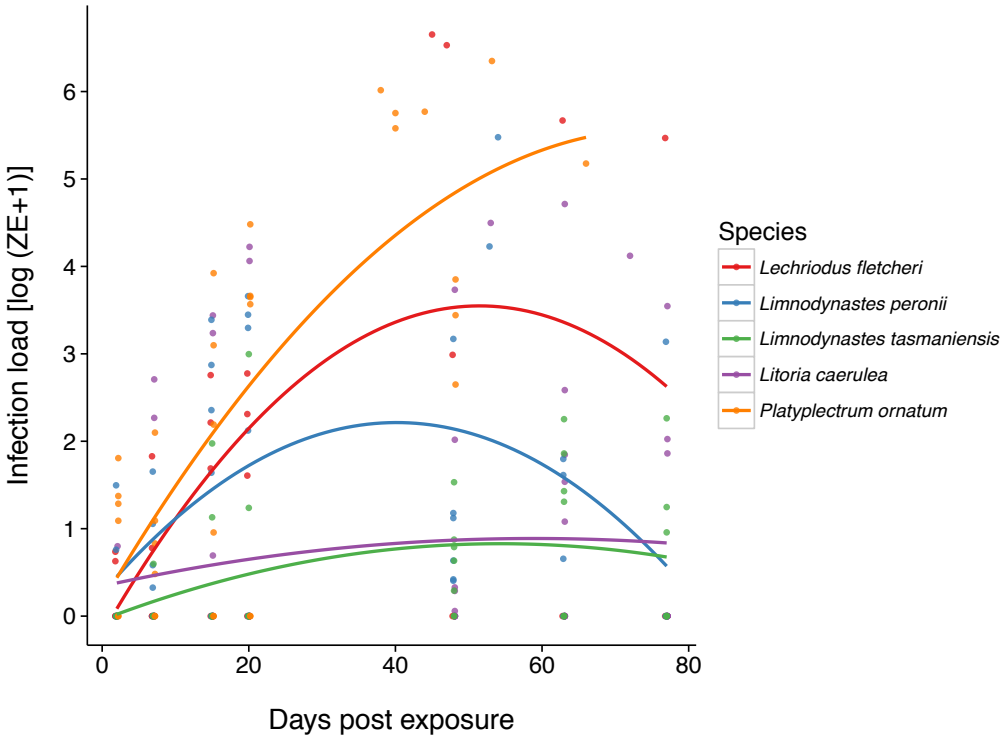

Figure 3.3 Change in infection load (log [zoospore equivalents (ZE)+1]) after exposure to *Batrachochytrium dendrobatidis* (Bd) in five frog species found in Southeast Queensland, Australia. Curves are quadratic polynomial smoothing functions fit by species**.**

Table 3.2 Statistical results from a phylogenetic linear mixed model (implemented in ASReml-R) examining the change in infection load (log (zoospore equivalents [ZE]+1)) during the experimental period in infected frogs only of five species of Australian frog exposed to *Batrachochytrium dendrobatidis* (Bd). Fixed effects were the interaction between *Species* and *Days post exposure,* and *Days post exposure*^{2}, and random effects were *Frog ID* nested in *Species,* and a variance-covariance matrix created from the phylogeny, to take into account correlated error from repeated measures on the same individual, and phylogenetic non-independence. s.e. = standard error, d.f. = degrees of freedom, bold p-values are significant, λ = phylogenetic signal

Fixed effects	**Estimate**	**s.e.**	**F**	**d.f.**	***p***
Intercept	0.070	0.25	51.55	1, 126.6	0.78
Species: Days post exposure (*Lechriodus fletcheri*)	NA	NA	17.85	4, 201.9	**1.5×10^{-12}**
Species: Days post exposure (*Limnodynastes peronii*)	-0.19	0.052			
Species: Days post exposure (*Limnodynastes tasmaniensis*)	-0.21	0.050			
Species: Days post exposure (*Litoria caerulea*)	-0.21	0.047			
Species: Days post exposure (*Platyplectrum ornatum*)	0.21	0.068			
Days post exposure	0.44	0.078	30.31	1,187.4	**6.6×10^{-9}**
Days post exposure^2	-0.012	0.0041	25.0	1,187.9	**4.9×10^{-3}**
Random effects	**Variance**	**s.e.**	**χ^2**	**d.f.**	***p***
Phylogeny	1.01×10^{-7}	1.24×10^{-8}	-7.6×10^{-7}	1	1
Frog ID nested in Species	0.71	0.25	NA	NA	NA
λ = 5.92×10^{-8}, se = 7.45×10^{-9}					

Table 3.3 Statistical results from a phylogenetic linear mixed model (implemented in ASReml-R) examining the natural log of sloughing duration (min) in five species of Australian frog exposed to *Batrachochytrium dendrobatidis* (Bd). Fixed effects were *days post exposure*, and *group* (control, clinical, infected and uninfected) and random effects were *Frog ID* nested in *Species,* and a variance-covariance matrix created from the phylogeny, to take into account correlated error from repeated measures on the same individual, and phylogenetic non-independence. s.e. = standard error, d.f. = degrees of freedom, bold p-values are significant, λ = phylogenetic signal

Fixed effects	**Estimate**	**s.e.**	**F**	**d.f.**	***p***
Intercept	2.14	0.055	2247.0	1, 2.7	**5.30×10^{-5}**
Days post exposure	0.0015	0.0012	1.56	1, 604.3	0.19
Group (Control)	NA	NA	1.67	3, 42.7	0.21
Group (Clinical)	0.086	0.070			
Group (Infected)	0.084	0.066			
Group (Uninfected)	-0.066	0.071			
Random effects	**Variance**	**s.e.**	**χ^2**	**d.f.**	***p***
Phylogeny	1.01×10^{-7}	1.16×10^{-9}	-7.65×10^{-7}	1	1
Frog ID nested in Species	6.09×10^{-2}	6.53×10^{-3}	11.82	1	**0.00059**
λ = 9.39×10^{-8}, s.e. = 3.03×10^{-9}					

Change in infection load with sloughing

To assess Bd load before and after sloughing, swabbing of individual frogs occurred between 12 and 386 min before (mean: 103.3 ± 102.9 s.d. min) and 1 and 356 min after (mean: 56.9 ± 84.1 s.d. min) each sloughing event. Bd load decreased in all species following sloughing (all species: F= 50.8, d.f. = 1, 43.1, $p = 8.34 \times 10^{-9}$, see Table 3.4 for PLMM details, Figure 3.4a). The percent change in Bd load (log[ZE+1]) from before to after sloughing was not significantly different between species over time (F= 0.38, d.f. = 4, 2.9, $p = 0.81$, Table 3.5, Figure 3.4b), but was marginally positively associated with the *time between (min)* the sloughing event and the after swab (F= 4.02, d.f. = 22, $p = 0.057$; Figure 3.5). This indicated that the sooner the frog was swabbed after sloughing, the greater the percent decrease in Bd load was observed. In *Lim. tasmaniensis* and *Lim. peronii,* species that experienced low mortality rates after Bd exposure, sloughing sometimes resulted in a 100% reduction in Bd load, and infection clearance occurred in those individuals.

Table 3.4 Statistical results from a phylogenetic linear mixed model (implemented in ASReml-R) examining the change in infection load (log (zoospore equivalents [ZE]+1)) after sloughing in five species of Australian frog exposed to *Batrachochytrium dendrobatidis* (Bd). Fixed effects were *before or after sloughing*, and random effects were *Frog ID* nested in *Species, days post exposure*, and a variance-covariance matrix created from the phylogeny, to take into account correlated error from repeated measures on the same individual, and phylogenetic non-independence. s.e. = standard error, d.f. = degrees of freedom, bold p-values are significant, λ = phylogenetic signal

Fixed effects	**Estimate**	**s.e.**	**F**	**d.f.**	***p***
Intercept	2.96	0.68	24.76	1, 3.5	**1.01×10^{-2}**
Before or After	0.82	0.11	50.81	1, 43.1	**8.34×10^{-9}**
Random effects	**Variance**	**s.e.**	**χ^2**	**d.f.**	***p***
Phylogeny	3.71	0.96	2.26	1	0.13
Frog ID nested in Species	1.61	0.45	9.77	1	**0.0018**
Days post exposure	3.48	0.98	37.08	1	**1.13×10^{-9}**
λ = 0.38, s.e. = 0.23					

Table 3.5 Statistical results from a phylogenetic linear mixed model (implemented in ASReml-R) examining the percent change in infection load (log (zoospore equivalents [ZE]+1)) after sloughing in five species of Australian frog exposed to *Batrachochytrium dendrobatidis* (Bd). Fixed effect were the interaction between *days post exposure* and *species*, and random effects were *Frog ID* nested in *Species, days post exposure*, and a variance-covariance matrix created from the phylogeny, to take into account correlated error from repeated measures on the same individual, and phylogenetic non-independence. s.e. = standard error, d.f. = degrees of freedom, bold p-values are significant, λ = phylogenetic signal

Fixed effects	**Estimate**	**s.e.**	**F**	**d.f.**	***p***
Intercept	-26.24	14.24	8.40	1, 2.3	0.19
Species: Days post exposure (*Lechriodus fletcheri*)	NA	NA	0.38	4, 2.9	0.81
Species: Days post exposure (*Limnodynastes peronii*)	-4.03	3.25			
Species: Days post exposure (*Limnodynastes tasmaniensis*)	-2.37	3.46			
Species: Days post exposure (*Litoria caerulea*)	-2.03	3.21			
Species: Days post exposure (*Platyplectrum ornatum*)	-4.58	8.32			
Days post exposure	2.48	3.11	0.065	1, 10.5	0.80
Random effects	**Variance**	**s.e.**	**χ^2**	**d.f.**	***p***
Phylogeny	2.53×10^{-5}	5.82×10^{-3}	-7.6×10^{-7}	1	1
Frog ID nested in Species	1.20	355.0	NA	NA	NA
$\lambda = 8.94 \times 10^{-6}$, s.e. = 0.00072					

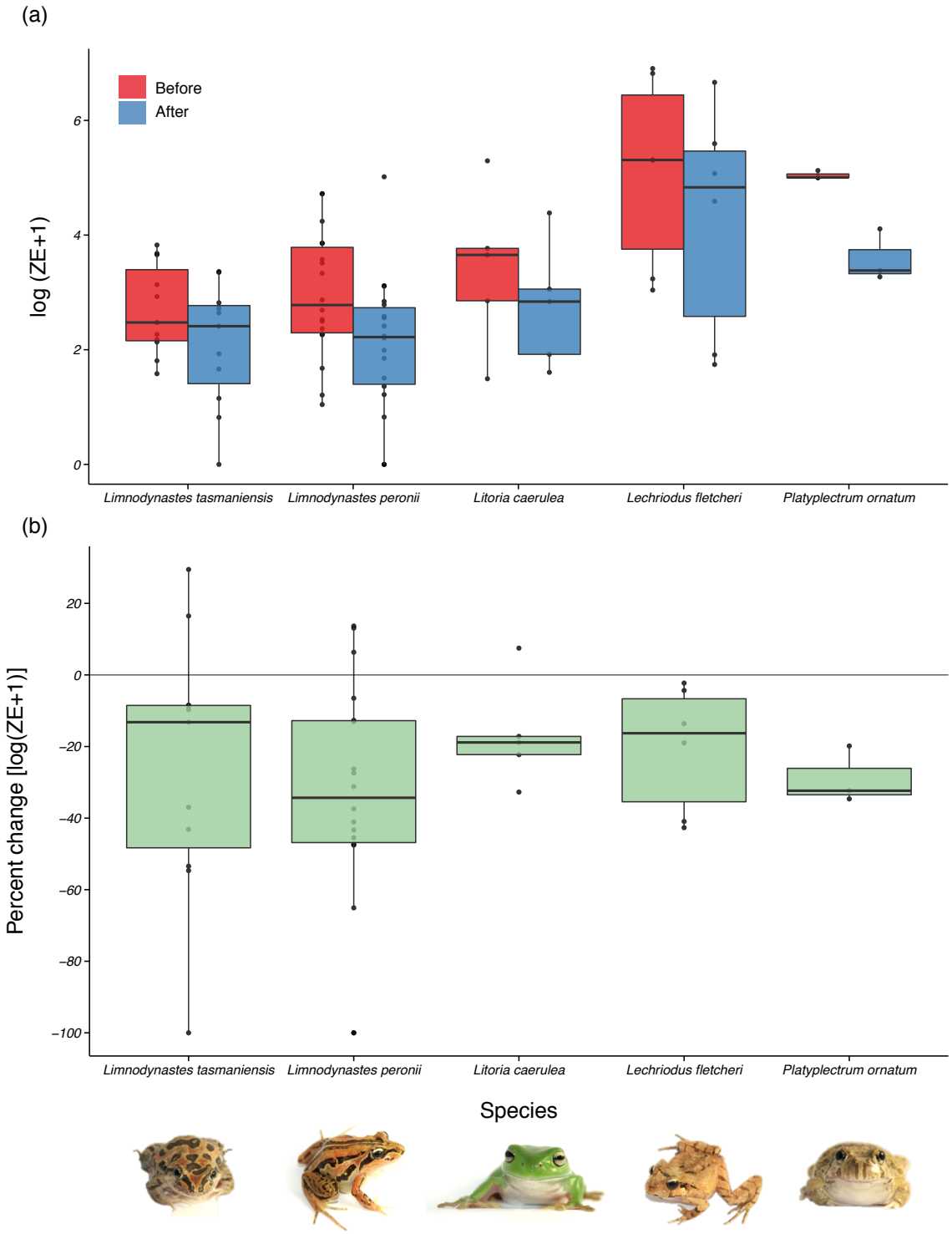

Figure 3.4 (a) Boxplots of infection load (log [zoospore equivalents (ZE)+1]) before and after sloughing, and (b) percent change (log [ZE+1]) in Bd load from before to after sloughing in five Australian frog species infected with the pathogen *Batrachochytrium dendrobatidis* (Bd). The centre line is the 50th percentile, top and bottom of box represent 75th and 50th percentile, and whiskers extend to extreme data points (no more than 1.5 times the interquartile range).

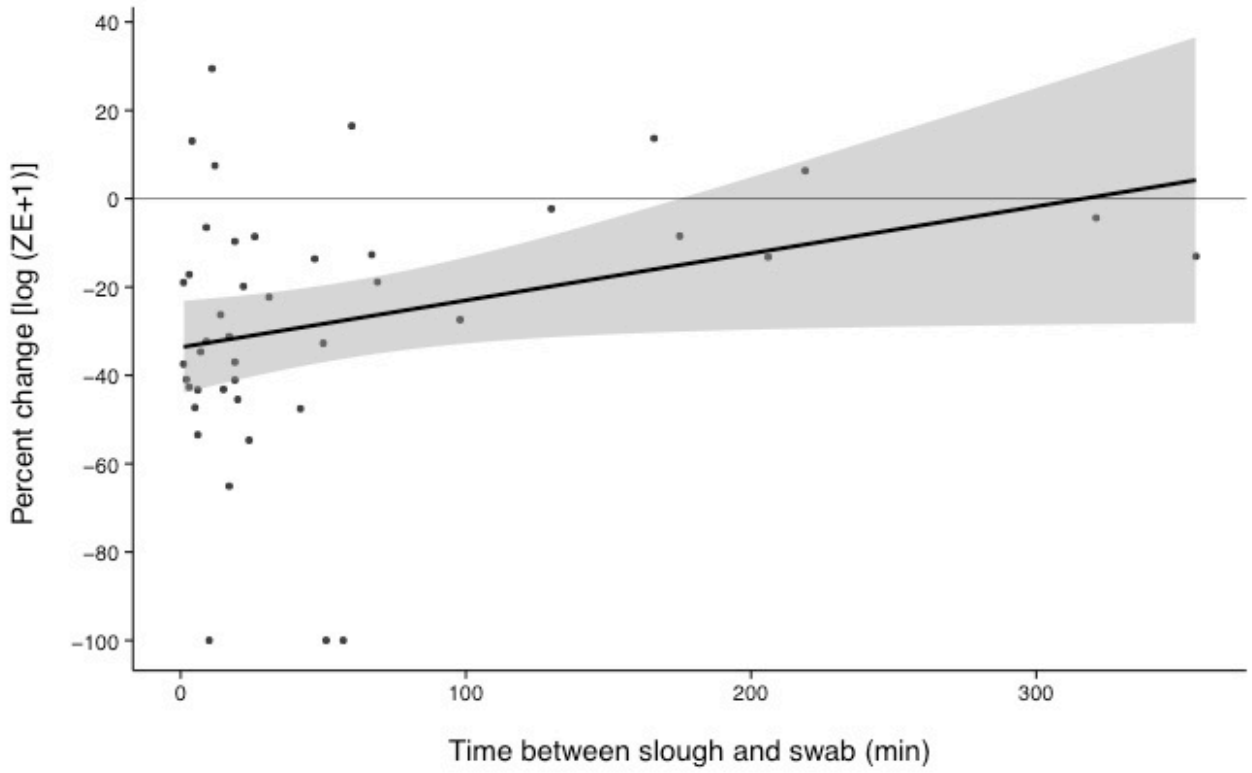

Figure 3.5 The association between the percent change in infection load (log [zoospore equivalents (ZE)+1]) and the time (min) between a sloughing event and the 'after' swab, in frogs infected with the pathogen *Batrachochytrium dendrobatidis* (Bd). Shaded area indicates standard error.

Change in sloughing rate with Bd infection

On average, IMI decreased in clinically infected *Lit. caeruela* and *Lec. fletcheri*, and in non-clinically infected *Lim. tasmaniensis,* indicating an increase in sloughing rate over time in these groups (Figure 3.6, Table 3.6, Appendix 3.1). There was no difference in sloughing rate amongst groups in *Lim. peronii* and *P. ornatum* (Figure 3.6, Table 3.6, Appendix 3.1). Furthermore, infected frogs demonstrated an increase in sloughing rate with infection load, as demonstrated in *Lit. caerulea* previously (Figure 3.7, Table 3.7, Ohmer et al. 2015). Interestingly, sloughing rate in *Lim. tasmaniensis* increased at lower Bd infection loads than other species (Figure 3.7), and this species demonstrated the lowest susceptibility to chytridiomycosis (0% mortality).

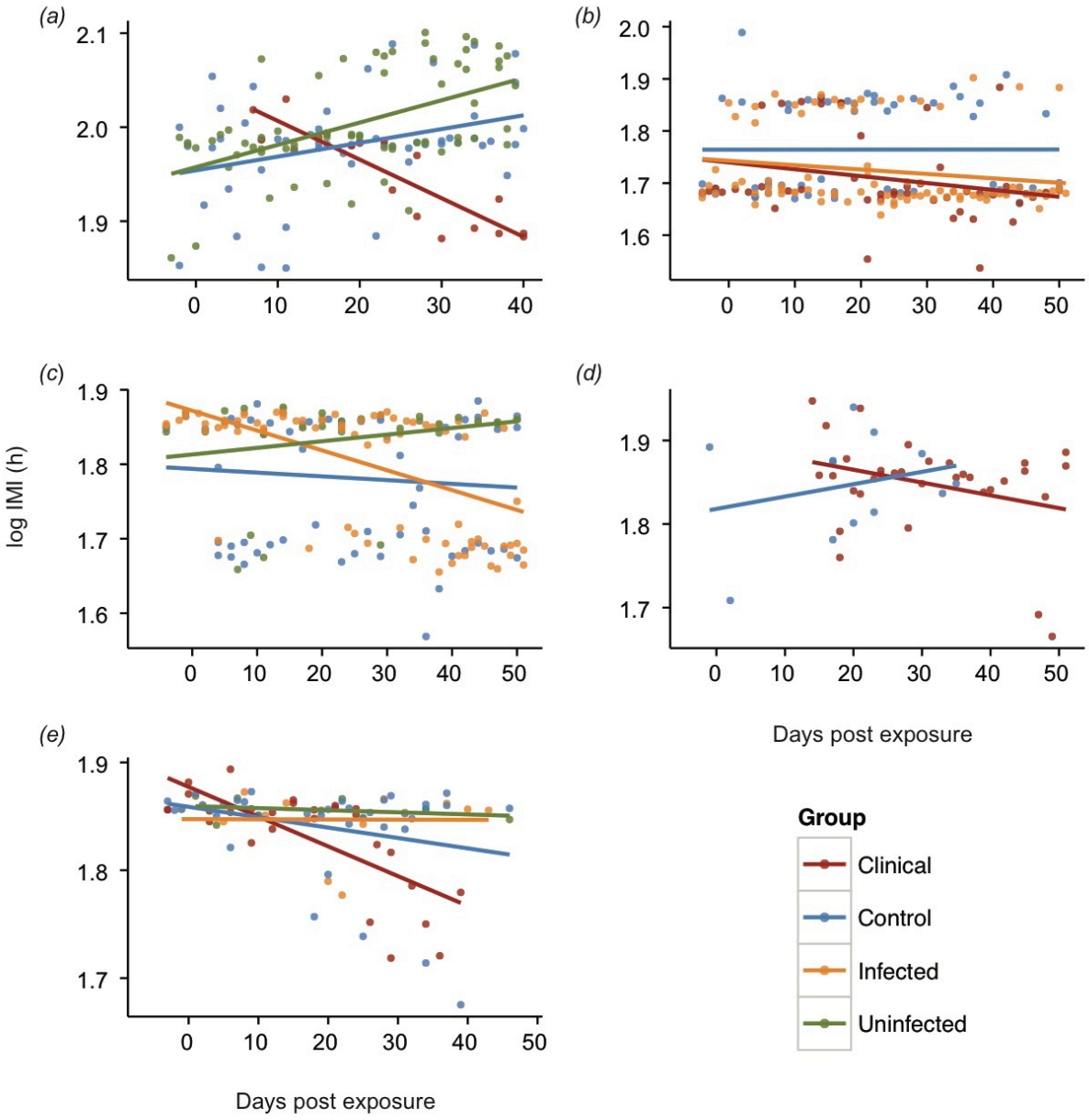

Figure 3.6 Log intermoult interval (IMI, h) for the first 40 days post exposure in *Litoria caerulea* (a), and the first 50 days post exposure in (b) *Limnodynastes peronii*, (c) *Lim. tasmaniensis* (d) *Platyplectrum ornatum*, and (e) *Lechriodus fletcheri.* Colours indicate group based on disease outcome (control, clinical, infected, or uninfected). Data for *Lit. caerulea* is truncated before the third experimental exposure to *Batrachochytrium dendrobatidis* (Bd) because exposure groups changed. Longitudinal data was sparser for *P. ornatum* due to their burrowing behaviour.

Table 3.6 Statistical results from a phylogenetic linear mixed model (implemented in ASReml-R) examining intermoult interval (IMI) over time in five species of Australian frog exposed to *Batrachochytrium dendrobatidis* (Bd). Fixed effects were *days post exposure*, *days post exposure*^{2}, and *group* (control, clinical, infected and uninfected) and random effects were *Frog ID* nested in *Species,* and a variance-covariance matrix created from the phylogeny, to take into account correlated error from repeated measures on the same individual, and phylogenetic non-independence. s.e. = standard error, d.f. = degrees of freedom, bold p-values are significant

Fixed effects	**Estimate**	**s.e.**	**F**	**d.f.**	***p***
Intercept	73.56	9.0	78.4	1, 3.7	**1.50×10^{-3}**
Days post exposure	0.44	0.12	13.91	1, 604.3	**2.10×10^{-4}**
Days post exposure^2	-0.009	0.002	19.92	1, 606.9	**9.62×10^{-6}**
Group (Control)	NA	NA	2.32	3, 42.7	**8.89×10^{-2}**
Group (Clinical)	-2.98	2.75			
Group (Infected)	0.47	2.78			
Group (Uninfected)	5.70	2.76			
Random effects	**Variance**	**s.e.**	**χ^2**	**d.f.**	***p***
Phylogeny	1.79	156.5	2.42	1	0.12
Frog ID nested in Species	2.88	9.75	68.26	1	**1.11×10^{-16}**
$\lambda = 0.58$, s.e. = 1.32					

Table 3.7 Statistical results from a phylogenetic linear mixed model (implemented in ASReml-R) examining the change in intermoult interval (IMI) with infection load of *Batrachochytrium dendrobatidis* (Bd), in zoospore equivalents (ZE). Fixed effects were *Bd load* (log[ZE+1]), and random effects were *Frog ID* nested in *Species* and a variance-covariance matrix created from the phylogeny, to take into account correlated error from repeated measures on the same individual, and phylogenetic non-independence. s.e. = standard error, d.f. = degrees of freedom, bold p-values are significant

Fixed effects	**Estimate**	**s.e.**	**F**	**d.f.**	***p***
Intercept	1.90	0.055	1194	1,3.9	**5.04×10^{-6}**
Bd load (log[ZE+1])	-0.016	0.0035	20.79	1,145.6	**1.08×10^{-5}**
Random effects	**Variance**	**s.e.**	**χ^2**	**d.f.**	***p***
Phylogeny	1.89	0.006	2.28	1	0.13
Frog ID nested in Species	0.054	3.2×10^{-4}	0.66	1	0.42
$\lambda = 0.64$, s.e. = 0.17					

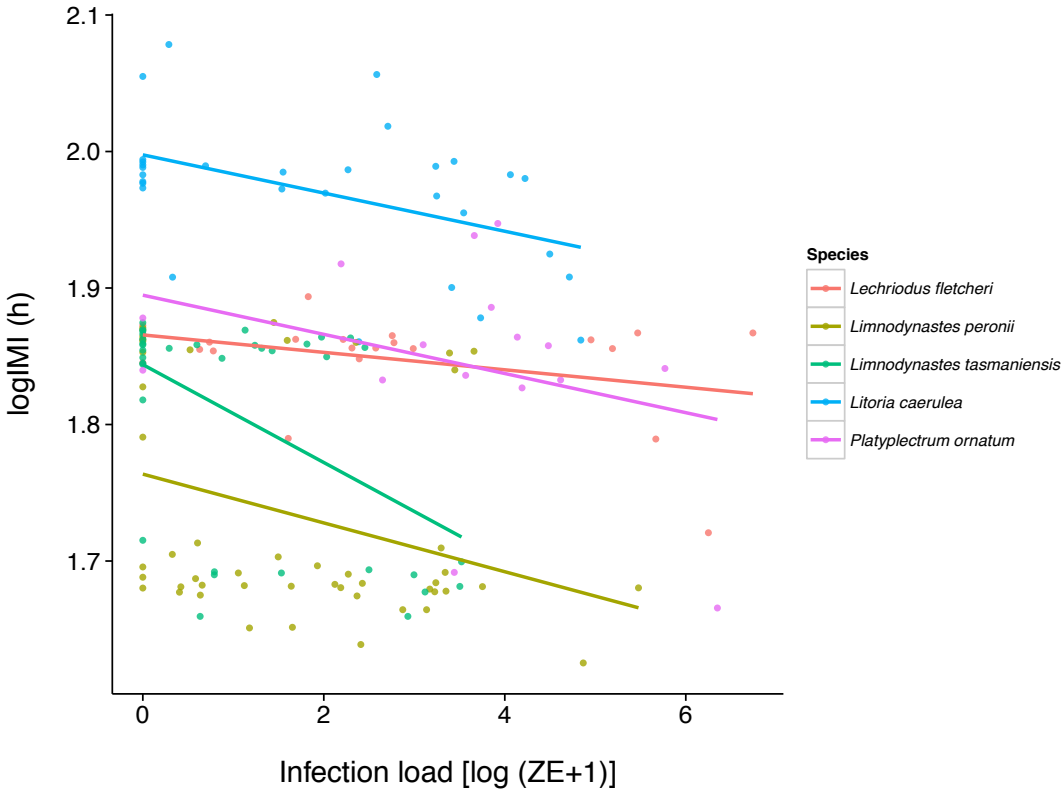

Figure 3.7 Change in log intermoult interval (IMI, h) with *Batrachochytrium dendrobatidis* (Bd) infection load (log [ZE+1]) in infected individuals of five frog species.

Discussion

Amphibians regularly shed their skin, and the importance of this frequent, sometimes daily, process has heretofore been overlooked. By comparing the sloughing rates and infection loads across five frog species with different susceptibilities to Bd infection, we were able to demonstrate that skin sloughing does indeed reduce Bd load on the ventral skin surface, and can even result in infection clearance. However, this decrease in Bd load on the skin surface is only temporary in susceptible species, and many individuals still developed clinical disease. Infection intensity, mortality, and skin sloughing rates varied across host species, as did the timing of increase in those sloughing rates. It is likely that sloughing is a double-edged sword: sloughing reduces Bd load, and can aid in clearance of infection in some individuals, yet, skin sloughing is a physiologically vulnerable time for amphibians (Jørgensen 1949), and an increase in sloughing rate at high infection loads may be more detrimental than beneficial.

Utilising a refined methodology (live video feeds) to swab frogs as soon as possible after sloughing, and avoiding the confounds of the swabbing action itself by swabbing only half the frog, we found that the mechanical action of removing the *stratum corenum* does indeed reduce Bd loads

on the skin surface across all species. In *Lim. peronii* and *Lim. tasmaniensis*, species that experienced low mortality rates after Bd exposure, sloughing sometimes resulted in 100% reduction in Bd load, and eventual clearance of infection. This indicates that sloughing can act as an immune defence to limit Bd infection load. However, in species that suffered high mortality rates, such as *P. ornatum* and *Le. fletcheri*, sloughing did not permanently reduce Bd loads, and infection intensity continued to increase. These host-dependent differences may be a reflection of the variation in Bd growth patterns on an amphibian's skin (Van Rooij et al. 2012). If the growth of Bd remains epibiotic, as seen in skin explants of the tolerant species *Xenopus laevis* (Van Rooij et al. 2012), then sloughing may be more effective at removing encysted zoospores and resulting zoosporangia. This may be the case in *Lim. tasmaniensis*, in which 71% of individuals became infected, but almost all individuals cleared infection by the end of the experimental period, and no clinical signs of infection were observed (e.g. inappetance, weight loss, or abnormal skin shedding). In the context of this experimental infection, *Lim. tasmaniensis* may be considered a species resistant to Bd infection, given that it can reduce pathogen colonisation and/or invasion until clearance (Schneider & Ayres 2008).

Organisms can defend themselves from a pathogen via two mechanisms, which are not mutually exclusive: resistance and tolerance (Schneider & Ayres 2008; Råberg 2014). While resistance mechanisms prevent infection or limit pathogen growth, tolerance mechanisms limit fitness effects of a given pathogen burden. Strictly speaking, because skin sloughing can limit pathogen growth, it would be defined as a resistance mechanism in competent host species that are able to limit the invasion of Bd in the epidermis via other immune defences, such as antimicrobial skin peptides or antimicrobial metabolites (Woodhams et al. 2007a; Harris et al. 2009; Conlon 2011). However, in susceptible hosts, it would appear that sloughing is not enough to remove Bd infection on its own, and this may be due to the invasive nature of Bd growth in these species (Van Rooij et al. 2012). Sloughing may also play a role in resistance to Bd colonisation. If sloughing occurs very soon after Bd exposure, it may rid the host of the pathogen completely before invasive Bd growth occurs. Yet, Van Rooij et al (2012) found that invasive germ tubes were seen as soon as two hours post exposure in *Lit. caerulea*, and by 16 hours post exposure, chytrid thalli were growing intracellularly (Van Rooij et al. 2012). In a previous experimental exposure, however, no association was found between Bd exposure and the timing of first slough and infection outcome in *Lit. caerulea* (Ohmer et al. 2015).

Sloughing could also be seen as playing a role in allowing host tolerance to Bd infection, by regulating Bd infection spread and thereby limiting the health effects of infection (Schneider & Ayres 2008). In some host species tolerant of high infection loads, such as *Pseudacris regilla* in

North America, it has been hypothesised that tolerance arises from localised infections isolated to certain patches on the skin, despite intracellular growth (Reeder et al. 2012). Mechanisms that restrict Bd from spreading to other skin areas are not known, but sloughing could play a role in preventing spread by removing zoospores released onto the skin from nearby infected areas.

It was previously found that frogs infected with Bd have increased sloughing rates, but only at high infection loads (Ohmer et al. 2015). We discovered a similar trend in this study, with infected individuals of all species increasing their sloughing rates, except in *Lim. peronii* and *P. ornatum*. The lack of sloughing rate plasticity in *Lim. peronii* may be due to the already fast baseline sloughing rate, or perhaps a lower number of replacement cell layers in the epidermis precludes sloughing plasticity (Greenspan et al. 2012). In *P. ornatum*, however, the lack of a significant increase in sloughing rate in infected frogs may be attributable to the difficulty in observing these burrowing animals at all times, thereby reducing the ability to detect a longitudinal trend. Most interestingly, the least susceptible species, *Lim. tasmaniensis*, increased sloughing rates at much lower infection loads, and eventually cleared infection. This indicates that sloughing may serve as an effective immune response and not only as a baseline defence in this species, because sloughing rate increased before a high level of cutaneous Bd growth on the skin was reached. Despite evidence that Bd avoids or evades the immune response and/or results in immunosuppression in some species (Rosenblum et al. 2012; Fites et al. 2013), an increased sloughing rate in *Lim. tasmaniensis* may indicate an immune response, although the mechanisms underlying this response are unknown.

Resistance to infection can be costly, as an increased immune response can result in not only damage to the pathogen, but also the host (termed immunopathology; reviewed in Ayres & Schneider 2012; Medzhitov et al. 2012). While we have shown that skin sloughing can reduce Bd loads and even clear infection in some hosts, sloughing itself leaves amphibians physiologically vulnerable. It has been shown that skin permeability to water and sodium increases during the sloughing process, temporarily disrupting physiological homeostasis (Jørgensen 1949). In frogs with severe chytridiomycosis, water and electrolyte imbalances are symptoms of clinical disease (Voyles et al. 2009; Voyles et al. 2012). Furthermore, infected frogs experience high cutaneous water loss rates during sloughing (Appendix A), and may be slow to rehydrate (Carver et al. 2010, corroborating findings of dehydration in severely infected frogs (Rosenblum et al. 2009; Voyles et al. 2012). Thus, an increase in sloughing rate at advanced infection stages may cause more harm than good, exacerbating imbalances in fluid and electrolyte levels (Ohmer et al. 2015). In *Lim. tasmaniensis*, however, sloughing rate increased at lower infection loads, perhaps limiting the negative effects of increased sloughing while increasing the rate of pathogen clearance.

The five Australian frog species exposed to Bd in this experiment demonstrated wide variation in susceptibility to Bd infection and chytridiomycosis. However, this variation may not be suggestive of Bd infection in wild populations of these species. In this study, frogs were kept in high-humidity conditions that were not indicative of typical conditions experienced by all of these species in the wild. In particular, *P. ornatum* is often found in dry or semi-arid conditions far from a permanent water source (Anstis 2013), thus high inherent susceptibility to disease under optimal conditions for the pathogen (as in this study) may not relate to population-level effects in an environmental context. This has been demonstrated in *Lit. caerulea*, which is typically very susceptible to Bd infection and chytridiomycosis in laboratory settings (Voyles et al. 2009; Ohmer et al. 2015), but has not undergone significant declines in the wild (Berger et al. 1998; Berger et al. 2004; Commonwealth of Australia 2006). Unexpectedly, adult *Lit. caerulea* collected for use in the current study demonstrated low infection prevalence and low mortality rates after exposure to Bd, in contrast to Ohmer et al. (2015). This may be due to the fact that frogs were collected from a population only 15 km north of previous records of *Lit. caerulea* with Bd infection (Murray et al. 2010), despite all animals being Bd negative upon collection. While there is currently no direct evidence of individuals from populations with long histories of Bd infection evolving resistance (Bataille et al. 2015), repeated exposures to Bd and subsequent heat treatment has conferred increased disease resistance in at least one species in the laboratory (McMahon et al. 2014). In addition, *Lit. caerulea* in this study had been in captivity for a shorter period of time than in our previous study (two months versus one year), which may also have contributed to the differences in Bd susceptibility (immunocompetence) observed. For example, immune defences such as the cutaneous bacterial community have been shown to be significantly different in captive animals, and this may influence disease susceptibility (Becker et al. 2014b). Finally, when comparing *Lit. caerulea* to the other three species, it is important to keep in mind that they were collected as adults, while the other three were raised from spawn in captivity. Although there are no data on how rearing history influences subsequent Bd susceptibility in amphibians, it cannot be ignored as a potential factor contributing to the observed susceptibility differences across species. Therefore, this caveat should be kept in mind when comparing susceptibility across species.

In order to best utilise models of host extinction risk following Bd exposure, a better understanding of the host-pathogen relationship for model parameterisation is required (Louca et al. 2014). Demonstrating that sloughing can reduce Bd loads on both susceptible and resistant hosts has implications for understanding the epidemiology of this pathogen in wild populations. In establishing that sloughing can regulate Bd growth, this cyclic process can be built into patterns of Bd growth on individual hosts, and the effects can be modelled at a population-level. In addition,

these findings have implications for interpreting swab results collected from individuals at a single time point. In demonstrating that skin sloughing can indeed reduce Bd load on the epidermis in multiple frog species, sometimes up to 100 %, we indicate the potential for false negatives, or an underestimation of actual infection load, if swabbing occurs shortly after sloughing. Many studies have reported frogs gaining and losing infection over short time scales (Briggs et al. 2010; Reeder et al. 2012; Ohmer et al. 2013), and this may be in part due to amphibian skin sloughing.

Differences in susceptibility to Bd, a generalist pathogen implicated in the decline or extinction of over 200 amphibian species worldwide (Skerratt et al. 2007), may be linked to inherent differences in the amphibian epidermis. This study demonstrates that amphibian skin sloughing, which varies in rate across species and increases with temperature (Meyer et al. 2012; Cramp et al. 2014) and disease progression (Ohmer et al. 2015), can also regulate Bd growth. The efficacy of the regulation of Bd growth is host-dependent, however, and indicates a key difference in the role of sloughing as a skin immune defence mechanism in susceptible versus tolerant or resistant hosts. This work has significant conservation implications, as it may improve our predictions of host-specific responses to Bd in wild populations, allowing for better conservation planning.

Appendix 3.1 Statistical results from linear mixed effects models examining the change in intermoult interval (IMI) after sloughing in five species of Australian frog exposed to *Batrachochytrium dendrobatidis* (Bd). Fixed effects were the interaction between *group* (control, clinical, infected, uninfected) and *days post exposure*, and *Frog ID* was included as a random factor to take into account correlated error from repeated measures on the same individual. s.e. = standard error, s.d. =standard deviation, d.f. = degrees of freedom, CI= confidence intervals, bold p-values are significant

Litoria caerulea

Fixed effect	**estimate**	**s.e.**	**t-value**	**d.f.**	***p***	**CI**	
						2.5%	**97.5%**
Intercept	1.97	0.016	124.17	121	**< 0.0001**	1.92	1.99
Group (Clinical)	0.089	0.034	2.65	14	**0.019**	$1.87x10^{-2}$	0.16
Group (Uninfected)	0.006	0.021	0.30	14	0.77	$-3.74x10^{-2}$	0.05
Days post exposure	0.002	0.0004	4.55	121	**< 0.0001**	$9.20x10^{-4}$	0.002
Groups (Clinical)*Days post exposure	-0.006	0.0008	-7.20	121	**< 0.0001**	$-7.12x10^{-3}$	-0.004
Groups (Uninfected)*Days post exposure	0.0008	0.0005	1.74	121	0.084	$-9.25x10^{-5}$	0.002
Random effects:	**s.d.**				**Residual**		
Frog ID	0.033				0.031		

Limnodynastes tasmaniensis

Fixed effect	**estimate**	**s.e.**	**t-value**	**d.f.**	***p***	**CI**	
						2.5%	**97.5%**
Intercept	1.79	0.025	71.66	160	**< 0.0001**	1.74	1.84
Group (Infected)	0.086	0.033	2.57	8	**0.033**	0.01	0.16
Group (Uninfected)	0.016	0.043	0.38	8	0.71	-0.081	0.11
Days post exposure	-0.0006	0.0005	-0.12	160	0.91	-0.0011	0.001
Groups (Infected)*Days post exposure	-0.003	0.0006	-3.72	160	**0.0003**	-0.004	-0.001
Groups (Uninfected)*Days post exposure	0.001	0.0009	1.21	160	0.23	-0.0006	0.0028
Random effects:	**s.d.**				**Residual**		
Frog ID	0.039				0.062		

Limnodynastes peronii

Fixed effect	**estimate**	**s.e.**	**t-value**	**d.f.**	***p***	**CI**	
						2.5%	**97.5%**
Intercept	1.77	0.026	68.58	179	**< 0.0001**	1.72	1.81
Group (Clinical)	-0.022	0.041	-0.54	6	0.61	-0.12	0.076
Group (Uninfected)	-0.019	0.034	-0.55	6	0.60	-0.10	0.063
Days post exposure	0.0003	0.0006	0.39	179	0.69	-0.001	0.001
Groups (Clinical)*Days post exposure	-0.0018	0.0009	-1.82	179	0.071	-0.0036	0.0011
Groups (Uninfected)*Days post exposure	-0.001	0.0008	-1.33	179	0.18	-0.0027	0.0005
Random effects:	**s.d.**				**Residual**		
Frog ID	0.033				0.074		

Lechriodus fletcheri

Fixed effect	**estimate**	**s.e.**	**t-value**	**d.f.**	***p***	**CI**	
						2.5%	**97.5%**
Intercept	1.86	0.011	162.53	78	**< 0.0001**	1.84	1.88
Group (Clinical)	0.019	0.017	1.14	3	0.34	-0.032	0.0069
Group (Infected)	-0.011	0.021	-0.51	3	0.64	-0.075	0.0053
Group (Uninfected)	-0.019	0.034	0.057	3	0.96	-0.071	0.0073
Days post exposure	-0.0009	0.0005	-2.06	78	**0.042**	-0.0018	-7.38×10^{-5}
Groups (Clinical)*Days post exposure	-0.0018	0.0007	-2.51	78	**0.014**	-0.0032	-4.43×10^{-4}
Groups (Infected)*Days post exposure	0.0009	0.0008	1.14	78	0.26	-0.0006	0.00025
Groups (Uninfected)*Days post exposure	0.0007	0.001	0.77	78	0.44	-0.0011	0.00026
Random effects:	**s.d.**				**Residual**		
Frog ID	0.0055				0.034		

Platyplectrum ornatum

Fixed effect	**estimate**	**s.e.**	**t-value**	**d.f.**	***p***	**CI**	
						2.5%	**97.5%**
Intercept	1.82	0.036	51.24	33	**< 0.0001**	1.75	1.88
Group (Clinical)	0.076	0.046	1.65	8	0.14	-0.025	0.18
Days post exposure	0.0014	0.0016	0.92	33	0.36	-0.0016	0.004
Groups (Clinical)*Days post exposure	-0.0029	0.0018	-1.63	33	0.11	-0.0064	0.0006
Random effects:	**s.d.**				**Residual**		
Frog ID	0.011				0.054		

CHAPTER 4

A phylogenetic investigation of the variation in sloughing frequency and epidermal thickness between anuran species demonstrating differences in susceptibility to a cutaneous disease

Abstract

Amphibian skin is the first barrier encountered by invading pathogens, including the devastating cutaneous pathogen, *Batrachochytrium dendrobatidis* (Bd). Yet, given the role of amphibian skin in physiology and immune defence, the structure and function of this organ is highly variable across anurans. Furthermore, the frequency of amphibian skin sloughing or shedding differs across species, and has been shown to reduce Bd infection load on the skin (Ohmer et al. 2015). In order to investigate this variation in skin-associated traits, we examined the relationship between susceptibility to chytridiomycosis and skin structure and function between anuran species within a phylogenetic context. We measured the sloughing rates of 21 frog species from Australia (9), Central and South America (11), and Southeast Asia (1). In addition to measuring sloughing rates, we measured epidermal thickness and the number of replacement layers in preserved specimen of seventeen of these species. Utilising a phylogenetic linear mixed model framework, we assessed the contribution of these skin turnover traits to overall evidence of Bd-driven declines, based on evidence from the IUCN Red List, published papers, grey literature, and personal communications. We found that sloughing rate demonstrates high phylogenetic signal, but was not associated with evidence of Bd-driven declines, or other skin characteristics, within this subset of species. This is the first comparison of sloughing rate across a wide range of amphibian species, and creates the first database of amphibian sloughing behaviour. Given the strong phylogenetic signal observed in sloughing rate, approximate sloughing rates of related species may be predicted based on phylogenetic position. As sloughing can reduce fungal infection loads on the skin surface, understanding variation in sloughing rate may help to explain differences in the severity of infection in genera with relatively slow skin turnover rates (e.g. *Atelopus*). A clear understanding of epidermal turnover in amphibian genera particularly affected by Bd may help focus conservation mitigation efforts.

Introduction

Amphibian skin is the first barrier encountered by invading pathogens (Richmond et al. 2009; Rollins-Smith et al. 2011). Given the importance of amphibian skin for a multitude of physiological functions, this organ is highly diverse in its form and function (Feder 1992; Duellman

& Trueb 1994; Wells 2010). The 'typical' amphibian demonstrates highly permeable skin that leaves the organism vulnerable to desiccation in terrestrial environments, and permeable to water and electrolytes in aquatic habitats (Jørgensen 1997). However, amphibians have developed a number of physiological, structural, and behavioural mechanisms to overcome these challenges (Toledo & Jared 1993; Jørgensen 1997; Wells 2010). Anurans are adapted to a wide variety of habitats and ecological niches, and morphofunctional properties of their skin have conferred tolerance to thermal and moisture extremes in many species (Toledo & Jared 1993). Adaptations of amphibian skin have allowed for decreased water loss (waxy lipids: McClanahan et al. 1978; cocoon formation: Withers 1995), increased water uptake (vascularised drink patch: McClanahan & Baldwin 1969; Christensen 1974; Viborg et al. 2006; skin sculpturing and water channelling: Lillywhite & Licht 1974), thermal regulation via evaporative water loss (mucous glands: Lillywhite 1971; Lillywhite & Licht 1975), and even potentially novel methods of thermoregulation and UV protection via coloration change (concentration of iridophores: Rudh & Qvarnström 2013). Solely in terms of the amphibian integument, the diversity of form and function of this organ would indicate that amphibians are not uniform hosts for cutaneous pathogens.

The chytridiomycete fungus *Batrachochytrium dendrobatidis* (Bd) is a generalist pathogen that is affecting amphibians on a global scale (Fisher et al. 2009a; James et al. 2015). Found on over 500 amphibian species and counting, never before has a single pathogen threatened such a wide diversity of species within a single class of vertebrates (Skerratt et al. 2007; Olson et al. 2013). In post-metamorphic amphibians, this pathogen is restricted to the keratinised layers of an amphibian's skin, and despite this localisation, infection can result in the disease chytridiomycosis, and mortality (Berger et al. 1998; Voyles et al. 2009). However, the degree to which host species are susceptible to infection and subsequent clinical disease is highly variable, and is likely the combined result of host and pathogen ecology and biology, community structure, and the thermal and hydric environment (Fisher et al. 2009b; James et al. 2015). Given that this pathogen is entirely cutaneous in post-metamorphic animals, and amphibian skin is important for a range of physiological and immune defence functions, understanding the variation in skin structure and function across amphibian species is paramount.

A key aspect of amphibian skin biology is the nature of its continual renewal via routine moulting or sloughing. During this process, the outer keratinised layer of skin, or *stratum corneum*, is shed in one piece via a series of limb and body movements, after which the shed skin is routinely eaten (Larsen 1976). It is assumed that this process transpires in most amphibians, and it is thought to occur anywhere from daily (Castanho & de Luca 2001) to once a week or fortnightly (Budtz & Larsen 1973; Meyer et al. 2012). Sloughing primarily acts in skin renewal, but may also play a role

in controlling cutaneous microbial populations (Meyer et al. 2012; Cramp et al. 2014), and even regulating Bd load on the skin (Chapter 3). It has thus been suggested that sloughing rate, or the rate of epidermal turnover, could contribute to the susceptibility of amphibians to chytridiomycosis (Greenspan et al. 2012; Ohmer et al. 2015; Chapter 3). However, most of the research on amphibian sloughing occurred prior to the discovery of Bd, and only with a limited number of common laboratory species (Larsen 1976). Thus, there is a very poor understanding of the variation in skin sloughing rates among amphibians, and whether this trait demonstrates phylogenetic signal.

Furthermore, the structure of amphibian skin is highly variable across species, particularly in terms of its thickness, sculpturing, number of mucosal and peptide glands, and the presence of additional structures postulated to play a role in the resistance of amphibian skin to cutaneous water loss (e.g. calcified dermal layer; Toledo & Jared 1993; Wells 2010). Epidermal thickness, and in particular the number of replacement layers in the epidermis, may correlate with the rate of epidermal turnover, and could indicate the level of 'moulting plasticity' within individuals or species (Greenspan et al. 2012). Given amphibians infected with Bd demonstrate an increase in sloughing rate (Ohmer et al. 2015), more epidermal layers may provide the flexibility to increase sloughing rates without physiological harm. In addition, skin thickness has been hypothesised to relate to the ecological habit of the amphibian (Toledo & Jared 1993), and may reflect the overall permeability of the skin to water. Understanding the variation in epidermal thickness across species could provide important insight into innate variation in disease susceptibility to this very generalist pathogen.

In order to create the first database of amphibian structure and function, we utilised a recently developed non-invasive method to analyse sloughing rates in captive amphibians (Ohmer et al. 2015), focusing on anurans. The number of amphibian species in captivity for conservation reasons continues to grow as species at risk of extinction are collected for captive breeding programs (Griffiths & Pavajeau 2008; Gratwicke et al. 2015). In addition to their direct purpose, these programs are an excellent resource for better understanding the biology and ecology of these species, via remote monitoring with infrared cameras. To capture a variety of amphibian species from two areas that have experienced severe amphibian declines, this comparison focused on frog species from Australia and Central and South America (Berger et al. 1998; Stuart et al. 2004; Menéndez-Guerrero & Graham 2013). Furthermore, utilising museum specimens, samples of ventral skin for each species were analysed for structural differences, namely epidermal thickness and the number of epidermal layers. Utilising a phylogenetic mixed model framework, to take into account non-independent evolutionary history amongst species, we examined the relationship of

these skin traits to characteristics of their life history, and the known susceptibility of these species to the disease chytridiomycosis.

Understanding how among-species differences in amphibian skin structure and turnover rates change within a phylogenetic context might help us better understand the drivers of susceptibility to cutaneous pathogens. Therefore, we investigated whether sloughing rate demonstrates phylogenetic signal across anuran species, and if this rate is influenced by body size, temperature, life history, or skin characteristics. Furthermore, activity time (nocturnality or diurnality) may influence anuran physiology because diurnal and nocturnal frogs experience opposite ends of daily thermal variation and subsequent potential thermal and water balance stress during active periods (Navas 1996; Navas et al. 2008). Given that sloughing behaviour is also physiologically challenging (Jørgensen 1949), the timing of the sloughing event was compared for diurnal and nocturnal species. Finally, both skin structure and sloughing rate were investigated as predictors of overall susceptibility to chytridiomycosis among the species measured. We hypothesised that species with a thinner epidermis and slower epidermal turnover would demonstrate greater evidence of Bd-driven declines in wild populations.

Methods

Measuring anuran sloughing rates

Frogs were filmed at zoos and captive breeding centres to determine sloughing rates (Table 4.1). Whenever possible, anurans were filmed individually, but never more than in groups of two per enclosure. Frogs were recorded continuously with twelve 600TVL Weatherproof infrared cameras (model EN-CI20B-65H, Eonboom Electronics Limited) at a frame rate of 1.52 frames per second (FPS). Video was recorded on either a 16 Channel H.264 Digital Video Recorder (DVR), model MDR688ZB (AU)-E. 600TVL, or a 4 Channel DVR, system model DVR-6204T, depending on the location. Monitoring amphibian sloughing via infrared video cameras has been shown to be successful previously (Ohmer et al 2015), and sloughing behaviour is easily recognisable when viewing recordings at 16x normal speed.

Table 4.1 Locations where sloughing rates were measured for each anuran species, as well as the temperature range and average temperatures during the measurement period, the light cycle, and specific husbandry notes.

Location	Species	N	Temp. range ˚C (mean)	Light cycle	Husbandry
The University of Queensland Brisbane, Australia	*Litoria caerulea*	10	15.0 - 23.0 (18.8)	12L:12D	Damp paper towels Plastic cup or PVC pipe for shelter Crickets (5 per week)
	Limnodynastes peronii	3			
	Limnodynastes tasmaniensis	4			
	Platyplectrum ornatum	4			
	Lechriodus fletcheri[1]	3			
	*Rhinella marina**	20	25.0 (constant)		Moistened wood chips Standing water at one end Crickets fed *ad libitum*
Taronga Zoo Sydney, Australia	*Litoria infrafrenata*	8	17.2 - 26.2 (20.7)	12L:12D	Aquarium stones for substrate Water dish PVC pipe for shelter Crickets fed *ad libitum*
	Litoria vereauxii alpina	8	20.0 - 22.0 (21.7)		
	Litoria booroolongensis	7	21.0 - 24.5 (23.6)		
	Litoria aurea	8	21.0 - 24.5 (23.2)		
Balsa de los Sapos Quito, Ecuador	*Atelopus (spumarius-pulcher complex)*	10	17.6 - 23.3 (20.4)	9L:15D	Damp paper towels for substrate and shelter Crickets fed *ad libitum*
			17.6 - 23.3 (20.4)		
	Gastrotheca riobambae	10	16.6 - 26.6 (20.5)		
	Epipedobates tricolor	10	18.1 - 24.2 (21.6)		
	Gastrotheca pseustes	6	18.1 - 24.2 (21.6)		
	Ceratophrys stolzmanni	10	16.7 - 22.7 (20.0)		
	Hyloscirtus pantostictus	4	17.0 - 22.2 (20.8)		
	Espadarana callistomma	4	16.4 - 22.2 (19.1)		
	Atelopus elegans	8			
San Diego Zoo Global San Diego, USA	*Dendrobates tinctorius*	3	20.0 - 25.0 (22.4)	15L:9D	Live plants (1-2), moss substrate PVC pipe for shelter Crickets fed *ad libitum* Water dish
	Dendrobates auratus	1			
	Polypedates otilophus[1]	4			

*This species was not filmed. Sloughing rate was measured by marking the dorsal surface with waterproof zinc, and observing toads twice daily for the removal of the mark

[1]Frogs filmed of these species were subadults

Filming enclosures were relatively simple at all locations to aid viewing of behaviours, with a few exceptions. In general, amphibians were provided with a damp substrate of crumpled paper towels, as well as a plastic cup or half a plastic PVC pipe for shelter. Given amphibian husbandry varied depending on institutional husbandry practices, deviations from these conditions are noted in Table 4.1. Amphibians were filmed for two to three weeks at each location, and temperatures were monitored with Thermochron iButtons (© Maxim Integrated). Given the variation in temperature requirements across species, average temperature over the entire filming period was used for analyses.

At each location, the mass and snout-vent length (SVL), sex (if known), and unique colouration characteristics (if filmed in pairs) for each individual was recorded. At the *Balsa de los Sapos*, a facility dedicated to amphibian conservation at the Pontificia Universidad de Católica del Ecuador, frogs were swabbed at the start of filming to determine Bd infection status, given a recent case of Bd infection in the facility. Swabs were analysed with standard qPCR techniques by Allan Pessier at the San Diego Zoo.

Videos were analysed by multiple 'viewers', and sloughing events and times were corroborated by at least one other 'viewer'. Analysed videos were utilised to calculate intermoult intervals (IMI), or the time between sloughing events, as well as slough duration and timing analyses.

IMI values for *Rhinella marina* were not obtained from video footage, but rather via the traditional marking and observation method. A small about of non-toxic waterproof zinc cream (Key-Sun Laboratories Pty Ltd, NSW, Australia) was applied to the dorsal surface and animals were checked twice-daily to record the disappearance of a mark (indicating sloughing had occurred). Marks were reapplied once the disappearance of a mark occurred. This method works well for terrestrial toads that have fairly dry skin (Meyer et al. 2012), but does not provide information on the duration and timing of the sloughing behaviour, which was therefore not available for this species.

Epidermal structure

Ventral skin samples were collected from preserved specimen (fixed in 10% neutral buffered formalin, stored in 70% ethanol) at museums and captive breeding centres. All skin samples were taken from the ventral pelvic area, given this area is most often infected with Bd (Berger et al. 2005c). Samples were taken from one to five individual specimens per species. Samples were processed, embedded, sectioned at 5 μm thickness, and stained with haematoxylin and eosin. Samples that were preserved poorly, or demonstrated evidence of skin abnormality

(disease, etc) were excluded (see Appendix 4.1 for the list of specimen utilised in analyses). Images were analysed in ImageJ (version 1.48, Rasband 1997) to determine epidermal thickness and the average number of epidermal cell layers per species.

Phylogenetic relationships

A phylogenetic tree of all species analysed was obtained from the Open Tree of Life (Hinchliff et al. 2015), accessed via the R package 'rotl' (Michonneau et al. 2015). Closest relatives were used for sub-species or species complexes not found within the tree (*Atelopus sp.* [spumarius-pulcher complex] and *Litoria verreauxii alpina*), and Grafen's arbitrary branch lengths were used for tree creation (Grafen 1989).

Susceptibility measures

To categorise species based on their known susceptibility to chytridiomycosis, two criteria were utilised. First, species were classified based on the evidence for chytridiomycosis-related declines, as cited in the IUCN Red List (IUCN 2015), published papers, grey literature, and personal communications (this classification scheme was modelled after Pedersen et al. 2007): (1) Direct evidence of Bd-driven declines in published literature, (2) Chytridiomycosis listed as a threat in the IUCN Red List, or (3) No evidence of Bd-driven declines. In addition, for a subset of species in which published experimental Bd infection studies existed, average mortality rates were utilised as a susceptibility measure (Appendix 4.2).

Species life history classification

Given skin characteristics, such as skin resistance to evaporative water loss, have been associated with life history in amphibians (Tracy et al. 2010), species were classified by dominant life history type (B: burrowing, A: arboreal, T: terrestrial, SA: aquatic/ semi-aquatic), ecological group (S: stream associated, P: pond breeding, E: ephemeral water breeder, T: terrestrial breeder, or a combination of two, e.g. E/T or E/P), and prevailing activity time (either nocturnal or diurnal). These classifications were based on those utilised by Murray et al (2011) and species were categorised based on information from online databases (AmphibiaWeb (2015) and the IUCN Red List (IUCN 2015)), as well as personal communication with experts.

Statistical analyses

All statistical analyses were performed in R (R Core Team 2013). Phylogenetic linear mixed models (PLMMs) were implemented using restricted maximum likelihood estimation (REML) in ASReml-R (Butler et al. 2009), which can account for multiple measurements on the same

individuals over time, and phylogenetic non-independence between species (function 'asreml', package 'asreml'). All models included individual *Frog ID* nested within *Species* and a phylogenetic variance-covariance matrix constructed from the phylogenetic tree, as random effects. A Wald type F-test was used to test for the significance of fixed effects (Kenward & Roger 1997), and the significance of random effects were determined using likelihood ratio tests (Self & Liang 1987, Tobias et al. 2014). Phylogenetic heritability, which is equivalent to the more widely-used λ (Pagel 1999), was used as an estimate of phylogenetic signal, and was calculated as the proportion of the variance in the trait, conditioned on the fixed effects (Wilson 2008), which is explained by the relationship among taxa as given by the phylogeny (Housworth et al. 2004). Approximate standard errors for the estimate of phylogenetic heritability were calculated using the R pin function (White 2013).

Given that temperature affects sloughing rates (Cramp et al 2014), we first tested the effect of the average, minimum, and maximum temperatures experienced by the amphibians during the filming periods on sloughing rate. Next, the relationship between sloughing rate and a) slough duration and SVL, b) ecological group, ecological habit, and activity time, c) skin thickness, and d) the number of epidermal layers was tested. Finally, we assessed the relationship between average sloughing rate (by species) and the evidence for Bd-driven declines or average precent mortality from published studies, taking into account ecological group, behaviour time, and skin thickness. All continuous variables were log or natural-log transformed to meet the requirement of normality, and models were reduced using likelihood ratio tests (Self & Liang 1987; Tobias et al. 2014).

Results

Sloughing behaviour and IMI

Overall, sloughing rates were measured for 21 anuran species from eight different families, originating from Asia (1), Central/South America (11), and Australia (9). On average, 59.7 sloughing events were recorded per species, with a minimum of 3 and a maximum of 359. In general, species that sloughed more often allowed for the recording of more sloughing events. Intermoult interval (IMI) ranged from 22 h (daily) to 480 h (every 20 days), with a mean interval of 119.5 h across all species (Figure 4.1).

Sloughing behaviour, or how the *stratum corneum* was physically removed during the sloughing process, was fairly similar across the anurans studied, although the duration and timing of the behaviour differed substantially. Generally, sloughing behaviour followed previous reports (Larsen 1976; Ohmer et al. 2015), in which a series of fore and hind limb movements, side contractions, and opening and closing of the mouth, aided the movement of the *stratum corneum*

into the mouth. Generally, all animals inflated with air before the sloughing process began, presumably to facilitate the splitting of the *stratum corneum* on the dorsum. Of note, burrowing species demonstrated slightly different sloughing behaviour, which involved less physical pushing of the skin with fore and hind limbs, given the comparably short length of their legs, and more lateral movement of the entire body back and forth. In addition, some species consistently sloughed from an elevated position, usually from a wall of the enclosure (e.g. *Atelopus* spp.) Furthermore, the timing of sloughing was strongly related to the activity pattern of that species as observed on the videos, with primarily nocturnal species typically sloughing in the early evening or at night, and primarily diurnal species sloughing in the early morning ($F=29.45$, d.f. $=1, 8.1$, $p= 0.0006$, Table 4.2, Figure 4.2).

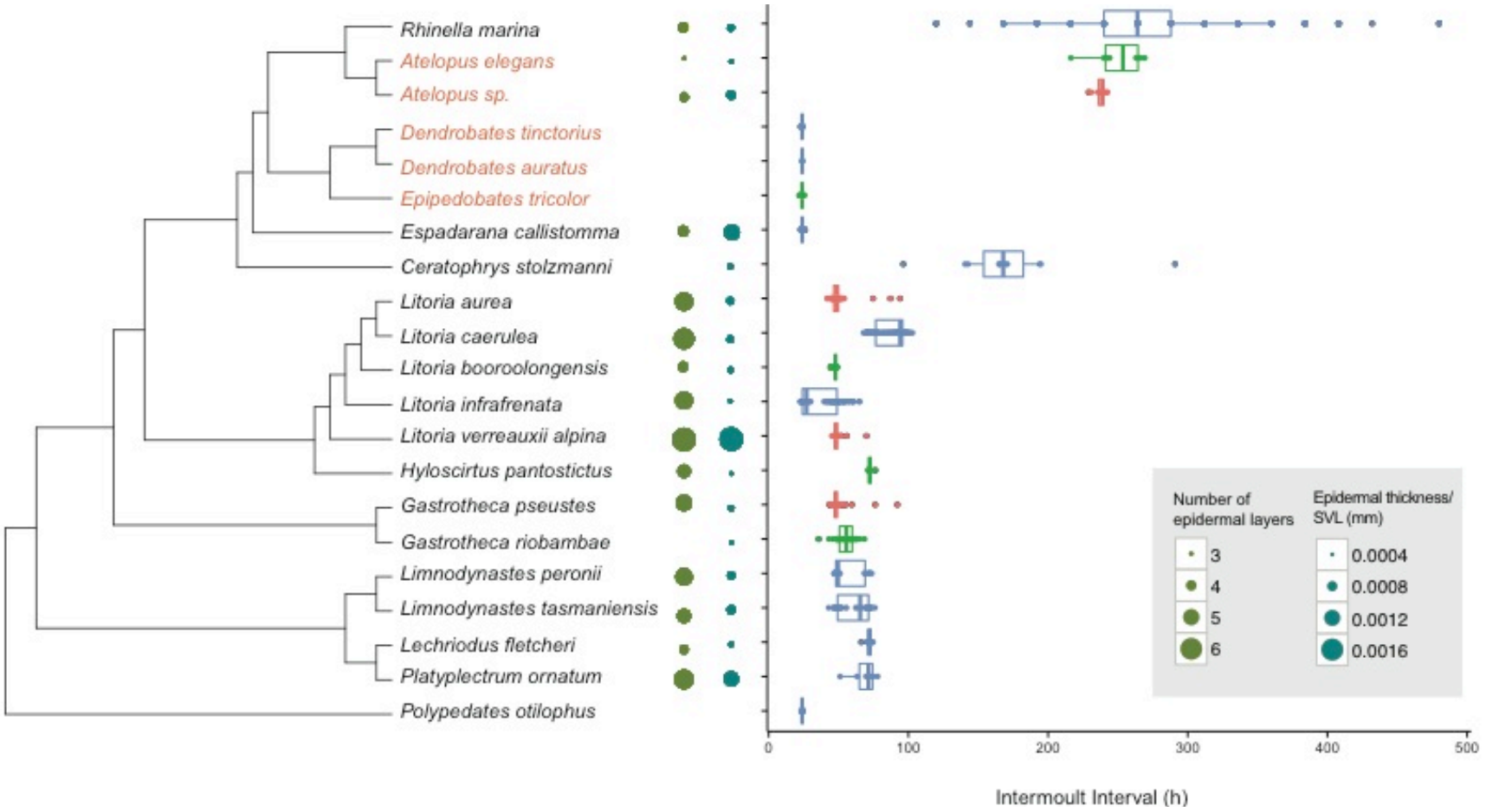

Figure 4.1 Phylogenetic relationships between 21 frog species for which intermoult interval, or the time between sloughing events, was measured (represented by boxplots on right). Boxplot colours indicate the evidence for *Batrachochytrium dendrobatidis* (Bd)-driven declines, categorised by direct evidence of Bd-driven declines (red), Bd-driven declines indicated by the IUCN Red List (green), and no evidence of Bd-driven declines (blue). Green circles represent the average number of epidermal layers in museum specimen for those species, while teal circles represent average epidermal thickness divided by snout vent length of the specimen (mm). Species names in orange indicate diurnal species, while the remainder are nocturnal species. Branch lengths are Grafen's arbitrary branch lengths.

Table 4.2 Statistical results from a phylogenetic linear mixed model (implemented in ASReml-R) examining the time of day sloughing events occurred versus the activity time for 20 anuran species (sloughing time of day was not available for *Rhinella marina*). Random effects included Frog ID nested in Species, and a variance-covariance matrix created from the phylogeny, to take into account correlated error from repeated measures on the same individual and phylogenetic non-independence. s.e. = standard error, d.f. = degrees of freedom, bold p-values are significant

Fixed effects	**Estimate**	**s.e.**	**F**	**d.f.**	***p***
Intercept	7.8	2.0	116.1	1, 3.7	**0.0007**
Activity time	9.0	1.7	29.5	1, 8.1	**0.0006**
Random effects	**Variance**	**s.e.**	**χ^2**	**d.f.**	***p***
Phylogeny	0.43	5.0	1.9	1	0.17
Frog ID nested in Species	0.18	0.73	20.5	1	**5.8×10^{-6}**
$\lambda = 0.62$, s.e. = 0.25					

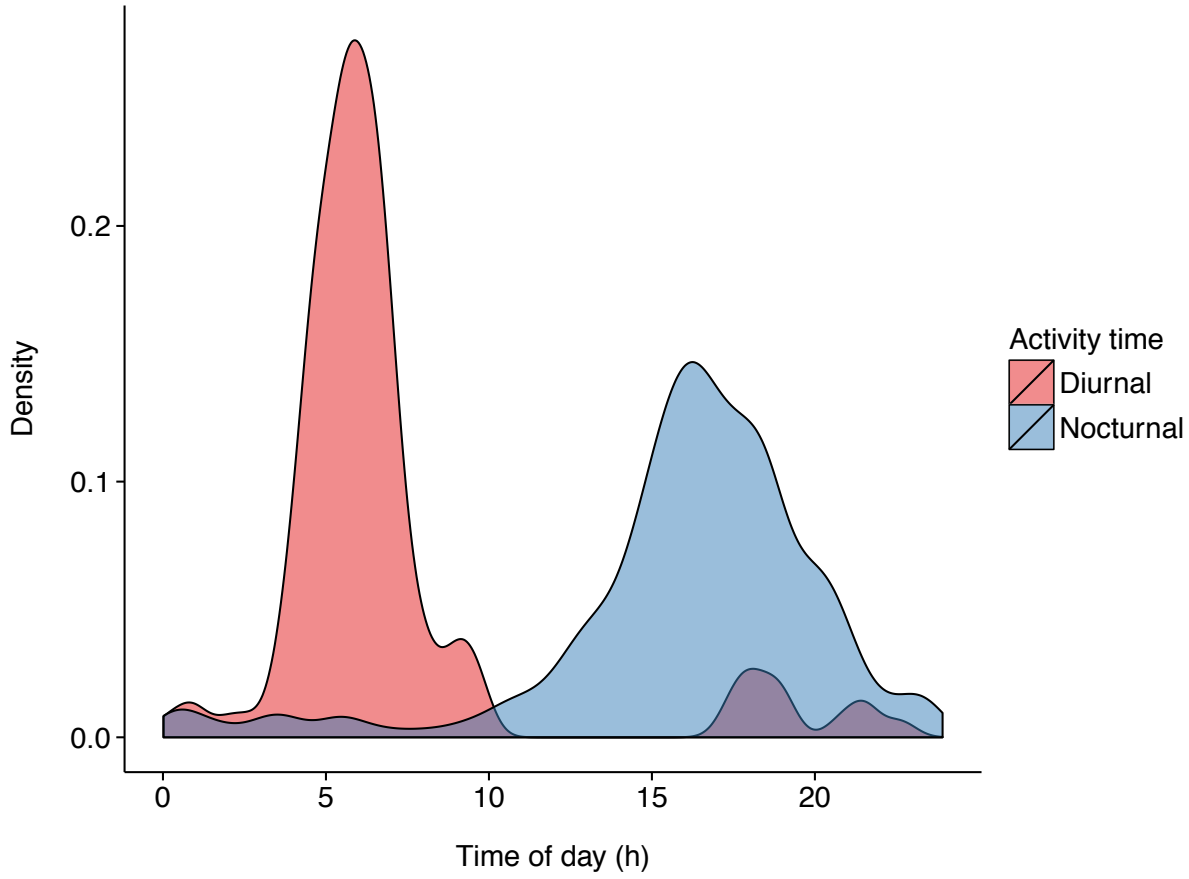

Figure 4.2 Density plot of the time of day sloughing events occurred for diurnal (n = 5) and nocturnal (n = 15) anuran species.

Bd status of frogs

Two of the nine species filmed at the *Balsa de los Sapos* tested positive for Bd. Bd prevalence was 20 % in *Gastrotheca pseustes* (N= 6) and 30 % in *Ceratophrys stolzmanni* (N=10). Sloughing rate was consistent across infected and uninfected individuals, and infection load was very low in both species (*G. pseustes*: 7.2 ± 13.4 s.d.; *C. stolzmanni*:16.0 ± 3.5 s.d. zoospore equivalents [ZE]). Previous work indicates that sloughing rates do not increase in Bd infected animals until high infection loads are reached (Ohmer et al. 2015), and these animals were not demonstrating clinical signs or variation in behaviour. Captive frogs at Taronga Zoo and the San Diego Zoo are tested on a regular basis, and no records of chytridiomycosis had occurred in either facility around the filming times (Peter Harlow, Allan Pessier *pers. comm.*). All frogs filmed at The University of Queensland were regularly tested for Bd infection, and all remained Bd negative.

Skin structure

After removing skin samples that were poorly preserved or diseased, skin structure was analysed based on multiple sections from 1-4 specimen per species (mean: 2.6). Ventral epidermal thickness across amphibian species ranged between 9.4-75.3 μm (mean: 31.4 ± 14.4 s.d.). Epidermal thickness, standardised by SVL of the specimen (mm), was positively correlated with the number of epidermal layers ($F=9.5$, d.f.=1, 11.9, $p = 0.0096$, Figure 4.3), which ranged between 2-7 layers (mean: 4.8 ± 1.0 s.d.). The structure of the ventral skin varied across species, but generally followed the structure of the typical anuran epidermis, containing 1-2 layers of *stratum corneum,* 1-4 layers of *stratum granulosum,* and 1-2 layers of *stratum basal.* The dermal layer varied greatly in thickness across species, and contained mucosal and peptide glands within the *stratum spongiosum*, followed by the *stratum compactum.* The greatest variation existed in the number of mucous and peptide glands, the amount of melanin and melanophores in the *stratum spongiosum*, and the sculpturing of the ventral skin (Figure 4.4 and Figure 4.5).

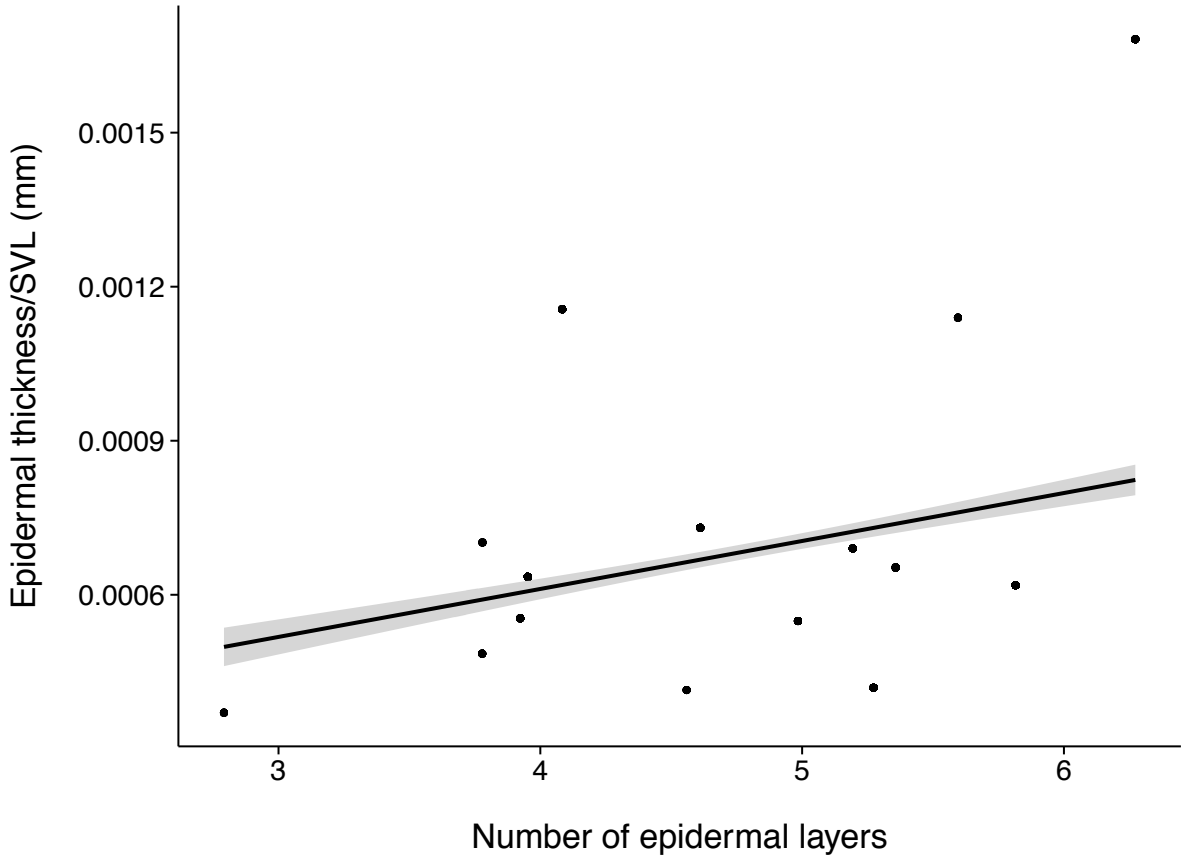

Figure 4.3 A positive association between epidermal thickness/SVL (mm) and the number of epidermal layers in 15 anuran species, measured in museum specimen. It was not possible to ascertain the number of epidermal layers in two species. Shaded area indicates standard error.

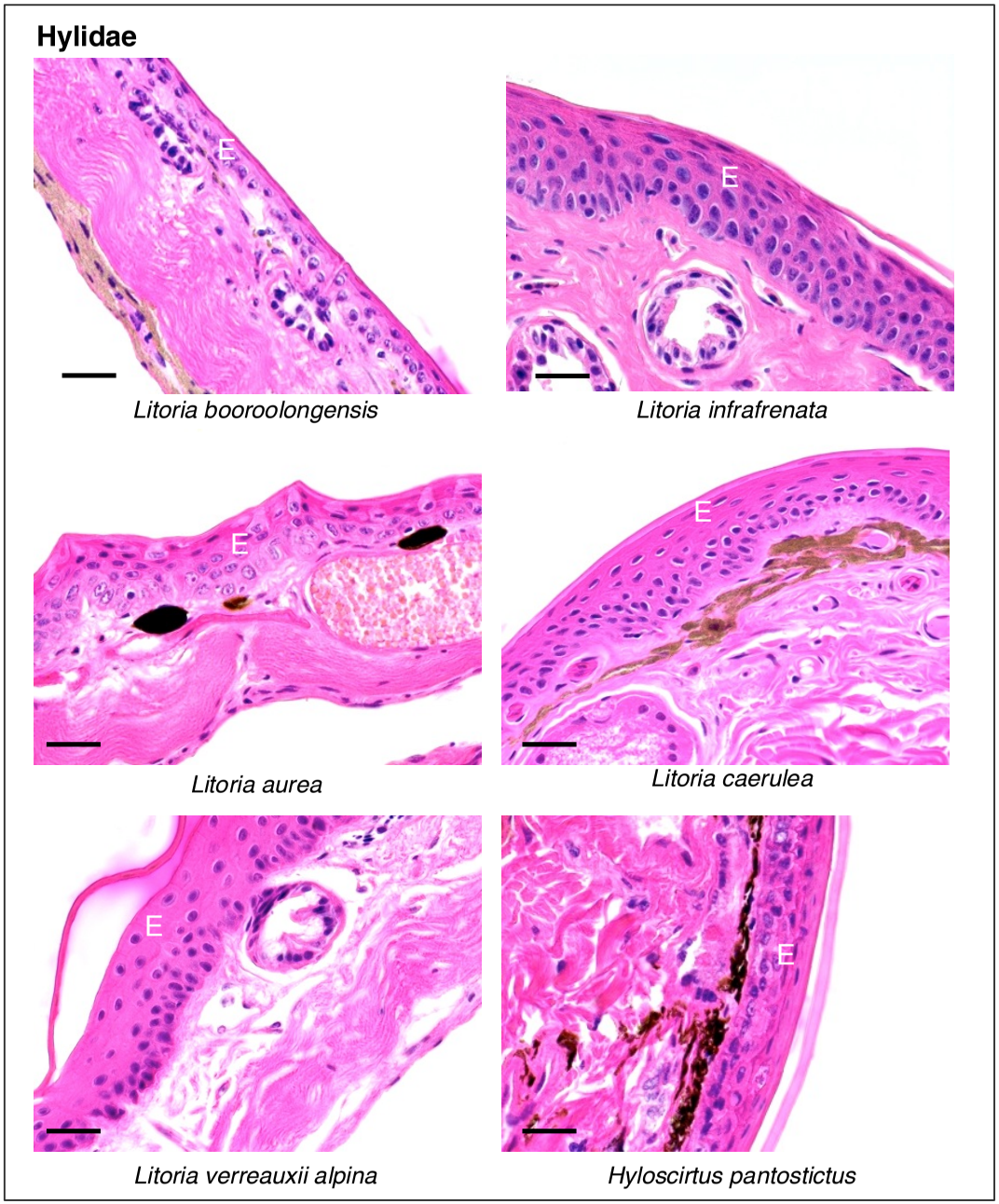

Figure 4.4
Examples of epidermal structure for species in the Hylidae and Bufonidae families. Skin sectioned at 5 µm and stained with haematoxylin and eosin. E = epidermis, brown colouration indicates melanin, scale bar = 50 µm

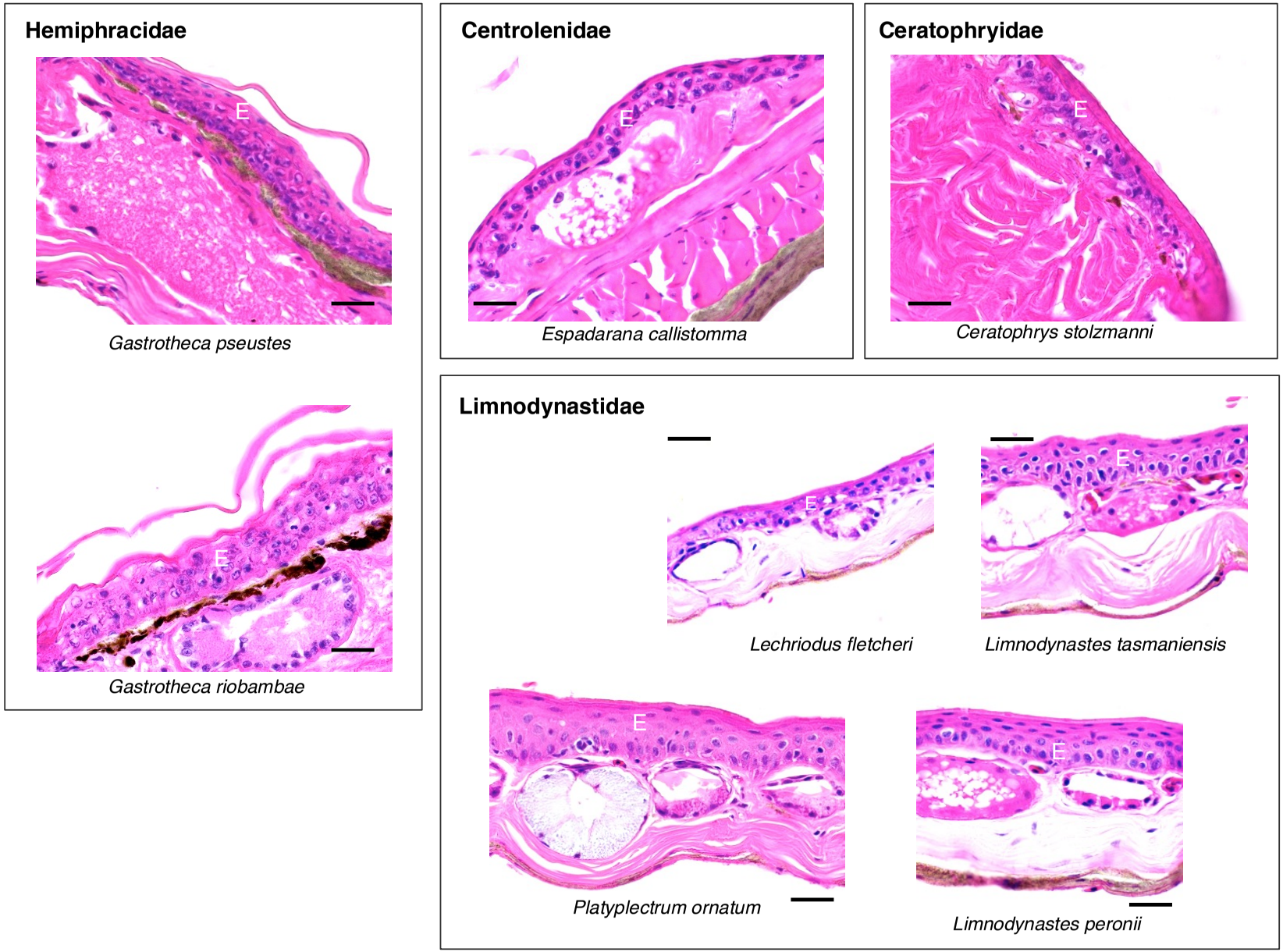

Figure 4.5 Examples of epidermal structure for species in the Hemiphracidae, Centrolenidae, Ceratophrynidae, and Limnodynastidae families. Skin sectioned at 5 µm and stained with haematoxylin and eosin. E = epidermis, brown colouration indicates melanin, scale bar = 50 µm

Relationship between IMI and other life history and skin structural traits

Overall, IMI across all species was not influenced by mean, minimum, or maximum temperature during the measurement periods of each species (mean: F = 0.01, d.f. = 1, 17.1, p = 0.91, min: F = 0.02, 17.1, d.f. = 1, 17.1, p = 0.88, max: F = 1.4, d.f. = 1, 16.7, p = 0.25; Table 4.3). Furthermore, there was no relationship between IMI and SVL (F = 0.001, d.f. = 1, 151.5, p = 0.97, Table 4.3), epidermal thickness (F = 0.51, d.f.=1, 16.5, p = 0.49, Table 4.3), or number of epidermal layers (F = 2.4 x 10^{-4}, d.f. = 1, 14.3, p = 0.99, Table 4.3). However, IMI did demonstrate very strong phylogenetic signal in all of these models (lambda = 0.97, s.e. = 0.009), with species in the family Bufonidae demonstrating the longest IMIs between 7-20 days, and species within Dendrobatidae almost exclusively sloughing every 24 h, demonstrating some of the shortest IMIs. IMI was positively associated with slough duration when average temperature was taken into account (F = 0.37, d.f. = 1, 17.2, p = 0.001, Figure 4.6, Table 4.4), indicating that frogs that slough less often take longer to complete the sloughing process. Across all species, the duration of the sloughing event varied between 2 and 92 min (mean: 8.2 ± 7.5 s.d.). IMI was also significantly different across ecological group (F = 9.1, d.f .= 4, 12, p = 0.00013, Figure 4.7), between nocturnal and diurnal species within the stream-associated breeders (F=6.7, d.f.=1,12, p=0.023, Figure 4.7), and across ecological habit (F = 3.58, d.f. = 3, 12, p = 0.047, Table 4.5). In this model, phylogenetic signal of IMI is low (lambda = 1.4 x 10^{-6}, s.e. = 4.9 x 10^{-7}, Table 4.5). Estimates of lambda are conditioned on the fixed effects, so it is likely that once the fixed effects of ecological group and habit are accounted for, there is no phylogenetic signal remaining. Ecological group and habit likely also demonstrate high phylogenetic signal.

Table 4.3 Statistical results from phylogenetic linear mixed models (implemented in ASReml-R) examining the effects of temperature during the measurement period, SVL, and skin characteristics on log intermoult interval (logIMI [h]), in 21 species of anuran. Random effects included *Frog ID* nested in *Species*, and a variance-covariance matrix created from the phylogeny, to take into account correlated error from repeated measures on the same individual and phylogenetic non-independence. s.e. = standard error, d.f. = degrees of freedom, bold p-values are significant

Temperature

Fixed effects	**Estimate**	**s.e.**	**F**	**d.f.**	***p***
Intercept	6.7	3.1	36.9	1, 17.1	0.047
Mean temp	0.78	7.3	1.78	1, 17.1	0.91
Max temp	-3.8	3.3	1.37	1, 17.1	0.26
Min temp	-0.65	4.3	0.56	1, 16.7	0.88
Random effects	**Variance**	**s.e.**	**χ^2**	**d.f.**	***p***
Phylogeny	0.45	0.0085	12.06	1	**0.0005**
Frog ID nested in Species	0.25	2.7×10^{-4}	139.1	1	**0**
$\lambda = 0.97$, s.e. = 0.009					

SVL

Fixed effects	**Estimate**	**s.e.**	**F**	**d.f.**	***p***
Intercept	1.69	0.32	35.4	1, 29.7	1.1×10^{-5}
logSVL (mm)	0.78	7.3	0.001	1, 151.5	0.97
Random effects	**Variance**	**s.e.**	**χ^2**	**d.f.**	***p***
Phylogeny	4.74	0.0082	15.78	1	**7.1×10^{-5}**
Frog ID nested in Species	0.25	2.7×10^{-4}	134.7	1	**0**
$\lambda = 0.97$, s.e. = 0.008					

Epidermal thickness/SVL

Fixed effects	**Estimate**	**s.e.**	**F**	**d.f.**	***p***
Intercept	1.33	0.77	36.9	1, 16.2	0.10
Epidermal thickness/SVL	-0.16	0.22	0.51	1, 16.5	0.49
Random effects	**Variance**	**s.e.**	**χ^2**	**d.f.**	***p***
Phylogeny	39.9	0.084	10.71	1	**0.001**
Frog ID nested in Species	0.26	3.1×10^{-4}	128.3	1	**0**
$\lambda = 0.97$, s.e. = 0.011					

Number of epidermal layers

Fixed effects	**Estimate**	**s.e.**	**F**	**d.f.**	***p***
Intercept	1.80	0.40	31.6	1, 13.2	0.00058
No. Epidermal layers	0.0008	0.051	2.42×10^{-4}	1, 14.3	0.99
Random effects	**Variance**	**s.e.**	**χ^2**	**d.f.**	***p***
Phylogeny	43.5	0.10	5.82	1	**0.016**
Frog ID nested in Species	0.27	3.4×10^{-4}	127.9	1	**0**
$\lambda = 0.97$, s.e. = 0.011					

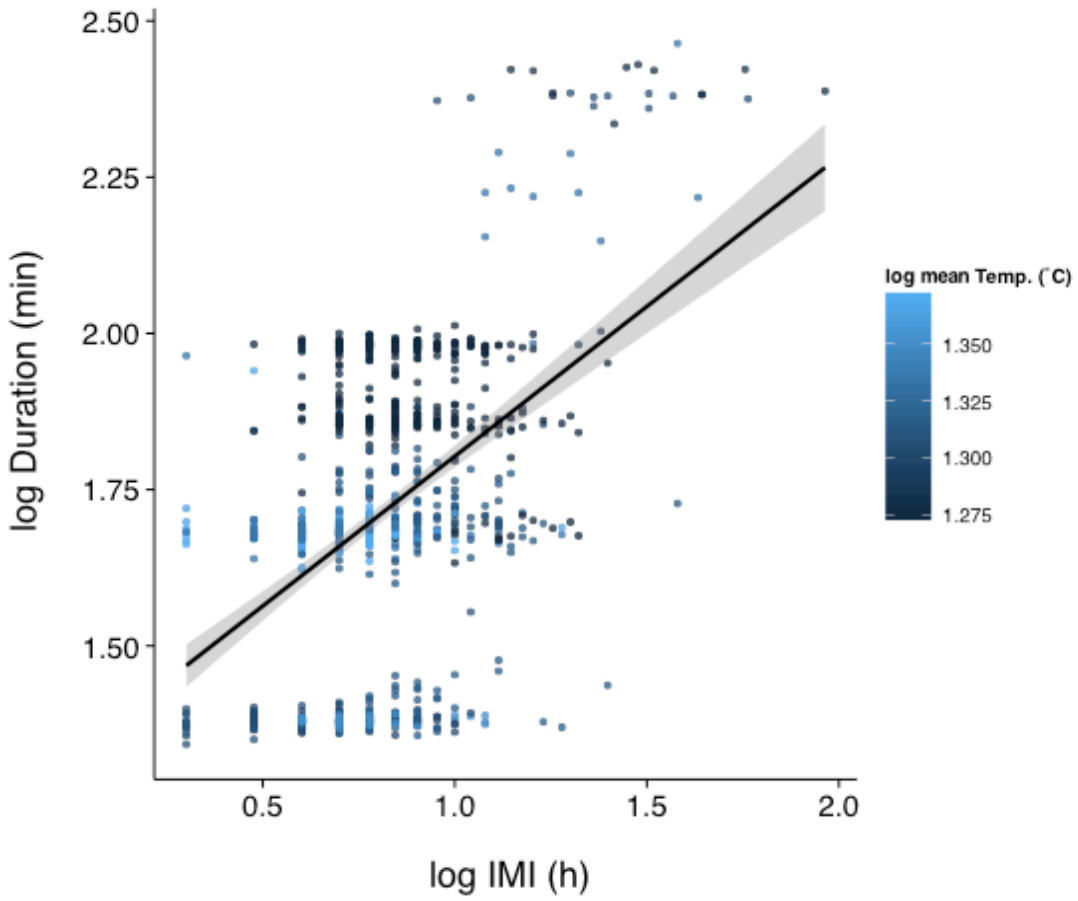

Figure 4.6 Relationship between slough duration and intermoult interval (IMI) across 20 amphibian species, with colours indicating log mean temperature (˚C) during the measurement period for each species. Shaded area indicates standard error.

Table 4.4 Statistical results from a phylogenetic linear mixed model (implemented in ASReml-R) examining the association between log slough duration and log intermoult interval (logIMI [h]), in 20 species of anuran (slough duration was not available for *Rhinella marina*), taking into account mean temperature during the measurement period (˚C). Random effects included *Frog ID* nested in *Species*, and a variance-covariance matrix created from the phylogeny, to take into account correlated error from repeated measures on the same individual and phylogenetic non-independence. λ = phylogenetic signal, s.e. = standard error, d.f. = degrees of freedom, bold p-values are significant

Fixed effects	**Estimate**	**s.e.**	**F**	**d.f.**	***p***
Intercept	3.13	2.49	45.65	1, 17.2	0.23
log Duration	0.063	0.02	10.89	1, 657	**0.0006**
log Mean Temp	-1.15	2.49	0.37	1, 17.2	0.55
Random effects	**Variance**	**s.e.**	**χ^2**	**d.f.**	***p***
Phylogeny	31.1	0.0068	12.02	1	**0.0005**
Frog ID nested in Species	0.032	1.6×10^{-4}	2.79	1	0.095
λ = 0.97, s.e. = 0.011					

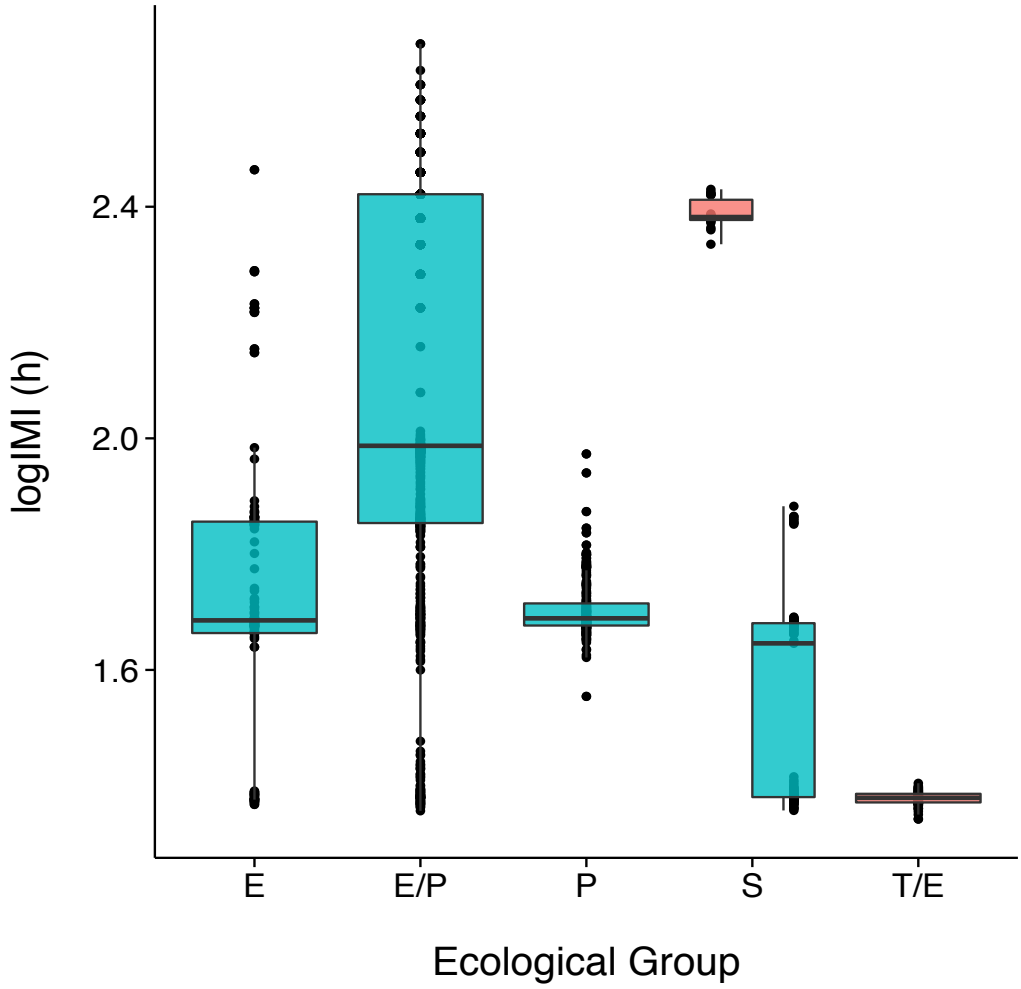

Figure 4.7 Intermoult interval (IMI) across amphibian species categorised by ecological group (E: ephemeral water breeders, P: pond-associated breeders, S: stream-associated breeders, T: terrestrial breeders). Diurnal (peach) and nocturnal (teal) stream-associated breeders demonstrated significantly different sloughing rates, whereas all terrestrial/ephemeral breeders in this study were diurnal. The centre line is the 50th percentile, top and bottom of box represent 75th and 50th percentile, and whiskers extend to extreme data points (no more than 1.5 times the interquartile range).

Table 4.5 Statistical results from a phylogenetic linear mixed model (implemented in ASReml-R) examining the association between ecological group (S: stream-associated, T: terrestrial, P: pond-associated, or E: ephemeral breeders), ecological habit (T: terrestrial, A: arboreal, SA: aquatic/semi-aquatic, B: burrowing), and activity time (nocturnal or diurnal), and log intermoult interval (logIMI [h]), in 21 species of anuran, taking into account mean temperature during the measurement period (˚C). Random effects included *Frog ID* nested in *Species*, and a variance-covariance matrix created from the phylogeny, to take into account correlated error from repeated measures on the same individual and phylogenetic non-independence. λ = phylogenetic signal, s.e. = standard error, d.f. = degrees of freedom, bold p-values are significant

Fixed effects	**Estimate**	**s.e.**	**F**	**d.f.**	***p***
Intercept	1.97	0.28	1626	1, 12	3.3×10^{-14}
Ecological habit A	NA	NA	3.58	3,12	**0.047**
Ecological habit B	0.60	0.21			
Ecological habit SA	0.049	0.17			
Ecological habit T	0.32	0.14			
Ecological group E/P	NA	NA	9.14	4,12	**0.0013**
Ecological group P	0.048	0.17			
Ecological group S	0.10	0.17			
Ecological group T/E	-0.91	0.25			
Activity time	-0.54	0.21	6.74	1,12	**0.023**
Random effects	**Variance**	**s.e.**	**χ^2**	**d.f.**	***p***
Phylogeny	1.2×10^{-5}	2.7×10^{-9}	-9.3×10^{-7}	1	1
Frog ID nested in Species	0.25	2.7×10^{-4}	135.7	1	**0**
$\lambda = 1.4 \times 10^{-6}$, s.e. = 4.9×10^{-7}					

IMI, skin characteristics, and susceptibility

Evidence for Bd-related declines in the wild was not significantly associated with average IMI by species, but was significantly associated with ecological group (F= 4.16, d.f.= 4, 8.8, p = 0.04, Table 4.6), with stream and pond-associated breeders demonstrating greater evidence of Bd-driven declines. In addition, average percent mortality was only marginally associated with IMI when ecological group and the number of epidermal layers were taken into account (F=42.05, d.f.=1,3, p = 0.059, Table 4.7), but this only included a subset of species (N=11) for which published and unpublished Bd exposure studies exist.

Table 4.6 Statistical results from a phylogenetic linear mixed model (implemented in ASReml-R) examining the association between log intermoult interval (logIMI [h]), ecological group (S: stream-associated, T: terrestrial, P: pond-associated, or E: ephemeral breeders), and the interaction, with the evidence for *Batrachochytrium dendrobatidis* (Bd)-driven declines in 21 species of anuran. Random effects included a variance-covariance matrix created from the phylogeny, to take into account correlated error from phylogenetic non-independence. λ = phylogenetic signal, s.e. = standard error, d.f. = degrees of freedom, bold p-values are significant

Fixed effects	**Estimate**	**s.e.**	**F**	**d.f.**	***p***
Intercept	1.37	2.45	26.84	1, 2.9	0.078
Ecological group E: log IMI	NA	NA	3.03	4, 7.9	0.086
Ecological group E/P: log IMI	-0.77	1.56			
Ecological group P: log IMI	42.49	15.90			
Ecological group S: log IMI	-1.96	1.53			
Ecological group T/E: log IMI	116.1	280.7			
logIMI	0.71	1.36	0.27	1, 7.6	0.62
Ecological group E	NA	NA	4.09	4, 8.8	**0.036**
Ecological group E/P	1.76	2.95			
Ecological group P	-73.38	27.33			
Ecological group S	3.05	2.94			
Ecological group T/E	-159.8	387.1			
Random effects	**Variance**	**s.e.**	**χ^2**	**d.f.**	***p***
Phylogeny	5.44	0.65	1.87	1	0.17
λ = 0.85, s.e. = 0.23					

Table 4.7 Statistical results from a phylogenetic linear mixed model (implemented in ASReml-R) examining the association between log intermoult interval (logIMI [h]), ecological group (S: stream-associated, T: terrestrial, P: pond-associated, or E: ephemeral breeders), and the number of epidermal layers, with average morality rate from exposure to *Batrachochytrium dendrobatidis* for 11 species of anuran. Random effects included a variance-covariance matrix created from the phylogeny, to take into account correlated error from phylogenetic non-independence. λ = phylogenetic signal, s.e. = standard error, d.f. = degrees of freedom, bold p-values are significant

Fixed effects	**Estimate**	**s.e.**	**F**	**d.f.**	***p***
Intercept	-224.9	80.6	183.4	1, 3	**0.023**
Ecological group E	NA	NA	25.48	3, 3	**0.012**
Ecological group E/P	-65.59	11.4			
Ecological group P	-10.71	15.77			
Ecological group S	-39.22	16.33			
Ecological group T/E	NA	NA			
logIMI	125.23	42.05	12.95	1, 3	0.059
No. of epidermal layers	17.32	6.94	20.63	1, 3	0.088
Random effects	**Variance**	**s.e.**	**χ^2**	**d.f.**	***p***
Phylogeny	2.3×10^{-6}	2.5×10^{-4}	-5.99×10^{-8}	1	1
λ = 7.43×10^{-10}, s.e. = 1.97×10^{-9}					

Discussion

Amphibian skin is a highly diverse organ, and adaptations of generally permeable amphibian skin have contributed to the extraordinary range of habitats and ecological niches they fulfil (Wells 2010). Understanding the variation in basic skin structure and function across a variety of species within a phylogenetic context can provide insight into the effects of these differences on susceptibility to a generalist cutaneous pathogen, such as Bd. Across 21 amphibian species from eight different families, we found substantial variation in the rate of epidermal turnover, with some species exclusively sloughing every day, to other species demonstrating larger individual variation and sloughing as slowly as every other week. This variation demonstrated high phylogenetic signal, indicating that a species' sloughing rate tended to be more similar to closely related species than to distantly related species (Blomberg et al. 2003). Overall, intermoult interval (IMI) was not influenced by body size, temperature, or skin characteristics such as epidermal thickness or the number of epidermal layers. Interestingly, IMI was different across ecological groups and habits, and between nocturnal and diurnal species of the stream-associated breeders, but the number of species in each of these groups may prevent further interpretation. Finally, the evidence for disease-related declines across all species was not associated with IMI, but again demonstrated significant differences across ecological groups, with stream and pond-associated breeders demonstrating greater evidence of Bd-related declines. Within the species examined, there was no clear association between sloughing rate and susceptibility to chytridiomycosis. However, an understanding of epidermal turnover in amphibian genera particularly affected by Bd, such as *Atelopus*, may help focus conservation efforts.

Within the range of amphibians studied, concentrated in Australia and Central/South America (and one species from Asia), we found IMI demonstrated very strong phylogenetic signal. This may indicate that the control and regulation of sloughing behaviour is strongly phylogenetically conserved, despite differences in habitat and ecological group among related species. While the animals utilised in this study have varying rearing histories, given the small intraspecific variation in sloughing rate, and the high phylogenetic signal, it would appear that this trait is not highly evolutionarily labile. Species in the Dendrobatidae and Centrolenidae families exhibited fast sloughing rates, with sloughing occurring every day. Conversely, species in the Bufonidae family demonstrated the longest intermoult intervals. This family demonstrates varying degrees of dependence on environmental moisture, and wide variation in thermal tolerances, which correlate with range size, from the range-restricted species in the genus *Atelopus*, to the invasive and range-expanding *Rhinella marina* (Van Bocxlaer et al. 2010). Of note, species in the genus *Atelopus* have been particularly devastated by the disease chytridiomycosis, with disease-related

declines occurring across the genus in Central and South America (La Marca et al. 2005). If sloughing rate demonstrates strong phylogenetic signal, it is likely that other *Atelopus* species demonstrate relatively slow epidermal turnover as well. This has implications for understanding the progression of disease in these species, as well as the regulation of cutaneous symbiotic bacteria.

Previous work has demonstrated that sloughing can regulate not only Bd load (Chapter 3), but also cultivable cutaneous microbial communities (Meyer et al. 2012; Cramp et al. 2014). Given the potential importance of symbiotic cutaneous bacteria in innate immune defence against Bd infection (Harris et al. 2009), understanding the sloughing rates of imperilled amphibian species may aid conservation actions. For example, *Atelopus zeteki*, like many *Atelopus* species, has experienced widespread declines in Central America due to epidemics of chytridiomycosis, and consequently has been bred in captive assurance colonies to avoid extinction in the wild (La Marca et al. 2005; Gratwicke et al. 2015). The realisation that this species may have relatively slow skin turnover sheds light on why they may be considered Bd 'supershedders' (DiRenzo et al. 2014), reaching high infection intensities and developing clinical chytridiomycosis quickly. While previous attempts to inoculate *A. zeteki* with beneficial bacteria active against Bd were ineffective (Becker et al 2011), knowledge of sloughing rates could help increase chances of success. Understanding that these species likely slough infrequently may help pinpoint when to best bioaugment their skin with beneficial anti-Bd bacteria (Becker et al. 2011, Becker et al. 2015), as addition of the bacteria immediately after sloughing may allow for colonisation when resident bacterial populations are low (Cramp et al. 2014).

IMI was not associated with temperature, amphibian body size (SVL), epidermal thickness or the number of epidermal layers. Although sloughing rate is positively correlated with temperature within a species (Meyer et al. 2012; Cramp et al. 2014), the range of mean temperatures experienced by each species in this study was fairly narrow (18.8 - 25˚C), which helped fortify the comparison across species. Thus, the trends observed across species were likely not an artefact of differences in temperature during the measurement period. Furthermore, sloughing rate was not explained by amphibian body size, or skin structure. Interestingly, IMI was positively associated with the duration of the sloughing event, when taking into account mean temperature. Frogs with high rates of skin turnover may have evolved a coincidently fast sloughing behaviour to compensate for the frequency of this physiologically vulnerable period. During sloughing, amphibian skin increases in permeability to both water and electrolytes (Jørgensen 1949; Appendix A), and the risk of predation is likely greater, thus high rates of sloughing may be physiologically stressful. For example, green tree frogs (*Litoria caerulea*) have been shown to have the highest rates of cutaneous water loss during the sloughing period (Appendix A). Conversely,

sloughing rate and duration may be inherently linked traits, but the genetic mechanisms governing sloughing are unknown.

Sloughing rate was significantly different across ecological group, habit, and activity time, with ephemeral/pond breeders demonstrating slower epidermal turnover than terrestrial/ephemeral breeders, and diurnal stream breeders demonstrating higher sloughing rates than nocturnal stream-associated breeders. This categorisation is interesting, but may be more related to the number of species in each of these categories in this study, and the number of species in each ecological group per family. Also, it may be that there is high phylogenetic signal in ecological group and habit as well, as has been demonstrated in other macroecological variables in amphibians (Cooper et al. 2008). Thus, a larger sample size across a greater range of amphibian species may be needed to tease apart variation in IMI across ecological group and habit.

Interestingly, the activity time of each species was associated with the time of the sloughing event, with nocturnal species sloughing in the evening or at night, and diurnal species sloughing in the early morning. Sloughing usually occurred before the daily activity period began in all species, which may be adaptive in that frogs would usually slough in day or night refuges (depending on whether they are nocturnal or diurnal), and then commence the active period. Frogs typically select refuges that reduce the rates of cutaneous water loss (Schwarzkopf & Alford 1996; Long & Prepas 2012), and frogs demonstrate high rates of evaporative water loss during sloughing (Appendix A). Thus, sloughing within a refuge could allow frogs to potentially avoid thermal or hydric extremes during the sloughing period, or reduce risk of predation.

We did not find an association between evidence of Bd-driven declines and sloughing rate or skin structure, although we did find greater evidence of Bd-driven declines in pond and stream-associated breeders. Ecological group and reliance on water has demonstrated a strong correlation with Bd-related declines in previous work (Bielby et al. 2008; Murray et al. 2011), so this finding is not surprising. However, the classification of amphibians in this study based on Bd-driven declines is geographically biased by the available information for each species, with more publications and Bd exposure studies for Australian species compared with species from South America and Asia. Thus, additional information regarding susceptibility to chytridiomycosis in each species studied will help further elucidate the role of sloughing rate or skin structure in that susceptibility. Regardless, given the high level of phylogenetic signal in sloughing rate, this work provides the first framework for predicting the sloughing rates of related species, which previously was a little known aspect of amphibian skin physiology and behaviour. In doing so, we may be able to better customise models of Bd growth on amphibian skin, taking into account how often resident and potentially pathogenic organisms are removed from the skin via sloughing for a particular species.

The understanding of basic amphibian biology and physiology within a phylogenetic context can inform conservation efforts. We demonstrate there is strong phylogenetic signal in amphibian skin sloughing rates, and this can improve our understanding of cutaneous disease progression in focal species for conservation mitigation strategies.

Appendix 4.1 List of specimen used for measuring epidermal thickness and the number of epidermal layers, using standard histological techniques.

Institution	Species	Institution ID	Collection date	SVL	Locality Name
Balsa de los Sapos	*Atelopus sp. (spumarius-pulcher complex)*	T2993	23/05/2014	42.03	Captive animal
Balsa de los Sapos	*Atelopus sp. (spumarius-pulcher complex)*	T3264.1	18/06/2012	31.77	Captive animal
Balsa de los Sapos	*Atelopus sp. (spumarius-pulcher complex)*	T3264.2	18/06/2012	32.87	Captive animal
Balsa de los Sapos	*Gastrotheca riobambae*	T3003	28/05/2011	34.93	Captive animal
Balsa de los Sapos	*Gastrotheca pseustes*	T1676	15/05/2013	51.92	Captive animal
Balsa de los Sapos	*Gastrotheca pseustes*	T1691	03/01/2013	48.68	Captive animal
Balsa de los Sapos	*Gastrotheca pseustes*	T1568	19/07/2013	42.06	Captive animal
Balsa de los Sapos	*Gastrotheca pseustes*	T1159	07/01/2013	46.76	Captive animal
Balsa de los Sapos	*Espadarana callistomma*	T3280.1	15/10/2012	21.16	Captive animal
Balsa de los Sapos	*Espadarana callistomma*	T3280.2	15/10/2012	17.46	Captive animal
Balsa de los Sapos	*Espadarana callistomma*	T3280.3	15/10/2012	20.01	Captive animal
Balsa de los Sapos	*Espadarana callistomma*	T3280.4	15/10/2012	18.39	Captive animal
Balsa de los Sapos	*Hyloscirtus pantostictus*	T2386	13/03/2013	55.4	Captive animal
Balsa de los Sapos	*Hyloscirtus pantostictus*	T2905	10/12/2012	58.36	Captive animal
Balsa de los Sapos	*Atelopus elegans*	T3221	23/11/2012	27.93	Captive animal
Balsa de los Sapos	*Atelopus elegans*	T3273	23/07/2012	27.0	Captive animal
Queensland Museum	*Litoria booroolongensis*	J28315	22/02/1977	41.51	Boonoo Boonoo, 28° 53' South, 152° 6' East, New South Wales/Australia
Queensland Museum	*Litoria booroolongensis*	J31331	19/09/1975	44.64	Coxs R, near Lithgow, 33° 29' South, 150° 9' East, New South Wales/Australia
Queensland Museum	*Litoria infrafrenata*	J92930	18/09/2013	105.26	Jardine River Crossing, south side, 11° 6' 22" South, 142° 17' 00" East, Cape York Peninsula/Cape York/Queensland/Australia
Queensland Museum	*Litoria infrafrenata*	J92979	14/09/2013	99.08	Jardine River Crossing, south side, 11° 6' 16" South, 142° 16' 58" East, Cape York

					Peninsula/Cape York/Queensland/Australia
Queensland Museum	*Litoria infrafrenata*	J86965	16/09/2008	100.24	Wattle Hill, 12° 32' 2" South, 143° 11' 10" East, Cape York Peninsula/Cape York/Queensland/Australia
Australian Museum	*Litoria verreauxii alpina*	R.166555	19/05/1965	32.5	Rules Point Road, Long Plain, 35° 39' S, 148° 34' E, New South Wales/Australia
Australian Museum	*Litoria verreauxii alpina*	R.166538	19/05/1965	31.09	Rules Point Road, Long Plain, 35° 39' S, 148° 34' E, New South Wales/Australia
Australian Museum	*Litoria verreauxii alpina*	R.166550	19/05/1965	33.43	Rules Point Road, Long Plain, 35° 39' S, 148° 34' E, New South Wales/Australia
Australian Museum	*Litoria verreauxii alpina*	R.166551	19/05/1965	33.06	Rules Point Road, Long Plain, 35° 39' S, 148° 34' E, New South Wales/Australia
Australian Museum	*Litoria aurea*	R.150000	09/11/1996	53.68	New Caledonia, Mt. Koghis, 22° 10' 43" S, 166° 30' 20" E, New Caledonia
Australian Museum	*Litoria aurea*	R.153967	03/04/1997	79.6	HOMEBUSH BAY, Pond GE5, 33° 51' S, 151° 5' E, New South Wales/Australia
Australian Museum	*Litoria aurea*	R.150429	Unknown	62.16	Somersby, Somersby Electricity Commission, near Gosford, 33° 22' S, 151° 17' E, New South Wales/Australia
The University of Queensland	*Lechriodus fletcheri*	LF3	17/09/2015	33.75	Captive bred, spawn collected in Southeast Queensland, Australia
The University of Queensland	*Limnodynastes peronii*	LP11	15/07/2015	40.95	Captive bred, spawn collected in Southeast Queensland, Australia
The University of Queensland	*Limnodynastes peronii*	LP9	15/07/2015	41.2	Captive bred, spawn collected in Southeast Queensland, Australia
The University of Queensland	*Limnodynastes tasmaniensis*	LT5	15/07/2015	34.1	Captive bred, spawn collected in Southeast Queensland, Australia
The University of Queensland	*Limnodynastes tasmaniensis*	LT10	15/07/2015	31.9	Captive bred, spawn collected in Southeast Queensland, Australia
The University of Queensland	*Limnodynastes tasmaniensis*	LT6	15/07/2015	30.6	Captive bred, spawn collected in Southeast Queensland, Australia
The University of Queensland	*Platyplectrum ornatum*	O9	17/09/2015	33.2	Captive bred, spawn collected in Southeast Queensland, Australia
The University of Queensland	*Platyplectrum ornatum*	O2	16/07/2015	37.1	Captive bred, spawn collected in Southeast Queensland, Australia

The University of Queensland	*Litoria caerulea*	A14	09/09/2013	61.93	Collected from Dalby, Queensland, Australia
The University of Queensland	*Litoria caerulea*	A25	10/09/2013	72.62	Collected from Dalby, Queensland, Australia
The University of Queensland	*Litoria caerulea*	A26	10/09/2013	74.9	Collected from Dalby, Queensland, Australia
The University of Queensland	*Litoria caerulea*	A28	09/09/2013	66.21	Collected from Dalby, Queensland, Australia
The University of Queensland	*Rhinella marina*	R22	22/05/2015	106.0	Collected from the University of Queensland, Australia
The University of Queensland	*Rhinella marina*	R9	29/06/2015	90.0	Collected from the University of Queensland, Australia

Appendix 4.2 Filming, life history, and location details of the amphibian species for which sloughing rate was measured. Total days indicate the total monitoring days.

Species	Family	N	Native distribution	Activity time	Institution	Total days
Atelopus elegans	Bufonidae	8	Colombia/Ecuador	Diurnal	Balsa de los Sapos	25
Atelopus sp. (spumarius-pulcher complex)	Bufonidae	10	Ecuador	Diurnal	Balsa de los Sapos	21
Ceratophrys stolzmanni	Ceratophryidae	10	Ecuador/Peru	Nocturnal	Balsa de los Sapos	23
Dendrobates auratus	Dendrobatidae	1	Central America	Diurnal	SDZG	23
Dendrobates tinctorius	Dendrobatidae	3	French Guiana/Suriname/Brazil	Diurnal	SDZG	23
Epipedobates tricolor	Dendrobatidae	10	Ecuador	Diurnal	Balsa de los Sapos	18
Espadarana callistomma	Centrolenidae	4	Ecuador	Nocturnal	Balsa de los Sapos	30
Gastrotheca pseustes	Hemiphractidae	6	Ecuador	Nocturnal	Balsa de los Sapos	23
Gastrotheca riobambae	Hemiphractidae	10	Ecuador	Nocturnal	Balsa de los Sapos	21
Hyloscirtus pantostictus	Hylidae	4	Colombia/Ecuador	Nocturnal	Balsa de los Sapos	10
Lechriodus fletcheri	Limnodynastidae	3	Australia	Nocturnal	UQ	61
Limnodynastes peronii	Limnodynastidae	3	Australia	Nocturnal	UQ	61
Limnodynastes tasmaniensis	Limnodynastidae	4	Australia	Nocturnal	UQ	61
Litoria aurea	Hylidae	8	Australia	Nocturnal	Taronga Zoo	22
Litoria booroolongensis	Hylidae	7	Australia	Nocturnal	Taronga Zoo	23
Litoria caerulea	Hylidae	10	Australia/Indonesia/Papua New Guinea	Nocturnal	UQ	64
Litoria infrafrenata	Hylidae	8	Australia/Indonesia/Papua New Guinea/Solomon Islands/Timon-Leste	Nocturnal	Taronga Zoo	26
Litoria verreauxii alpina	Hylidae	8	Australia	Nocturnal	Taronga Zoo	20
Platyplectrum ornatum	Limnodynastidae	4	Australia	Nocturnal	UQ	61
Polypedates otilophus	Rhacophoridae	4	Borneo/Sumatra	Nocturnal	SDZG	23
Rhinella marina	Bufonidae	20	South America	Nocturnal	UQ	329

Appendix 4.3 Ecological group and habit variables, number of intermoult intervals (IMI) measured, and Bd susceptibility data of all amphibian species for which sloughing rate was measured. Mean mortality rate was calculated from published exposure studies (see citations for details). Ecological group (S: stream-associated, T: terrestrial, P: pond-associated, or E: ephemeral breeders), ecological habit (T: terrestrial, A: arboreal, SA: aquatic/semi-aquatic, B: burrowing), evidence of Bd-driven declines (1: Direct evidence, 2: Bd-driven declines indicated by the IUCN Red List, 3: no evidence of Bd-driven declines

Species	Ecological Group	Ecological Habit	IMI N	Evidence of Bd-related declines	Mean mortality rate (N studies)	Citations
Atelopus elegans	S	T	12	2		La Marca et al. 2005; Ruiz & Rueda-Almonacid 2008; Flechas et al. 2012a
Atelopus sp. (spumarius-pulcher complex)	S	T	10	1		La Marca et al. 2005; Peña-Loyola 2007; Proaño-Bolaños et al. 2007; Salazar-Valenzuela 2007; A. Merino-Viteri *pers. comm.*
Ceratophrys stolzmanni	E	B	11	3		IUCN 2015
Dendrobates auratus	T/E	T	3	3	100 (1)	Nichols et al. 2001; Perez et al. 2014
Dendrobates tinctorius	T/E	T	11	3	100 (2)	Nichols et al. 2001; Daszak et al. 2004; Courtois et al. 2012
Epipedobates tricolor	T/E	T	50	2		Forzan et al. 2008; Spitzen-van der Sluijs et al. 2011
Espadarana callistomma	S	A	53	3		IUCN 2015
Gastrotheca pseustes	E	T	54	1		Ron et al. 2003; Korfel 2012; Manzano-Pasquel 2014
Gastrotheca riobambae	P	T	50	2		Manzano-Pasquel 2010, 2014
Hyloscirtus pantostictus	S	A	8	2		IUCN 2015
Lechriodus fletcheri	E	T	21	3	75 (1)	Ohmer unpublished (Chapter 3)
Limnodynastes peronii	E/P	SA	29	3	22 (2)	Stockwell et al. 2010; Ohmer unpublished (Chapter 3)
Limnodynastes tasmaniensis	E/P	SA	38	3	0 (2)	Woodhams et al. 2007a; Ohmer unpublished (Chapter 3)
Litoria aurea	P	SA	79	1	100 (2)	Stockwell et al. 2008; Stockwell et al. 2010
Litoria booroolongensis	S	T	46	2	14 (1)	NSW Office of Environment and Heritage 2012; Cashins et al. 2013
Litoria caerulea	E/P	A	215	3	59.67 (3)	Berger et al. 2005c; Voyles et al. 2007; Voyles et al. 2009; Ohmer et al. 2015
Litoria infrafrenata	E/P	A	127	3	0 (1)	Berger et al. 2004; Young et al. 2014
Litoria verreauxii alpina	P	T	48	1	97 (1)	Osborne et al. 1999; Hunter et al. 2009; Bataille et al. 2015 Brannelly et al. 2015; Scheele et al. 2015
Platyplectrum ornatum	E	B	6	3	100 (1)	Ohmer unpublished (Chapter 3)
Polypedates otilophus	E	A	23	3		Rowley et al. 2007; Winters et al. 2014
Rhinella marina	E/P	T	359	3		Alemu et al. 2008; Sanchez et al. 2008; Shine 2010

CHAPTER 5

General Discussion

The pathogenic fungus, *Batrachochytrium dendrobatidis* (Bd), has been implicated in the enigmatic declines of amphibians worldwide, and continues to pose a threat to hundreds of amphibian populations (Skerratt et al. 2007; Fisher et al. 2009b; Catenazzi 2015). With the incredible breadth of amphibian species and communities affected by this pathogen, there is concomitant variation in susceptibility to cutaneous infection with Bd and subsequent clinical disease (Searle et al. 2011b; Gahl et al. 2012; Ohmer et al. 2013; James et al. 2015). Thus, a better understanding of the structure and function of amphibian skin can provide important insights into the relationship between the amphibian host and cutaneous pathogens, such as Bd. In this thesis, the first comprehensive investigation of skin sloughing as a component of the innate immune system in amphibians, and its role in the progression and susceptibility of amphibians to the cutaneous disease chytridiomycosis, was conducted. Given that Bd only infects the skin of post-metamorphic amphibians, this thesis provides the foundation for understanding processes affecting the skin at the site of pathogen invasion, leading to significant findings that have advanced our understanding of the host-pathogen relationship.

Chapter 2 examined how sloughing interacts with Bd infection and the development of disease in the susceptible host *Litoria caerulea*. While amphibian skin sloughing rate had been hypothesised to increase with Bd infection intensity, this assertion had never been tested directly. This work provides the first in-depth investigation of amphibian sloughing utilising continuous infrared behavioural video monitoring. Skin sloughing rate was found to increase in frogs infected with Bd (Ohmer et al. 2015), and did not result in Bd infection reduction or clearance. This increase in sloughing rate may increase susceptibility to disease given sloughing itself can result in temporary imbalances in water and electrolyte homeostasis (Jørgensen 1949). This has implications for the pathogenesis of chytridiomycosis, and provides insights into the nature of skin dysfunction with disease progression.

Second, Chapter 3 investigated whether the act of sloughing reduces fungal load on the ventral skin surface, and if this differs in susceptible and resistant hosts. Utilising common anuran species from Southeast Queensland, skin sloughing was found to temporarily reduce Bd loads in all species, despite variation in susceptibility of these species to chytridiomycosis. This provides evidence that skin sloughing is an immune defence mechanism, but may not be effective at removing highly invasive Bd zoospores and zoosporangia in susceptible species. This work has the

potential to inform mathematical models of Bd growth on individual hosts, and explain patterns of infection gained and lost over time.

Finally, in chapter 4, sloughing rate and epidermal thickness was investigated across a range of species and anuran families in relationship to Bd-related declines. While no evidence of a relationship between these skin-related traits and declines was found, sloughing rate demonstrated high phylogenetic signal. This is the first database created of amphibian skin sloughing rates, and given the high phylogenetic signal observed in this trait, sloughing rates of focal species may be predicted. This can be informative for understanding the development of disease in certain species, particularly those that slough infrequently, and for conservation mitigation strategies.

Overall, the work contained in this thesis has advanced our understanding of amphibian skin sloughing and its interaction with a devastating pathogenic skin pathogen, as well as basic amphibian physiology and behaviour. There are a number areas related to this work that would be beneficial to investigate in the future, to expand our understanding of the host-pathogen relationship, and interspecific variation in susceptibility to disease.

Future directions

How does skin sloughing influence amphibian physiology?

Fundamental work on amphibian skin sloughing occurred in the first three-quarters of the 20th century (for a review, see Larsen 1976). This thesis provides the most recent update on what we understand about amphibian skin turnover, and highlights the need for a more complete understanding of its role in amphibian physiology. Jørgensen (1949) demonstrated that amphibian skin is 3-4 times more permeable to water and up to 20 times more permeable to ions up to 12 hours before, during, and immediately after the sloughing process, but we do not fully understand the cause for this increased permeability. Ion channels in the skin regulate the influx of water via an osmotic gradient that is driven by solute transport (Campbell et al. 2012). An investigation of how this process is affected by sloughing will help elucidate its effect on amphibian physiology in the healthy and diseased state. Experimental work indicates that Bd itself inhibits sodium epithelial channels in the skin, leading to hyponatremia (low blood sodium levels), presumably through reduced Na^+ absorption in an aquatic environment (Voyles et al. 2009, Campbell et al. 2012). Furthermore, the permeability of amphibian skin to evaporative water loss during the resting (non-sloughing) period is highly variable across species (Young et al. 2005), and increases with both Bd infection and during sloughing (see Appendix A). Thus, sloughing for animals clinically infected with Bd could be challenging for varying reasons, depending on whether they are in an aquatic or terrestrial environment when sloughing occurs. Investigating the mechanisms behind changes in

physiological homeostasis during sloughing in both healthy and Bd infected amphibians will further our understanding of the pathophysiology of chytridiomycosis.

How does sloughing interact with Bd and the frog skin microbiome?

By investigating the relationship between skin sloughing and Bd infection, skin sloughing is demonstrated to play a role in regulating cutaneous fungal pathogens, much as it does in regulating cutaneous bacterial populations (Meyer et al. 2012, Cramp et al. 2014). Given symbiotic bacterial populations likely form an important part of the innate immune system of amphibians, inhibiting Bd growth in culture and potentially producing antifungal metabolites that are active against Bd (Harris et al. 2006, Woodhams et al. 2007b, Lauer et al. 2007, Harris et al. 2009, Bletz et al. 2013), understanding how sloughing, Bd, and the skin microbiome interact would help solidify our understanding of amphibian immune defence mechanisms and improve mitigation efforts. The skin microbiome community is periodically 'reset' as skin sloughing occurs, indicating that this community is far from stable (Meyer et al. 2012, Cramp et al. 2014). As of yet, we do not fully understand how the skin microbiome rebuilds after a sloughing event. It may be that amphibians are re-inoculated from the environment, or that secretory glands, which extend through multiple skin layers to the skin surface, can harbour bacteria for replenishing the skin after sloughing occurs (Meyer et al. 2012, Lauer et al. 2007). This warrants further investigation, especially given the potential utilisation of beneficial anti-Bd bacteria for bioaugmentation of susceptible species (Bletz et al. 2013, Harris et al. 2009).

In addition, how does the introduction of Bd change skin bacteria persistence and re-colonisation? In *Rana sierrae*, a high elevation species that has experienced significant Bd-driven declines in North America, Bd infection resulted in the disturbance of skin bacterial communities in both wild and captive amphibians (Jani & Briggs 2014). However, in populations in which Bd had become enzootic, skin bacterial populations appeared to remain relatively stable with changes in Bd infection intensity, although the sample size was too small to make conclusions (Jani & Briggs 2014). Skin bacterial community diversity has also been found to be lower in populations of a susceptible Panamanian frog species where Bd has become endemic (Rebollar et al. 2016). Given sloughing rate increases at high Bd infection intensity (Ohmer et al. 2015), teasing apart the role of sloughing in regulating and/or disrupting bacterial communities on the skin of infected animals would be very informative. The next step would be to measure the diversity of bacterial communities before and after sloughing in infected and healthy amphibians, in order to relate the magnitude of community change with the outcome of infection. Furthermore, additional research into sloughing during periods of instability in skin microbial communities, such as immediately

after metamorphosis (Bletz et al. 2013), may reveal the importance of this process in disease outcome.

Furthermore, if bioaugmentation for enhanced protection against Bd infection was to be trialled for a particular species, understanding how often that species sloughs could be informative for ensuring the correct choice of probiotic species, as well the timing of introduction of that probiotic to the animal. Recent work has demonstrated that quorum sensing, a process in which bacteria need to reach a threshold density to trigger metabolite production, is an important process governing Bd inhibition *in vitro* (Bletz et al. 2013; Yasumiba et al. 2016). For amphibians that slough every day, it would be important to select probiotics that reach optimal density for metabolite production quickly. Alternatively, for species that slough infrequently, such as those in the genus *Atelopus*, it may be more effective to inoculate amphibians directly after sloughing occurs, when resident symbiont populations are low.

How does environmental temperature affect skin sloughing and its role as an immune defence?

The immune response of the amphibian host and the pathogenicity of the fungal pathogen are affected by environmental temperature (Fisher et al. 2009b, Rohr et al. 2013, James et al. 2015). As with most physiological processes in ectotherms, this is also true of sloughing rate (Meyer et al. 2012; Meyer et al. 2012). Furthermore, sloughing frequency does not appear to demonstrate thermal acclimation or compensation to temperature changes, thus amphibians are likely to experience variation in sloughing rate with the seasons (Meyer et al. 2012, Cramp et al. 2014). This may contribute to seasonal variation in infection intensity and prevalence, in that skin sloughing reduces Bd load on the ventral skin surface (Chapter 3), and a slower sloughing rate in conjunction with temperature-mediated reduced immune function (Raffel et al. 2006) may result in increased susceptibility to disease. Climate change is thought to not only bring changes to average overall temperatures experienced by organisms, but also temperature variability, and recent work has demonstrated that sudden drops in temperature increase the frequency of Bd infection and mortality in moist environments (Raffel et al. 2015). This is in line with the temperature variability hypothesis, which states that pathogens acclimate to changes in temperature faster than hosts, owing to their small size and faster metabolic rates (Rohr & Raffel 2010a). Future work should focus on the efficacy of skin sloughing in reducing Bd load at a range of temperatures, and how quickly sloughing rate changes with a sharp change in environmental temperature. In *L. caerulea,* the temperature coefficient (Q_{10}) of sloughing rate is close to 2 (1.86), indicating that sloughing rate almost doubles with every increase of 10˚C (Cramp et al. 2014). Examining Q_{10} values of sloughing rate for other species would further increase our understanding of the relationship between sloughing and its potential to regulate the growth of Bd on the skin surface in changing

environmental conditions. This would be very informative for predictive modelling of Bd growth on the skin at different temperatures, which would inform projections of how Bd infection grows and spreads within populations.

How do additional stressors affect skin sloughing rates?

The amphibian decline crisis is not solely the result of infectious diseases, such as chytridiomycosis, although this disease has resulted in unprecedented biodiversity loss (Skerratt et al. 2007). Multiple causes have been investigated (Ohmer & Bishop 2011), and many causes, including habitat loss and/or destruction, invasive species, and climate change are likely working synergistically or additively to lead to declines (Blaustein & Kiesecker 2002; (Collins & Storfer 2003). As anthropogenic change intensifies, amphibians are encountering increasing stressors above and beyond those occurring naturally (Blaustein et al. 2012), and this in turn may be related to increasing incidence of disease outbreaks (Fisher et al. 2012; (Rohr & Raffel 2010b). As skin sloughing not only plays a role as an immune defence mechanism (Chapter 3), but also represents a vulnerable period for amphibian physiology, understanding how skin sloughing rates change with various stressors other than disease may help elucidate the mechanisms for increased physiological stress in those situations. Skin sloughing rate is regulated hormonally (Larsen 1976; Barker Jørgensen 1988), and as such increased levels of circulating glucocorticoids in response to stressors (Blaustein et al. 2012) may influence sloughing rate. This stress response may be beneficial, if a faster sloughing rate is induced in the face of a cutaneous pathogen, or detrimental, if long-term stress increases sloughing rates that lead to loss of physiological homeostasis. It would be helpful to further investigate the hormonal regulation of amphibian skin sloughing, and the effects of multiple stressors on this regulation.

How is sloughing different in salamanders, and what implications does this have for understanding Batrachochytrium salamandrivorans *infection?*

While the focus of this thesis was anuran amphibians, both salamanders (Vazquez et al. 2009; Weinstein 2009; Becker & Harris 2010) and caecilians (Gower et al. 2013) have been found to be susceptible to Bd infection. Furthermore, another salamander-specific pathogen, *B. salamandrivorans* (Bsal) was recently discovered in Europe, and has the potential to wreak havoc on North American salamander species if it spreads (Martel et al. 2014). Thus, an understanding of skin shedding in salamanders would be beneficial to better understanding chytridiomycosis in this group of amphibians. Previous work has demonstrated that salamanders also appear to shed their skin more frequently when infected with Bd (based on shed skin found in enclosures), and brown spots characteristic of Bd infection in one species (Weinstein 2009) disappeared after skin had been shed. The author of this study hypothesised that under conditions less favourable for fungal growth,

skin shedding could result in clearance of the infection (Weinstein 2009). Thus, future work examining skin sloughing in uninfected and infected salamanders would advance our understanding immune defence mechanisms in this group of amphibians.

Conclusions

The host-pathogen interaction occurs within the context of physiological responses (Blaustein et al. 2012,Rohr et al. 2013). In terms of the generalist fungal pathogen Bd and its amphibian hosts, their interaction is tightly linked with the routine process of skin turnover, as this is the site of pathogen invasion. This thesis provides the basis for understanding the patterns of Bd infection on the skin before and after skin shedding, which is important given the great variation in skin sloughing rates across species (Chapter 4) and the potential for sloughing as an immune defence mechanism (Chapter 3). Amphibian skin is an incredibly important organ for water and ion balance, and gas exchange (Boutilier et al. 1992), and as such any perturbations to normal skin functioning can lead to the disruption of physiological functioning. This work has led to the first in-depth understanding of the interaction of amphibian skin turnover and sloughing behaviour with Bd infection, and provides additional pieces to the puzzle of variation in amphibian susceptibility to this generalist pathogen. Future work should examine the interactions of skin sloughing and Bd infection at different environmental temperatures or with additional stressors, and within the context of the amphibian skin microbiome. In doing so, we can improve our predictions of the risk of Bd-related declines for specific amphibian species and populations, and better focus and implement mitigation efforts.

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Appendix A

Rates of water loss increase in frogs infected with a pathogenic skin fungus

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Abstract

Batrachochytrium dendrobatidis (Bd) is a pathogenic fungus that causes the cutaneous, infectious disease chytridiomycosis and has been implicated in population declines of numerous anuran species worldwide. Proximate cause of death by chytridiomycosis is asystolic cardiac arrest as a consequence of severe disruption to electrolyte balance. Animals infected with Bd also experience a disruption to their skin sloughing regime indicating that core functions of the skin such as water retention may be severely impacted. This study examined how skin sloughing, body size and Bd infection interact to influence water loss rates in five Australian frog species: *Litoria caerulea, Limnodynastes peronii, Lechriodus fletcheri, Limnodynastes tasmaniensis* and *Platyplectrum ornatum*. Rates of water loss more than doubled during sloughing in four of the five species examined and smaller frogs had higher mass specific rates of water loss than larger frogs. When infected with Bd, frogs sloughed more frequently and water loss rates were 2-3 times higher than those of uninfected frogs. This indicates that hydric disequilibrium may be a significant factor contributing to the morbidity of severely Bd infected anurans, a symptom that is then exacerbated by an increased rate of sloughing. When taking size into account, smaller and/or juvenile anurans may be more at risk from dehydration from Bd infection, as they lose a larger amount of water and slough more frequently than adults. This may partially explain the higher morbidity rates for small and juvenile frogs infected with Bd.

Introduction

Amphibian skin not only provides the first layer of defence against infection, but also serves as a semipermeable surface across which osmotic and respiratory exchanges can occur (Mancini 2004; Campbell et al. 2012). The outermost layer of the skin, the *stratum corneum*, is composed of one to two thin layers of keratinised cells that allow an almost completely uninhibited flow of water from internal to external environments (Amey & Grigg 1995; Liu & Hou 2012). Consequently, many frogs are at risk of dehydration via evaporative water loss when they are away from aquatic or humid environments (Lillywhite 2006). To counteract the risk of dehydration, some arboreal anuran species have developed substantial resistance to water loss across the skin (Lillywhite 2006;

Wygoda 1984). These anurans often do not actively seek escape from hot, dry conditions but rather have evolved mechanisms to avoid desiccation and overheating (Amey & Grigg 1995). The waterproofing mechanism in the majority of these species is a cutaneous layer of lipids that can either occur in the skin or be excreted from skin glands and wiped over the body (Amey & Grigg 1995; Barbeau & Lillywhite 2005).

All amphibians maintain the integrity of their skin by regularly shedding, or sloughing, the *stratum corneum* (Alibardi 2003; Smith 1975) and with it, anything adherent to that tissue such as microbial flora and fauna (Meyer et al. 2012). Sloughing occurs when the *stratum corneum* separates from the layer beneath (*stratum granulosum*) and becomes the 'slough'. When the *stratum granulosum* keratinises, it becomes the new *stratum corneum* and the slough is shed from the body (Alibardi 2003; Smith 1975). Sloughing can alter electrolyte transport dynamics and osmotic movements across the skin in amphibians (Jørgensen 1949). In *Bufo bufo*, *Rana temporaria* and *Rana esculenta* large increases in water permeability and rate of ion loss have been observed during sloughing (Jørgensen & Larsen 1961). Although the separation of the *stratum corneum* and *stratum granulosum* begins approximately three hours before the slough is removed from the body in *B. bufo* (Jørgensen & Larsen 1961), changes in the rate of water uptake have been observed up to twelve hours before a sloughing event occurs (Ewer 1951; Jørgensen 1949).

Amphibians host a wide variety of microflora on their epidermis as typically moist amphibian skin naturally provides a suitable habitat for microbes to flourish on. However, only a subset of all microbes found in the environment is able to colonise amphibian skin (Cramp et al. 2014; Culp et al. 2007). Most cutaneous microbes are either commensal or mutualistic however some opportunistic microbes can become pathogenic (Cramp et al. 2014; Woodhams et al. 2011). Recently it has been shown that sloughing can substantially reduce the microbial load on the skin, suggesting that it is an important process in regulating the abundance of cutaneous microbes (Cramp et al. 2014; Meyer et al. 2012). The chytrid fungus, *Batrachochytrium dendrobatidis (*Bd*)*, is one such pathogenic microbe that can establish itself on the *stratum corneum* of anuran skin.

Amphibians infected with Bd can develop the cutaneous disease chytridiomycosis, which has been implicated in the decline and extinction of numerous frog species worldwide (Skerratt et al. 2007). Anuran species found in the rainforests of Australia and Central America have been particularly affected, with Bd being recognised as the primary cause of several extinctions (Berger et al. 1998; La Marca et al. 2005). Bd propagates via sporangia that form and release zoospores. When zoospores locate a suitable host, amphibian skin for example, they encyst and develop into new sporangia (Berger et al. 2005b; Longcore et al. 1999; Rosenblum et al. 2008). Bd zoospores invade the superficial layers of the epidermis and colonisation predominantly occurs on the ventral

surface and toes (Berger et al. 2005b) of anuran species. Heavily infected frogs slough more frequently, with the skin coming off in pieces, rather than as a whole (Ohmer et al. 2015). However, in frogs with heavy Bd infections, sloughing has no net effect on Bd load (Ohmer et al. 2015). This implies that the increased rate of sloughing, instead of decreasing infection, may in fact contribute to the loss of ionic and osmotic homeostasis in the skin in this susceptible species (Ohmer et al. 2015). The proximate cause of death from chytridiomycosis is asystolic cardiac arrest as a consequence of severe disruption to Na^+ and K^+ balance (Voyles et al. 2009). However animals infected with Bd also display evidence of dehydration (Voyles et al. 2012) suggesting that water loss may be a significant factor contributing to the morbidity of anurans with chytridiomycosis.

Previously, it has been demonstrated that there are ontogenetic differences in susceptibility to Bd, with juvenile anurans being more vulnerable to chytridiomycosis. In *Eleutherodactylus coqui* exposure to Bd induced very high mortality rates in froglets while adult frogs largely cleared infection and had survival rates indistinguishable from controls (Langhammer et al. 2014). The biggest physical difference between adult and juvenile frogs is body size, which also influences rates of water loss (Claussen 1969). Smaller frogs have a higher surface area to volume ratio and thus a higher rate of net water loss relative to body mass (Christian 1978; Tracy & Christian 2005; Withers et al. 1982).

Antemortem Bd infection in anurans is correlated with restricted electrolyte uptake, particularly Na^+, across the skin (Voyles et al. 2009). However, sloughing alone can alter electrolyte transport dynamics in amphibians (Jørgensen 1949) and heavy Bd loads increase sloughing rates in infected animals (Ohmer et al. 2015). This raises the possibility that altered skin electrolyte dynamics in chytridiomycosis infected frogs could result from changes in sloughing frequency (Ohmer et al. 2015). Moreover sloughing and body size can influence osmotic (water) movements across the skin in amphibians (Jørgensen 1949). Clearly the relationships between Bd infection, sloughing, skin function and body size are complex and not fully understood. Understanding the physiology behind host-pathogen relationships is critical for understanding the susceptibility of different species to disease and in doing so, to inform management decisions. Therefore, understanding how Bd affects core functions of the skin like rates of evaporative water loss, is essential. This study aimed to determine how sloughing, body size and Bd infection interact to influence rates of water loss in five Australian frog species, *Litoria caerulea, Limnodynastes peronii, Lechriodus fletcheri, Limnodynastes tasmaniensis* and *Platyplectrum ornatum*. It was hypothesised that water loss rates relative to body mass would be highest in smaller frogs, and that water loss rates would be highest during sloughing, compared to periods of active and inactive

behaviour. Finally, we hypothesised that animals infected with Bd would experience higher water loss rates than uninfected frogs, and that sloughing would exacerbate these effects.

Materials and Methods

Animal selection and maintenance

The primary study species was the green tree frog, *Litoria caerulea,* which is increasingly utilised as a model for evaporative water loss, sloughing and chytridiomycosis research (Lillywhite 2006; Ohmer et al. 2015). *Lit. caerulea* is a moderately waterproof species (Lillywhite 2006; Young et al. 2005) and in temperatures cycling over a naturalistic temperature range from 15 °C to 23 °C will slough approximately every 3 - 4 days (Ohmer et al. 2015). In attempt to determine how chytridiomycosis influences water loss rates in other species, data from an additional four Australian frogs species, *Limnodynastes peronii, Lechriodus fletcheri, Limnodynastes tasmaniensis* and *Platyplectrum ornatum,* infected with Bd as part of an unrelated study, were incorporated. These additional species are common in south eastern Queensland, Australia but have not yet demonstrated widespread evidence of declines as a result of Bd (Speare et al. 2006).

Animal capture and husbandry

Lim. peronii, Lec. fletcheri, Lim. tasmaniensis and *P. ornatum* were sourced as eggs and raised in captivity. Adult and juvenile *Lit. caerulea* were captured from non-protected areas (wet roads and vegetation) near Fernvale, Queensland, Australia. Animals were transported to The University of Queensland where they were transferred into either 5 or 10 litre plastic containers (depending on size) lined with wet paper towel and half of a PVC pipe to provide shelter. Enclosures were cleaned and frogs fed vitamin dusted crickets on a weekly basis. Lighting and temperature cycles mimicked natural conditions, with a 12 hour photoperiod from 0300 – 1500 (chronologically shifted to facilitate monitoring of sloughing), a minimum temperature of 15 °C at 0300 and a maximum temperature of 23 °C at 1500. Snout vent length (SVL) was measured using calipers (W77194 Calipers, Mitutoyo, West Heidelberg, Victoria, Australia) and body mass was measured using an electric balance (EJ-303 Compact Precision Balance, A&D Weighing, Melbourne, Victoria, Australia). All animals were tested for Bd infection prior to experimentation (see below).

Monitoring sloughing frequency

Frog behaviour was monitored continuously using infrared security cameras (HW242C Security Camera, K Guard Security, New Taipei City, Taiwan). Recordings were analysed to determine the timing and rate of sloughing and to predict future sloughs for each frog. Sloughing

was easily recognised from a suite of characteristic behavioural actions including limb movements and gaping behaviour. For the purposes of this study the total sloughing duration was measured from the first to the last mouth gape, and the time between successive sloughing events was termed the intermoult interval (IMI).

Rates of water loss

Following the methods of Amey & Grigg (1995) rate of water loss was determined by measuring body mass loss over time while the frog sat in a constant stream of dry air. The experimental setup is detailed in Figure A.1. Briefly, an air pump (HP40 Air pump, Techno Takatsuki, Osaka, Japan) pushed air through a plastic container in which a frog was positioned. The container was positioned on a top loading balance (EJ-303 Compact Precision Balance, A&D Weighing, Melbourne, Victoria, Australia) and changes in the mass of the frog were measured and logged every ten seconds (AD-1688 Weighing Data Logger, A&D Weighing, Melbourne, Victoria, Australia). A Powerlab (ML866, AD Instruments, Bella Vista, New South Wales, Australia) was used to simultaneously collect air flow measurements (ML140 Spirometer, AD Instruments, Bella Vista, New South Wales, Australia), changes in temperature (ML312 T-type Pod, AD Instruments, Bella Vista, New South Wales, Australia) and video recordings (c170 Webcam, Logitech, Brisbane, Queensland, Australia) of the experiment. Humidity was monitored in five minute increments with a humidity data logger (DS1923 Temperature/Humidity Logger, Maxim Integrated, Brisbane, Queensland, Australia).

For all frogs, rates of water loss were measured for 30 – 120 min immediately prior to sloughing, during sloughing and for a further 30-60 min after sloughing. Behaviour was monitored continuously throughout these measurement periods; animals were classed as active if they moved around during measurements, or inactive if they remained immobile during measurements. If defecation occurred during experimentation the experiment was aborted and repeated another day. Frogs were closely monitored to ensure that no individual lost more than 30 % (for controls) or 10 % (for infected frogs) of its original body mass during the experiment.

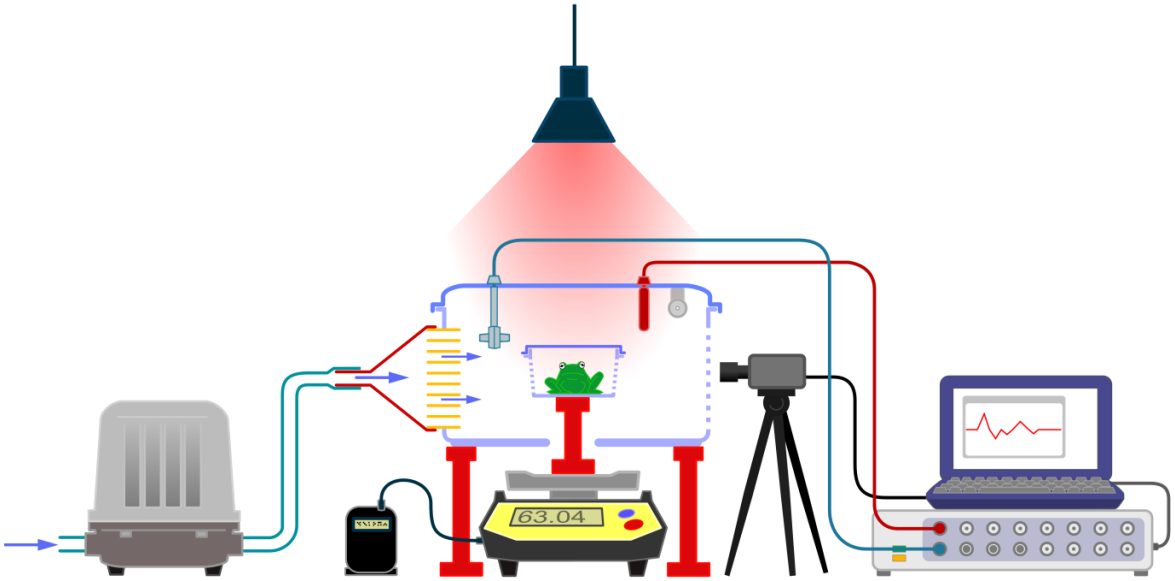

Figure A.1 The experimental setup used to measure water loss rates in five Australian frog species. Briefly, dry air was slowly pumped through a series of flow straighteners and into a container holding a frog which was positioned on a logging balance. Mass changes were recorded every 10 seconds. Air speed, temperature and frog activity levels were captured and recorded using an ADInstruments Powerlab. Humidity was recorded at 5 min intervals using a separate humidity data logger. All measurements were made under red light to reduce disturbance to the animals.

To examine how rates of water loss changed over the intermoult period in healthy frogs, water loss rates were measured every 24 hours throughout the entire sloughing cycle. Frogs (n = 7) were placed into the experimental setup and then rates of net water loss (NWL g h^{-1}) were determined using the equation:

$$NWL = \Delta M / \Delta T$$

Where ΔM represents the change in body mass (g) over the total measurement period (ΔT, in h).

Bd culturing

Two Australian Bd strains (*Waste point-Lverreauxii-2013-LB, RW, 2* and EPS4) were used for experimental infection. Cultures were kept at 4 °C. Four to seven days before the exposure date the strains was passaged onto 1 % agar, 0.25 % tryptone, 0.25 % tryptone-soy plates and kept at 21 °C. Zoospores were collected by flooding the plates with sterile distilled water for 30 min and gently stirring them. Zoospore suspension was collected and concentration calculated using a haemocytometer as detailed by (Boyle et al. 2004).

Bd exposure and infection detection

Throughout the course of this study frogs were exposed to Bd on four separate occasions. During all exposures frogs were randomly allocated into a control and an exposure group. A total of 15 Bd infected and 34 control animals were used (Table A.1). Animals in the exposure group were exposed to approximately 250 000 – 500 000 Bd zoospores in 40 mL of aged tapwater in 300 mL plastic containers for five hours (Berger et al. 2005b; Ohmer et al. 2013). Control frogs were exposed, using the same methods, to distilled water containing no zoospores. After exposure animals were placed back in their enclosures where sloughing rate was monitored continuously using video surveillance, and clinical signs of chytridiomycosis (Berger et al. 2005a) were assessed twice daily. Bd load was tested for by swabbing the ventral surface of the frogs with a sterile cotton swab (MW100, Medical Wire, Corsham, Wiltshire, UK). Swabbing involved firmly running a cotton swab three times over the frogs abdomen, sides, thighs, feet, webbing and toes (Kriger et al. 2006; Prunier et al. 2012; Retallick & Miera 2007). Swabs were extracted in 50 µL of Prepman Ultra (Applied Biosystems, Foster City, California, USA) and analysed in triplicate with quantitative PCR following Boyle et al. (2004) and Hyatt et al. (2007). Quantitative PCR was conducted in a Mini Opticon real-time PCR detection system (CFD – 3120, Bio-Rad, Greenslopes, Queensland, Australia) with a 15 µL reaction volume per well (Ohmer et al. 2015). Before experimentation began each frog was lightly shaken dry and swabbed to quantify Bd load. Experimentation began for Bd infected frogs once infection loads exceeded 400 zoospore equivalents (ZE) or animals displayed symptoms of chytridiomycosis. Clinical signs of chytridiomycosis included, but weren't limited to, discoloured skin, lack of appetite, sluggish behaviour, loss of righting reflex, abnormal posture, excessive sloughing of skin and the development of ulcers on thighs and toes (Campbell et al. 2012; Hyatt et al. 2010; Ohmer et al. 2015).

Table A.1 Sample sizes for frogs of each study species infected with *Batrachochytrium dendrobatidis* (Bd+) and those that were uninfected (Bd-).

Frog Species	Bd+	Bd-
Litoria caerulea	6	14
Limnodynastes peronii	2	3
Limnodynastes tasmaniensis	1	10
Lechriodus fletcheri	2	4
Platyplectrum ornatum	4	4

Controls and animals that failed to contract chytridiomycosis after exposure were grouped together for analyses. Frogs demonstrating severe clinical signs of chytridiomycosis were removed from the experiment and humanely euthanased. Euthanasia was conducted by immersing the entire animal in a neutrally buffered solution of 0.3 % tricaine methanesulfonate (MS-222).

Statistical analysis

All statistical analyses were conducted using the program R (R Core Team 2015). Mixed effects models (function lme, package nlme) were used to compare changes in water loss rate between Bd infected (Bd+) and uninfected (Bd-) frogs during active, inactive and sloughing (only *Lit. caerulea*) periods.

For the analyses comparing rates of water loss across species during active and inactive periods r*ate of water loss* was the response variable. Fixed effects were *treatment* (Bd+ or Bd-) and *SVL*. *Frog ID* and *species* were included as random effects. *Frog ID* was used as a random effect to account for multiple measurements on the same animal. *Species* was included to account for measurements being taken from five different species. *Wind speed*, *temperature* and *humidity* were also analysed as random effects however these variables were removed to improve model quality when no significance was found during active (Temperature: $F_{20} = 0.294$, $P = 0.594$, Air flow speed: $F_{31} = 0.536$, $P = 0.470$, Humidity: $F_{20} = 2.741$, $P = 0.113$) and inactive (Temperature: $F_{20} = 1.666$, $P = 0.212$, Air flow speed: $F_{31} = 0.976$, $P = 0.331$, Humidity: $F_{20} = 0.809$, $P = 0.379$) periods. For the analyses comparing rates of water loss during sloughing in *Lit. caerulea* alone, *species* was not included as a random factor. Models were fitted using maximum likelihood (ML).

To examine the effects of Bd load on rates of water loss, Bd load values were log + 1 transformed. Percent IMI represents how far each individual frog was through its intermoult interval at the time water loss was measured (approximately 24 hours after sloughing to 24 hours before sloughing). Percent IMI was calculated by dividing how many hours post sloughing the rate of water loss measurements were taken at by the total number of hours in the IMI for that individual. This calculation took into account any variation in IMI across individuals.

Results

Uninfected frogs

Most *Lit. caerulea* had a consistent sloughing rate with the average intermoult interval lasting 81.1 ± 14.1 h. The majority of all *Lit. caerulea* (N = 18) sloughed between 1530 h and 1830 h, just after the lights went out. Sloughing behaviour lasted, on average, for 6.5 ± 3.2 min and was as described by Ohmer et al. (2015). Body size had a significant effect on the rate of water loss

during sloughing ($F_{1,9}$ = 14.557, P = 0.004) (Figure A.2a) and when active during intermoult periods ($F_{1,7}$ = 10.443, P = 0.014), with smaller frogs losing more water relative to body mass than larger frogs. Amongst inactive *Lit. caerulea*, the rate of water loss increased significantly prior to sloughing ($F_{1,18}$ = 7.826, P = 0.012) (Figure A.2b). However this pattern was not seen in active *Lit. caerulea*, with rates of water loss varying largely and remaining consistently high (F_{18} = 0.23, P = 0.637). Water loss rates were significantly higher during sloughing when compared to inactive periods ($F_{1,29}$ = 35.903, P = <0.001), with sloughing frogs losing on average 110 % more water per unit body mass than inactive frogs. However, water loss rates were highest when frogs were active immediately after sloughing, losing on average 27% more water than sloughing frogs (Figure A.2c). A substantial period of activity after sloughing was a common occurrence.

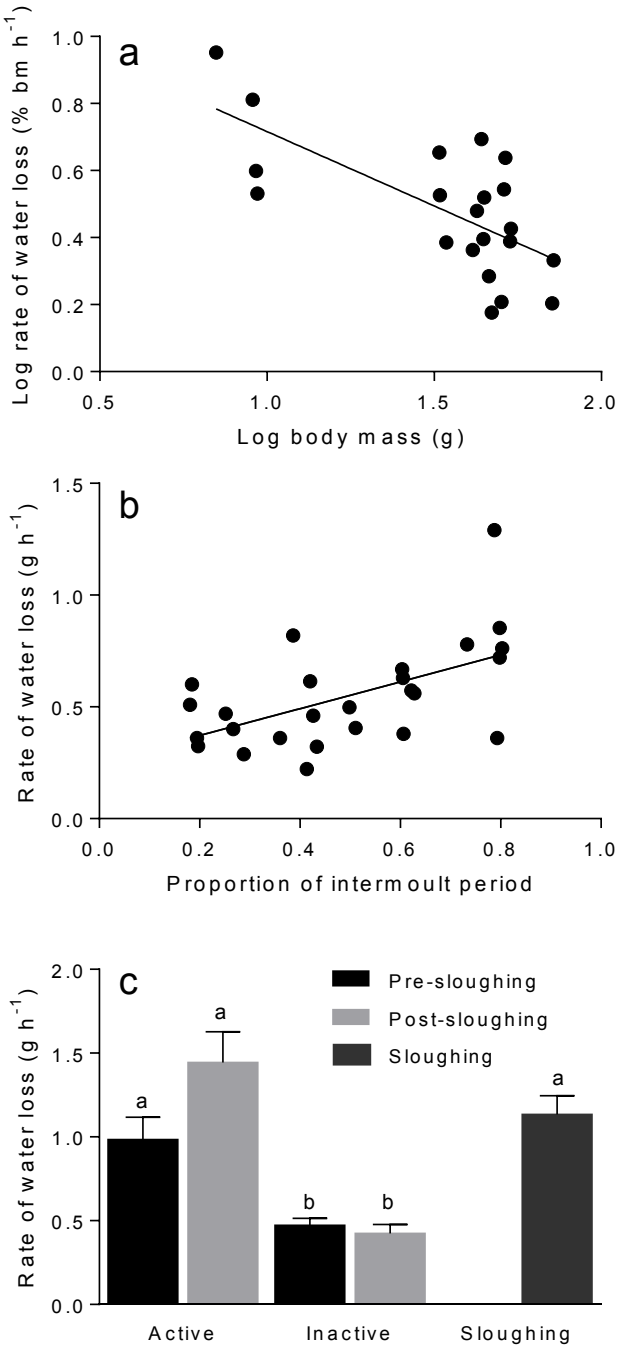

Figure A.2 (a) The effect of body size on rates of water loss in green tree frogs, *Litoria caerulea*. Body size had a significant effect on water loss rates with smaller frogs losing a larger amount of water per unit time than larger frogs. (b) Rates of water loss increased significantly as green tree frogs progressed through the intermoult period. Frogs had the highest rate of water loss in the hours immediately preceding sloughing. (c) Activity levels had a significant effect on water loss rates in green tree frogs. Frogs lost substantially more water when active, relative to when they were still. The process of sloughing increased rates of water loss by 3-fold (relative to inactive periods).

There were no differences in water loss rates amongst remaining frog species and so these were combined for the following analyses. As with green tree frogs, the rate of water loss when frogs were inactive was significantly lower than the rate of water loss when frogs were active (F_{81} = 95.824, P = <0.001; Figure A.3). Body size had a significant effect on the rate of water loss during the intermoult period of all species during inactive periods (F_{34} = 6.181, P = 0.018), with smaller frogs losing a higher percentage of their original body mass than larger frogs. SVL had no effect on water loss rates in active frogs (F_{34} = 2.376, P = 0.133), however variation amongst water loss rates was much higher in active frogs.

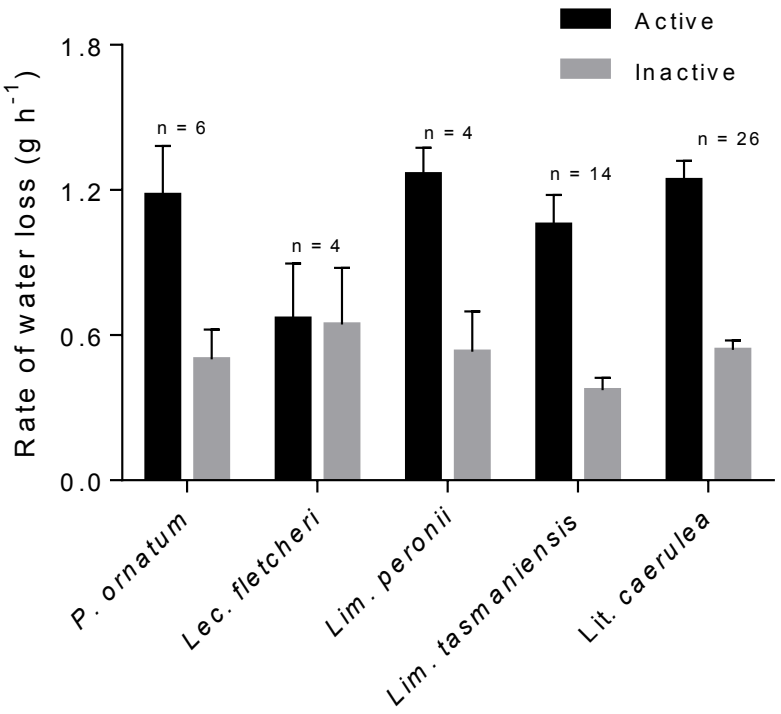

Figure A.3 Rates of water loss in 5 species of Australian frogs: *Platyplectrum ornatum, Lechriodus fletcheri, Limnodynastes peronii* and *Limnodynastes tasmaniensis*. *Litoria caerulea* data are provided again for comparison purposes. Across all species, rates of water loss were comparable and significantly affected by activity level.

Infected frogs

All frogs were Bd negative prior to the first experimental exposure to Bd. Control frogs remained healthy and Bd negative throughout the experimental period. Sloughing in Bd+ frogs was very erratic and difficult to observe in the experimental set up. Consequently water loss rates during the process of sloughing were only collected from six infected *Lit. caerulea*. Behaviour during sloughing differed from uninfected frogs, with Bd+ individuals often failing to remove the entire

slough and displaying drastically reduced limb movements. Sloughing duration was not significantly different from uninfected frogs (7.4 ± 4.2 min). Bd infection had a significant effect on water loss rates during sloughing in *Lit. caerulea* (F_{14} = 7.78, P = 0.015), with Bd+ frogs losing on average 120 % more water than Bd- frogs (Figure A.4). The average intermoult interval of Bd+ *Lit. caerulea* (N = 6) was 57 ± 7.2 h which is significantly lower than in uninfected *Lit. caerulea*.

The majority of Bd exposed *P. ornatum*, *Lec. fletcheri*, *Lim. peronii* and *Lim. tasmaniensis* became Bd+. However most *Lim. peronii* and *Lim. tasmaniensis* did not display severe signs of chytridiomycosis and were able to maintain a low level of Bd on the skin, or in some instances, completely cure themselves of infection. Bd infection had a significant effect on water loss rates in both active (F_{31} = 19.005, P = <0.001) and inactive (F_{31} = 22.906, P = <0.001) frogs, with infected frogs of all species losing on average 120 % (during activity) and 72 % (during inactivity) more water than uninfected frogs (Figure A.4). As Bd load increased, the rate of water loss significantly increased across all frog species during both active (F_{32} = 21.410, P = <0.001) and inactive (F_{32} = 10.759, P = 0.002) periods (Figure A.5).

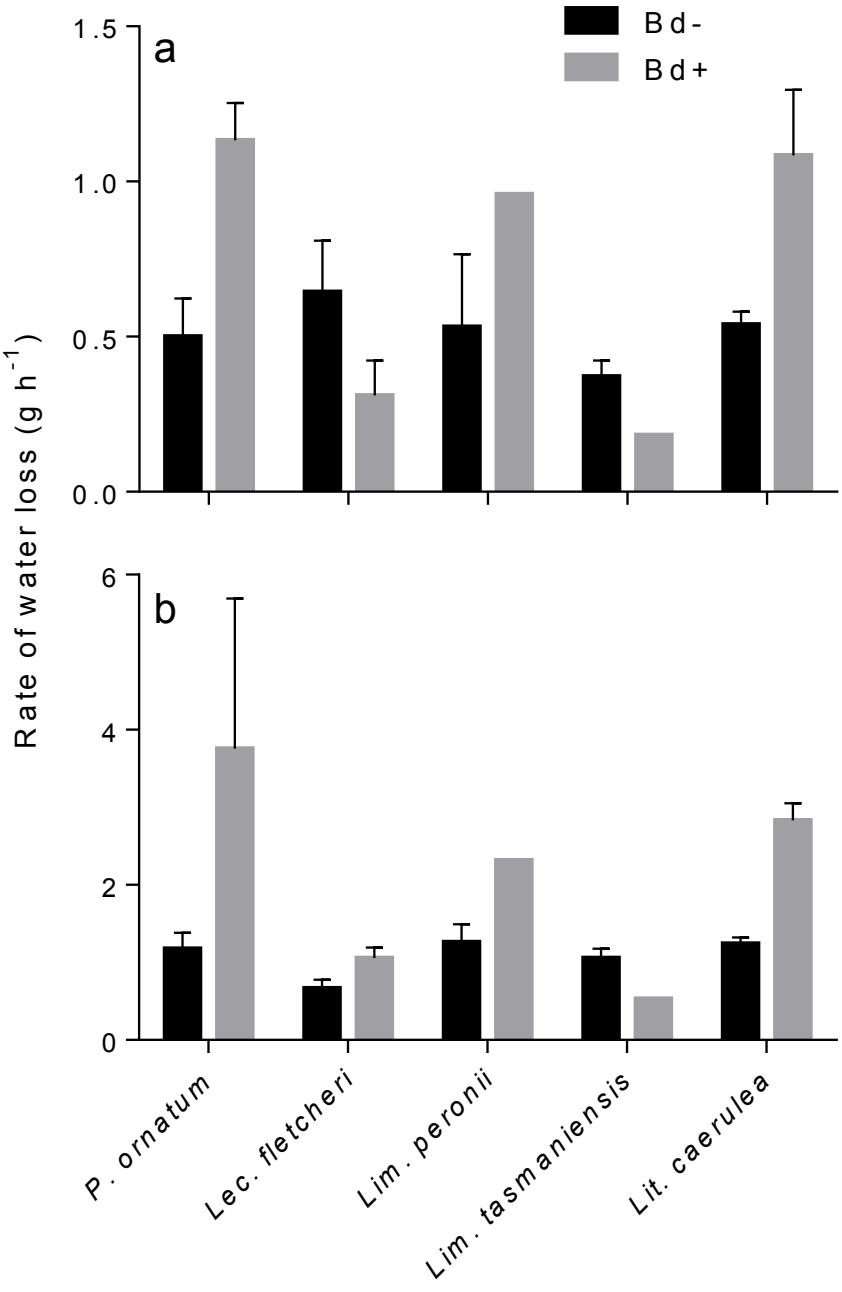

Figure A.4 The effect of infection with Bd on rates of water loss (a) while inactive, and (b) while active, in the Australian frogs, *Platyplectrum ornatum, Lechriodus fletcheri, Limnodynastes peronii*, *Limnodynastes tasmaniensis* and *Litoria caerulea*. Bd infection increased rates of water loss significantly with infected animals losing up to 120% more water than uninfected controls.

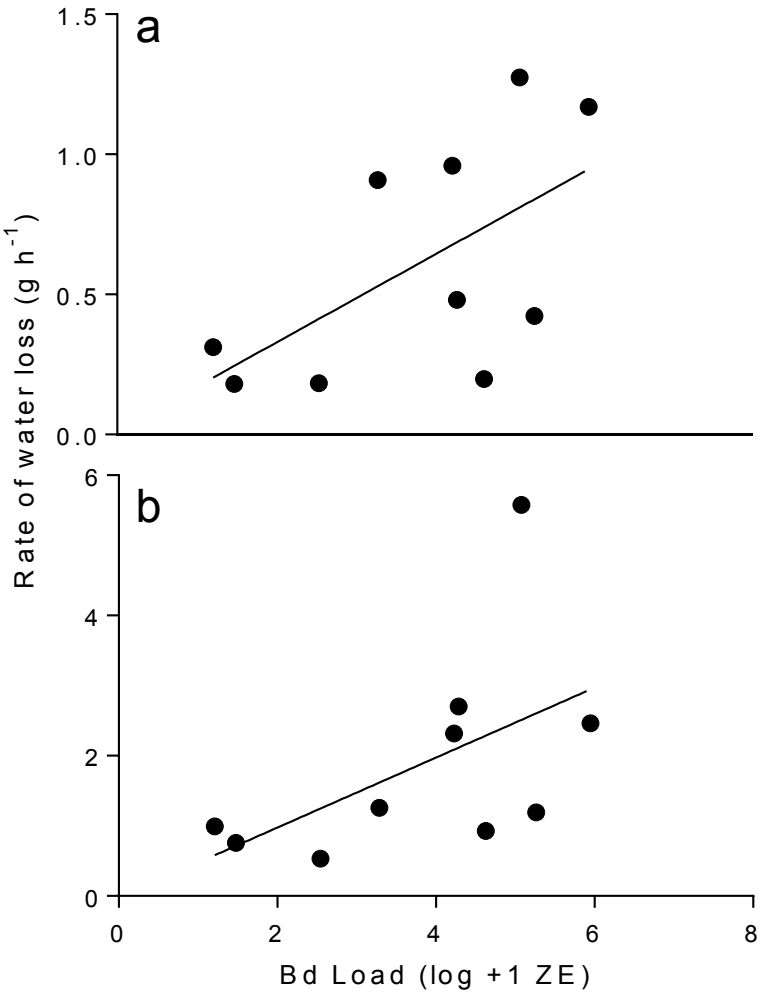

Figure A.5 The effect of Bd load on rates of water loss in frogs (all species). Rates of water loss increased significantly with Bd load both while active (a) and during periods of inactivity (b).

Discussion

Chytridiomycosis has been implicated in the decline and extinction of numerous frog species worldwide (Skerratt et al. 2007). Whilst cause of death by chytridiomycosis is suggested to result from restricted electrolyte uptake across the skin (Voyles et al. 2009), wild animals infected with Bd have also displayed evidence of dehydration (Voyles et al. 2012) suggesting that changes to water loss and/or uptake rates may also contribute to morbidity. This study showed that skin sloughing and infection with Bd substantially increase rates of water loss in five anuran species, with smaller frogs losing disproportionately more water per unit time than larger frogs. These results are consistent with the idea of a hydric imbalance in frogs with chytridiomycosis, and provide a possible mechanism through which this imbalance may occur (i.e. increased sloughing rate).

Behaviour and water loss rates

Across all species, frogs had a significantly higher rate of water loss when active, compared to when they were inactive. Altering behaviour is one of the simplest ways frogs can manipulate their rate of water loss, with many species employing a suite of behavioural, physiological and morphological strategies to minimising water (Shoemaker et al. 1992; Young et al. 2005). The type of habitat a frog utilizes will also influence level of activity, meaning different species (occupying different niches) will often have different activity patterns (Tracy et al. 2014). Differences in water loss rates amongst species may also reflect differences in body shape and levels of cutaneous resistance (Lillywhite 2006; Young et al. 2005). Whilst rates of water loss did not vary much between species in this study, species specific differences did occur when comparing the percentage of starting body mass lost over time, with smaller bodied species losing a larger percentage of their body mass than larger ones; however caution must be taken in extrapolating this finding more broadly given the relatively small number of species examined in this study.

Sloughing *Lit. caerulea* lost on average lost twice the amount of water than inactive frogs. When amphibians slough, the process of manipulating the shed skin from the body into the mouth involves a suite of relatively vigorous limb and mouth movements. The pattern of sloughing behaviour is relatively consistent across anuran species (M. Ohmer, pers. obs), generally consisting of the fore and hind limbs being used to push loose skin from dorsal and lateral skin surfaces up towards the head where gaping mouth movements draw the loose skin into the mouth. Therefore, it is likely that the physical movements that accompany sloughing would have contributed to the increased rate of water loss during this period. In addition, immediately after sloughing occurs the new *stratum corneum* is not completely keratinised and may provide a less restrictive barrier to water movement. Combined with the fact that most frogs became active immediately following sloughing, this may also explain why rates of water loss increased again after sloughing occurred (Figure A.2c). Of the species studied, the majority sloughed in the evening, which is also when these nocturnal species are typically most active (Gomez et al. 2006). Frogs, like most shedding species, are likely unable to actively repress sloughing behaviour, as sloughing is stimulated by a series of hormonal cues provided by the endocrine system (Barker Jørgensen 1988; Herman 1992; Jorgensen et al. 1965). Although the overall behavioural movements that accompany sloughing are relatively consistent across species (M. Ohmer, pers. obs), the duration of each sloughing event and the frequency of sloughing varies greatly amongst species (M. Ohmer, pers. obs), indicating that cumulative the effect of sloughing on water loss rates will most likely differ amongst species.

In green tree frogs, the rate of water loss changed throughout the intermoult interval, gradually increasing until a sloughing event occurred. This indicates that permeability of the skin to

water is altered even before sloughing occurs. Although rates of water loss during sloughing have not been measured before, previous work has shown that in *B. bufo* and *B. regularis,* changes in the rate of water uptake across the skin occur up to 12 hours before sloughing occurs (Ewer 1951; Jørgensen 1949). However in *B. bufo* the separation of the *stratum corneum* from the *stratum granulosum* only begins three hours before sloughing occurs Jørgensen & Larsen 1961 suggesting that changes in the permeability of the skin to water precede the separation and loss of the *stratum corneum*. Whether the timing of the separation of the *stratum corneum* from the *stratum granulosum* influences the rate of water loss across the skin in *Lit. caerulea* remains unclear. Further research at the cellular level is needed to fully understand how permeability of the skin is affected by the morphological changes to the skin layers immediately preceding sloughing.

Body size had a significant effect on rate of water loss in *Lit. caerulea*, with smaller frogs consistently losing a higher percentage of their original body mass. This is consistent with previous work showing that that larger frogs have a higher boundary layer resistance to water loss than smaller frogs (Shoemaker et al. 1992; Tracy & Christian 2005; Withers et al. 1982). This also means that smaller frogs are more susceptible to dehydration than their larger, adult counterparts.

Chytridiomycosis and water loss rates

In the majority of the frog species examined, Bd infection significantly increased rates of water loss. Although what stage a frog was through its intermoult interval was found to have a significant effect on the rate of water loss, the effect of Bd infection was substantially higher, with infected *Lit. caerulea* losing approximately double the amount of water of uninfected frogs. However, to have a significant effect on rate of water loss Bd infection load had to be high (on average $2.1 \times 10^5 \pm 3.4 \times 10^5$ ZE). This is consistent with Ohmer et al. (2015), in which the effect of infection with Bd on intermoult interval length in *Lit. caerulea* is Bd load dependent. Only once the minimum Bd zoospore threshold level (~7000 ZE) had been reached did intermoult interval change (Ohmer et al. 2015). In addition, Voyles et al (2012) found electrolyte and haematocrit levels only changed significantly in Bd infected *Rana muscosa* when zoospore levels were above 10000 ZE. In all cases Bd load has to be relatively high before clinical signs of chytridiomycosis can be observed. This suggests that low levels of Bd infection may not be sufficient to disturb skin function substantially and may explain why some species are able to resist developing chytridiomycosis if infection levels are kept low (Berger et al. 1998; Daszak et al. 2003; Lips et al. 2006). Consistent with this observation, Carver et al. (2010) found that Bd exposure had no significant effect on rate of water loss. This is most likely because the effects of Bd on water loss rates were measured just seven days after exposure to Bd which is unlikely to have allowed sufficient time to infection intensities high enough to disturb skin function.

An increased rate of evaporative water loss and increased sloughing rates associated with moderate to high Bd infection loads may contribute to recently observed increases in haematocrit and plasma protein levels in wild frogs with high Bd loads (Voyles et al. 2012). High haematocrit and protein levels are both indicative of dehydration (Billett 1990; Lee 2009; Voyles et al. 2012). Dehydration occurs when water loss rate exceeds water uptake rate, resulting in a net loss of water in the blood, and thereby elevating the concentration of red blood cells and plasma proteins in the blood. Moreover, water absorption rates are also reduced in Bd infected amphibians (Carver et al. 2010; Wardziak et al. 2013). Dehydration as a consequence of the severe disruption to skin water resistance, the reduced ability to take up water, and increased sloughing frequency, all of which accompany Bd infection, may contribute to morbidity in frogs suffering from chytridiomycosis.

Bd infection, sloughing and water loss rates

Heavy Bd infections substantially increased the amount of water lost during sloughing in *Lit. caerulea*. Although general activity also increased the rates of water loss in animals with Bd, in general Bd infected frogs had considerably lower levels of overall activity than uninfected frogs. Animals infected with Bd may therefore regulate behavioural levels so as to limit excessive water loss rates. Bd infection also increased the rate of sloughing in *Lit. caerulea*, a result that has been seen by previous studies (Ohmer et al. 2015). Therefore, Bd infection not only increases water loss rates during sloughing, but also increases the rate at which sloughing occurs in general, implying that increased sloughing rates could potentially contribute to morbidity in anurans with chytridiomycosis. This increase in the rate of sloughing may also exacerbate the effects of Bd infection, by continually disrupting osmotic and ionic homeostasis. Since sloughing cannot be actively controlled (Herman 1992), occurs more frequently when animals are infected with Bd (Ohmer et al. 2015) and increases rates of water loss, frogs infected with Bd are at a substantial risk of dehydration. Since smaller animals slough more frequently and lose more water relative to body mass, they are also more likely to develop an osmotic imbalance relatively suddenly and this may contribute to the explanation of why smaller frogs are more susceptible to Bd, and succumb quicker, than larger ones (Carey et al. 2006; Langhammer et al. 2014).

Conclusions

This study found that sloughing, activity levels and body size significantly influenced rates of water loss in five species of Australian frogs. These results were compounded by Bd infection, with rates of water loss increasing substantially in infected frogs. Given that frogs slough more frequently when infected with Bd and Bd infected animals have a higher rate of water loss and a

reduced water uptake capacity, it is likely that cutaneous osmotic dysfunction leads to dehydration and is a contributing factor in morbidity from chytridiomycosis.

Bd has had a substantial effect on the health and abundance of numerous frog species worldwide, with many species experiencing severe declines and extinctions (Berger et al. 1998; Hyatt et al. 2010; Skerratt et al. 2007). Comprehending the host-pathogen relationship and the effect of this pathogen on basic skin functions is important for understanding the variation in species susceptibility, and responses to, Bd infection.

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