Virulence and pathogenesis of chytridiomycosis: A lethal disease of amphibians

Thesis submitted by
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STATEMENT OF CONTRIBUTION

This research was co-supervised by Ross Alford, Lee Berger, Lee Skerratt and Rick Speare at James Cook University, Townsville, QLD. All advisers contributed on experimental designs, technical support and statistical analysis for the experimental research. They also provided produced editorial assistance. Additional assistance was provided many individuals. Details of their contributions can be found in the acknowledgments and preface.

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It is often said in academic circles that we “stand on the shoulders of giants”. When I moved half way around the world to begin this project I knew I was coming to work with the “big guys”, but I could not have imagined that I would have such a gigantic community so graciously support my work. Ross Alford never failed to provide thought-provoking ideas and intriguing insight on my research. “He-Lee” Skerratt and “She-Lee” Berger paved the path for this research and then helped me every step along the way. Despite his monstrous schedule, Rick Speare always found time to speak to me when I needed direction, address my research needs and even sit with me in the hospital when I was very unwell. Thank you to each of my advisers; I am forever grateful for your kindness, your support and your example. I have some enormous shoes to fill.

Where would a parasite be without her hosts? The work presented in the following chapters exists because of the energy and assistance of many individuals. For everything they have generously provided I am extremely grateful.

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ABSTRACT

Few fungi are highly virulent to terrestrial vertebrates. Yet the disease chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), is one of the causes of global amphibian declines. How a superficial skin fungus can cause catastrophic extirpations is perplexing. To date most investigations have focused on ecological aspects of the host-pathogen dynamic: understanding the seasonal dynamics of the disease, mapping the distribution of the pathogen and determining its impact on amphibian populations. Relatively few studies have considered the importance of differential virulence, and evolution of virulence, of *Bd*. Additionally, the mechanisms of pathogenesis in chytridiomycosis remain largely unresolved. I examined the growth and developmental response of *Bd* to different biotic and abiotic conditions over multiple generations with an underlying objective of understanding *Bd* virulence. I also used pathophysiological techniques to determine the cause of mortality in frogs with severe chytridiomycosis.

In some susceptible amphibian species severe disease is closely associated with high burdens of *Bd*. Therefore, rate of zoospore production is likely to be an important determinant of *Bd* virulence. I quantified zoospore densities in multiple isolates and examined growth and development of *Bd* in different nutrient and temperature conditions over multiple generations. In short term experiments *Bd* responds to different temperature and nutrient conditions by adjusting its life history. I found that, after multiple passages, *Bd* is phenotypically plastic in its response to low nutrient concentrations, but may have an adaptive response to long-term maintenance in low temperatures. Cultures that were originally derived from a single cryo-archived isolate and passaged in 0.2% tryptone TGhL (tryptone/gelatin hydrolysate/lactose media) for 24 passages had higher zoospore densities when inoculated into 1.6% tryptone TGhL, suggesting that *Bd* is phenotypically plastic in its response to nutrient conditions after 24 passages. In a reciprocol transplant experiment, cultures maintained in 4ºC for 20 passages released zoospores earlier and had a longer period of high zoospore densities

than cultures of the same isolate and passage history, but that were maintained at 23ºC. This pattern of early zoospore release was consistent for cultures maintained in low temperatures at 4ºC and at 23ºC, suggesting an adaptive response to lower temperatures.

The effects of serial passage on growth of *Bd* cultures were also examined. Two cultures that were originally derived from the same cryo-archived isolate, but had higher and lower passage histories, had different zoospore densities in *in vitro* experiments; after 50 passages cultures had significantly higher zoospore densities than cultures with a passage history of 10. These patterns of zoospore densities *in vitro* corresponded with differences in prevalence and intensities of infection in experimentally exposed *Litoria caerulea*. However, the differences in these response variables (prevalence and intensities of infection) were not significant and no mortality occurred in any experimental group. These results suggest that variation can exist within a single *Bd* isolate and that certain environmental conditions may exert selective pressures on *Bd*, which could influence the host-pathogen dynamic in important ways. For example, adaptive adjustments to low temperatures could enhance transmission and substantially alter the impact of the disease in amphibian populations. The practical applications of these results are that *Bd* may be evolving in particular ways due to long-term culturing practices, which should be a consideration for laboratory experiments aimed at understanding chytridiomycosis.

The pathophysiological changes associated with chytridiomycosis were investigated by tracking *Bd* infection in experimentally exposed *L. caerulea* and measuring a wide range of biochemical and physiological parameters. Infected *L. caerulea* that developed clinical signs of severe chytridiomycosis had the highest burdens of *Bd*. Ussing chamber tests which measure transepithelial current and resistance demonstrated that skin samples from experimentally infected *L. caerulea* had inhibited electrolyte (sodium and chloride) transport across the skin surface. Plasma electrolyte concentrations, including potassium, sodium, magnesium and chloride, were reduced in the terminal stages of disease. Surgically implanted biotransmitters that were continuously recording cardiac electrograms revealed that asystolic cardiac arrest (which can be triggered by shifts in electrolytes) was the terminal event in *L. caerulea* with severe chytridiomycosis.

Diseased frogs that received an electrolyte supplement became more active and lived longer than diseased frogs that received no treatment. Because I found no significant changes in haematocrit, albumin, total protein or body mass, it appears that the reductions in electrolyte concentrations were due to depletion from circulation rather than water uptake. It is the disproportionate loss of electrolytes compared with water that signifies an imbalance in osmotic homeostasis. Loss of electrolytes could occur via the skin or the kidney. Histological analysis of the kidney samples was inconclusive but the skin was severely damaged when assessed with histology and electrolyte transport (Ussing chamber) tests, suggesting that the skin is the primary organ involved in the extensive electrolyte shifts that lead to mortality. Amphibians can tolerate greater electrolyte fluctuations than other terrestrial vertebrates, but my results support the epidermal dysfunction hypothesis, which suggests that the disruption to cutaneous functioning, and the extent of electrolyte imbalance that occurs in severe chytridiomycosis, produce a life-compromising pathophysiology.

The unique importance of the skin in maintaining amphibian homeostasis and the ability of *Bd* to disrupt epidermal functioning are two key factors that help explain how mortality can occur in a wide range of amphibian species. Additionally, the ability of *Bd* to respond to a wide range of environmental conditions (temperatures and nutrient conditions) in ways that potentially alter the virulence and impact of chytridiomycosis, makes *Bd* a formidable pathogen. These disease characteristics, combined with the ability to spread rapidly and persist at low host densities, create a lethal suite of concomitant variables that, taken separately, might not be so devastating, but together are threatening amphibians worldwide.

PREFACE

The amphibian disease chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), was first described in 1998 and rapidly became a disease of interest for scientists and the general public alike. It is, perhaps, one of the better-known wildlife diseases due to pervasive media coverage. My personal interest in the plight of amphibians was piqued before I began my master's research at the University of Colorado. At the time, I was working on a series of amphibian projects in the Republic of Panama when an outbreak of chytridiomycosis extirpated the frogs and salamanders from the streams and ponds of El Parque Nacional de Omar Torrijos (Omar Torrijos National Park). The subsequent silence made a lasting impression on me. Without the normal orchestra of calling frogs, the quiet of the rainforest seemed eerie and abnormal.

Witnessing the disappearance of species, while devastating at a personal level, provided the motivation to understand the "why" and the "how"… or in other words, the mechanisms responsible for the little understood processes surrounding these losses of biodiversity. Disease is an important, although sometimes under-estimated, driver in biological systems and chytridiomycosis has brought considerable attention to the importance of emerging diseases and their potential threat to many organisms. The dramatic loss of amphibian biodiversity due to chytridiomycosis is accurately described by media clips as "catastrophic" and "tragic". However, it also provides the opportunity to study and better understand the underpinnings of biological processes that impact diversity and evolution of all species. These are the reasons why I focused on this research topic.

The research experiments described in this thesis were accomplished with consultation and collaboration with individuals from different scientific backgrounds. In many cases, their expertise made these multidisciplinary projects possible. Many of those who offered assistance were thanked, albeit too briefly, in the acknowledgements. Here I

summarize the scientific and technical contributions of collaborators in each of the investigations, as their assistance should be formally recognized and appreciated.

CHAPTERS FOUR AND FIVE

Scott Cashins and Erica Rosenblum contributed substantially to the experimental designs for each experiment. Lee Berger did the initial isolations and cryo-archiving of the *Bd* isolates. I refined the methods, performed all of the *in vitro* work with *Bd* (quantifying zoospore densities) as well as all of the *in vivo* work for exposure experiments. Stephen Garland performed the real time PCR for the swabs collected from experimental frogs. Rebecca Webb assisted with *Bd* isolate maintenance and culturing. Rick Speare assisted with trouble shooting in the laboratory. Ross Alford and Lee Skerratt assisted with interpretation and statistical analysis of the data. I wrote the manuscripts and I expect to have input from all co-authors.

CHAPTER SIX

Gerri Marantelli provided the captive bred *Litoria caerulea* for this project. Lee Berger and Rebecca Webb collected the skin swab and blood biochemical samples. Ruth Cambell performed the real time PCR on swab samples. Jeff Warner and Donna Rudd assisted with methods and analysis for the blood biochemistry. I analyzed the data, with help from Lee Skerratt, and wrote the manuscript for publication. Lee Berger and Sam Young edited multiple drafts of the manuscript and all co-authors commented on a final draft.

CHAPTER SEVEN

This chapter consists of multiple linked experiments aimed at understanding the pathophysiology of chytridiomycosis. It was a large undertaking and as such, it required considerable effort from many individuals. I was the primary person responsible for the experimental design, logistics, organization, animal husbandry, collection and analysis of the data and writing the manuscript. Rebecca Webb and Sara Bell assisted with animal husbandry. Lee Berger assisted with blood sample collections. Sam Young analysed the majority of plasma samples at James Cook University, Cairns, Australia, although some

blood and urine samples were analysed by Gribbles Pathology, Melbourne, Australia. Sam Young and I performed the wet-field surgeries to implant the biotransmitters. Lee Berger and Rebecca Webb did most of the epidermal histology. The renal histology was done with stains provided by University of Sydney with consultation from Kerrie Rose, Toronga Zoo. David Cook, Anuwat Dinudom and Craig Campbell consulted on the experimental design for the Ussing chamber experiments and Craig Campbell collected the majority of the data at the University of Sydney. Virginia Boone performed the haemolysis tests on *Bufo marinus* erythrocytes. Lee Skerratt and Ross Alford assisted me with the statistical analysis and Jeremy VanDerWal assisted with the R graphing and statistical software. Multiple drafts of the manuscript were edited by Lee Berger and Sam Young and were commented on by all co-authors.

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LIST OF ABBREVIATIONS

AAHL	CSIRO Australian Animal Health Laboratories
ANOVA	Analysis of variance
ATPase	Adenosine triphosphate
Bd	*Batrachochytrium dendrobatidis*
cm	Centimetres
d	Days
dH_2O	Distilled water
^oC	Degrees Celsius
DNA	Deoxyribonucleic acid
g	Grams
h	Hours
µg	Micrograms
µl	Microlitres
mg	Milligrams
ml	Millilitres
mm	Millimetres
m	Minutes
M	Molar = $g.L^{-1}$
mMol	Millimolar
MR cells	Mitochondrial rich cells
L	Litre
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
s	Seconds
SPE	Serial Passage Experiments
w	Weeks

CHAPTER ONE

General introduction and overview

Scope and aims

The principal objective of this research was to use experimental methods to investigate virulence and pathogenesis in a highly lethal disease of amphibians, chytridiomycosis. Although the focus of this study was on a single host-pathogen system, the project aimed to understand chytridiomycosis within the broader context of disease ecology. Host-pathogen dynamics are driven by biotic and abiotic interactions at many levels, including the interactive effects of multiple variables including pathogen virulence, host susceptibility, population dynamics and environmental conditions. As I could not address all these variables, I chose specific, testable hypotheses, then aimed my experimental work at testing these to increase our understanding of chytridiomycosis.

Thesis overview

Chapter two (Part I) provides definitions of terms and reviews the basic concepts of infectious disease and virulence. This is important because the definitions of these concepts vary according to the source. Chapter two (Part II) provides background information on the phenomenon of amphibian declines, chytridiomycosis and a review of the host and pathogen characteristics that are important for understanding the disease and its effects.

Chapter three describes the general methods used for working with the host (the frogs) and the pathogen (the fungus). The experimental work for this investigation involved a series of linked infection experiments and *in vitro* serial passage experiments. Most of the methods are well established and have been published or are commonly used in

disease research. However, in some cases techniques were modified to suit the objectives of various experiments. Chapters four to seven contain specialized methods that were used for the respective experiments.

Chapters four to seven detail the hypotheses, experimental designs, results and conclusions. These chapters are written in manuscript format and as such, they contain some repetition in background information (e.g., about amphibian declines).

Chapters four and five centre on the pathogen; both investigations concern differential growth and development of *Batrachochytrium dendrobatidis* (*Bd*), and the implications of this for virulence. Some evidence suggests that the virulence of *Bd* differs among isolates (Berger et al. 2005b, Briggs et al. 2005, Morgan et al. 2007, Retallick & Miera 2007, Fisher et al. 2009), but the determinants of this variability are unknown. To date very few investigations have focused on the evolution of *Bd* virulence and the possibility of host-pathogen co-evolution in chytridiomycosis. Pathogenesis may be closely tied to pathogen load (Carey et al. 2006), and therefore I hypothesized that differences in growth rate and zoospore densities might be an important feature in differential virulence among *Bd* isolates. Also, because pathogens are generally known to attenuate in culture, I hypothesized that serial culturing of *Bd* in laboratory conditions might be leading to altered virulence. To test these hypotheses I used multiple *in vitro* and *in vivo* experiments to quantify *Bd* zoospore densities between and within isolates.

A primary objective of the first study (Chapter four) was to investigate specific biotic and abiotic factors that might influence zoospore production in *Bd*. Two *in vitro* experiments manipulated temperature and nutrient availability and quantified *Bd* growth and zoospore densities over multiple culture generations. In the second study (Chapter five), the aim was to determine whether an isolate of *Bd* would change (and/or could be manipulated) during serial culturing and if culturing practices might influence virulence. This investigation consisted of three experiments. First, to characterise differences among *Bd* isolates, I examined growth and development (zoospore densities) in three *Bd* isolates actively being passaged in the laboratory. Second, to better understand how *Bd* might

respond to serial passage practices, I revived a single isolate from cryo-archives and compared growth and development between two cultures with different passage histories. Third, to see if differences in zoospore production between cultures corresponded with differential virulence, I performed an exposure experiment with frogs known to be susceptible to chytridiomycosis. In both studies I review the importance of zoospore production and discuss the importance of understanding *Bd* virulence.

Chapters six and seven bring the host and the pathogen together to understand pathogenesis, disease development and mortality. It has been hypothesised that *Bd* kills amphibians by disrupting the osmoregulatory processes of the skin, where *Bd* is predominantly found (Berger et al. 1998, Berger 2001, Puschendorf & Bolaños 2006a), but this hypothesis has not been tested. Chapter six examines the effect of chytridiomycosis on the plasma biochemistry of clinically diseased frogs. When significant electrolyte loss was discovered in terminal frogs, I refocused my aims. Chapter seven incorporates additional pathophysiological techniques to answer more specific questions about epidermal electrolyte transport and cardiac electrical functioning in clinically diseased frogs. The importance of electrolyte loss in the pathogenesis of chytridiomycosis was also examined with an experiment that provided an electrolyte supplement to diseased frogs. I hypothesized that an electrolyte replacement (especially sodium and potassium) at the onset of clinical signs of disease might mitigate the deleterious effects of electrolyte loss and thus, demonstrate the importance of electrolyte depletion in pathogenesis.

The final chapter (Chapter eight) provides a general discussion of the research findings and reviews current information on the management of chytridiomycosis for amphibian conservation. This is followed by specific recommendations for advancing virulence and disease research.

CHAPTER TWO

Literature review

Chapter overview

This chapter consists of two parts. Part I is focused on defining terms and providing background information on the concept of virulence. This is important because the definitions of disease terms vary according to the source. Take, for example, the term "disease". The roots of the word "disease" are Latin (Borror 1960); the prefix "dis" means separate or apart, with a negative connotation, and "ease" equates to comfort or pleasure (Borror 1960). So in the simplest terms, disease is a lack of comfort. However, most dictionary definitions are far more descriptive, complex and incorporate words such as "host" and "pathogen". The term "virulence" is used extensively in the disease literature but definitions vary widely. Understanding the current views of virulence is central to this investigation. This section reviews various definitions of virulence, provides historical background and considers current views on the concept of virulence.

Part II reviews the historical background of amphibian declines due to chytridiomycosis. It also provides information on the biology of the host and the pathogen with an emphasis on those aspects that are important for pathogenesis and virulence research. This includes epidermal anatomy, physiology and pathology in chytridiomycosis for the amphibians, and morphology, growth and development for *Batrachochytrium dendrobatidis*.

Part I. The concept of virulence

Introduction

The word virulence is derived from the Latin word "virulentus", meaning "full of poison" (Glare 1982). A general search for a contemporary explanation of the term virulence provides the following definitions:

1. the relative ability of a microbe to cause disease (Stedman 2000)
2. the degree of pathogenicity of a given pathogen (Vaughan & Morrow 1989)
3. the degree to which a disease-causing organism can affect the organism it attacks (Science 1997).

In various academic fields the term means different things. Microbiologists often refer to virulence as a composite product of two features: the ability to colonize a host (infectivity) and the severity of the disease produced (Read & Harvey 1994). Epidemiological modellers have quantified virulence as the mathematical rate of parasite-induced mortality, represented in mathematical disease models by the Greek small letter for alpha (Anderson & May 1978, May & Anderson 1978, Anderson & May 1979, May & Anderson 1979). Ecologists often focus on parasite-mediated effects on host fitness (including morbidity and the ability to reproduce), rather than specifically on mortality. Because many of the definitions of virulence appear to be focused on attributes of the pathogen, they could lead to the assumption that virulence is strictly a microbial property. However, this does not take into account the fact that disease development is also highly dependent on the host (Casadevall & Pirofski 1999, 2001). That is, disease is as much a consequence of host susceptibility or resistance (Read & Harvey 1994) as it is of the characteristics of the pathogen. A microbe that is highly virulent in an immunocompromised host may not cause disease in a healthy host. For example, the ubiquitous bacterium *Aeromonas hydrophila* causes disease in immunocompromised hosts (Wolff et al. 1980, Cailleaux et al. 1993, Simmaco et al. 1998). Therefore, virulence is best considered a dynamic variable that is dependent on host-microbe interactions and the effects of the environment on these interactions (Read & Harvey 1994, Levin 1996, Casadevall & Pirofski 1999, 2001).

The importance of clarifying the definition and concept of virulence is not merely academic. Understanding that virulence is a property of the host-pathogen system, rather than simply of the pathogen alone, strongly changes the focus of research aimed at answering questions such as: Why are some pathogens more virulent than others? Why do some diseases have greater impacts in certain geographical regions? Under what circumstances might we be able to intervene and diminish the impacts of disease on human and animal populations? The answers to such questions are important for a wide range of basic and applied biological issues (Levin 1996, Dybdahl & Storfer 2003). Resolving the evolutionary principles driving disease should provide insight into its role in generating biological diversity (Bull 1994, Dybdahl & Storfer 2003). It is also critical information in a variety of fields including medicine, agriculture and conservation management (Read & Harvey 1994, Ebert & Bull 2003).

A historical view of virulence- the conventional wisdom

It was historically believed that pathogens should evolve towards benignness or avirulence (in the sense of reduced pathogenicity, not reduced transmission) in any host species (Ewald 1983, Bull 1994, Nowak & May 1994, Ewald 1994). The logic was simply that damage to the host would be harmful to the pathogen: if the host dies, the pathogen dies too. This theory assumes pathogens that minimize their pathogenic effects will maximize their transmission rates by maintaining mobile, living hosts that provide opportunities for transmission. This idea has become so ingrained in medicine and the study of parasitology that it is referred to as the "conventional wisdom" and is sometimes used as the sole basis for hypotheses about host-pathogen biology today (Bull 1994, Levin 1996, Galvani 2003, Ewald 1994).

While the characteristics of some diseases are consistent with this hypothesis, many others are not, and there are likely many cases in which its assumptions are incorrect (Anderson & May 1978, 1979, May & Anderson 1978, 1979, Levin 1996, Ewald 1994). There is empirical evidence indicating evolution toward avirulent commensalisms is not

universal. For example, some very old parasites are highly virulent. Herre (1993) suggested that Panamanian nematodes, which have an ancient association with fig wasps, have evolved to be more virulent based on the population structures, and the corresponding transmission dynamics, of the host fig wasp species. By some estimates malaria has been plaguing hominids since the evolutionary split of humans and chimpanzees (Pennisi 2001). Yet today, some 8 million years later, malaria persists in multiple strains and with variable degrees of virulence, which, in some cases, are highly lethal (Snow et al. 2005). Furthermore, some diseases caused by one parasite may differ in severity based on geographical rather than temporal parameters. The parasite *Helicobactyer pylori* isolated from human populations in different geographic areas are genetically indistinguishable, but the severity of gastric disease caused by the parasite appears to vary by region (Chattopadhyay et al. 2002).

Proponents of conventional wisdom have defended its inconsistencies (Levin 1996). They have argued that perhaps 8 million years isn't long enough for the malarial pathogen to evolve toward benignness or that geographical variation in virulence may be the result of confounding variables (Levin 1996). Such defences of the conventional wisdom worked for a long time and few individuals took issue with the many contradictions. Paul Ewald (1994) suggested that in the absence of alternative hypotheses, the conventional wisdom was widely accepted because it was "too appealing to be rejected". However, evolutionary biologists have largely moved away from historical views of virulence.

An alternative model of virulence- the trade-off hypothesis

The field of evolutionary epidemiology applies the principles of evolution and natural selection to host-pathogen systems (Galvani 2003, Ewald 1994). This synthesis occurred only in the later part of the 20th century despite its importance for human health (Levin 1996, Ewald 1994, Stearns & Koella 2007). Roy Anderson and Robert May (1978, 1979) laid the groundwork for mathematical and theoretical modelling and thus spurred the

formation of evolutionary epidemiology. With attention on the potential applications for human interests (Bull 1994, Ebert & Bull 2003, Stearns & Koella 2007), the study of the evolution of virulence has advanced from a neglected topic to theoretical discussions to modelling of disease systems (Galvani 2003). Consequently, multiple alternative models of virulence have been generated and discussed as evolutionary biologists consider and test the evolutionary underpinnings of disease (Bull 1994).

A key advance in the study of virulence was the careful consideration of the relationship between parasite-mediated mortality and between-host transmission. Simply stated, the "trade-off" model (sometimes called the optimal virulence hypothesis) recognizes a necessary balance between virulence and transmission (Anderson & May 1982, Ewald 2004). This hypothesis implies that natural selection can maintain virulence over time. The optimal level of virulence will depend on the ecology of the host-parasite interaction and, more specifically, factors such as host density, host and parasite life spans and mode of transmission among others (Levin 1996). This model involves explicit assumptions: host mortality (virulence) is directly related to parasite reproduction and also, higher concentrations of the parasite increase the probability of transmission (Bull 1994, Ebert & Bull 2003).

A commonly cited example is the myxoma virus, which infects the European rabbit (*Oryctolagus cuniculus*) (Fenner & Ratcliffe 1965). A highly virulent parasite, the myxoma virus initially caused high mortality rates (~99%) in rabbit populations in Australia. In subsequent experimental infections in the laboratory, the recovered virus (from wild, surviving rabbits) had attenuated and did not cause comparable levels of mortality (down to ~70%). The results of this study were taken as evidence in support of an extricable link between transmission and virulence (Fenner 1959, Anderson & May 1982) and offered the promising prospective of virulence management (Ebert & Sokolova 2001, Ebert & Bull 2003). For example, Ewald (1994) discusses the importance of managing transmission rates for the virulence of human immunodeficiency virus (HIV) and human T-cell lymphotropic viruses (HTLVs) (i.e., preventative measures towards reducing sexual transmission should not only reduce prevalence, but also have an

evolutionary effect on viral virulence). However, the interpretations of the myxoma virus studies have been challenged and similar tests of the trade-off model in other host-pathogen systems have been less convincing (Ebert & Bull 2003). The resulting controversy has led to a characterisation of the model as "too simplistic" (Ebert & Bull 2003), and perhaps only applicable in single host-pathogen systems where the parasite cannot exist in the environment. Nevertheless, the trade-off hypothesis remains the fundamental basis for the study of virulence evolution (Day 2003).

Current views of virulence

Perhaps the principal shortcoming in previous models of virulence was addressing how natural selection operates for parasites. Previously, studies focused on host mortality rather than examining traits more directly related to parasite fitness (Bull 1994, Ebert & Bull 2003). Natural selection should optimise fitness, the measure of an organism's success at passing genetic information on to future generations (Stearns 1976, Roff 1992), for both the host and the parasite as independent organisms (Ewald 1994). For example, a host must balance the energetic cost of mounting an immune response against the risk of infection (optimal immune defence), as demonstrated in studies of foraging energetics and immune responses to parasitic infections in eider ducks (Hanssen et al. 2004, Houston et al. 2007). However, until recently factors influencing parasite evolution were overlooked, misunderstood or difficult to study (Bull 1994, Ebert & Bull 2003, Ewald 1994).

Parasite evolution should function under the same principles of natural selection governing the evolution of any organism (Bull 1994, Nowak & May 1994, Ebert & Bull 2003, Ewald 2004). These include the possibility of competition between parasite genotypes within a single host (Bull 1994, Ebert & Bull 2003). A basic assumption is that parasite fitness should be maximised by striking an optimal balance between the parasite's own within-host growth and reproduction (fecundity) and between-host transmission (persistence) (Day 2003, Dybdahl & Storfer 2003, Galvani 2003). The

distinction from previous hypotheses is the recognition of the importance of within-host evolutionary dynamics (Levine et al. 1970, Bull 1994, Ebert 1998). For example, in an oversimplified system such as direct transmission within a single host species, theoretical models predict a wide variety of possible virulence outcomes based on parasite population dynamics (Bull 1994). Competition among parasite strains within a single host should select for higher parasite reproductive rates, but this may or may not be directly linked to virulence when explicitly defined as host morbidity or mortality (Bull 1994) depending on inherent characteristics of the parasite (e.g., mutations rates of microparasites such as RNA viruses are very different from those of macroparasites such as parasitic worms).

The evolutionary dynamics of parasites and their adaptations within hosts have been investigated using serial passage experiments in which parasites are propagated under defined conditions (Ebert 1998). Serial passage experiments (SPEs) have been used for *in vitro* and *in vivo* investigations of a wide variety of parasites including viruses (Sabin & Schlesinger 1945, Zuckerman et al. 1994), bacteria (Cushion & Walzer 1984, Maisnier-Patin et al. 2002, Somerville et al. 2002), protozoa (Lecomte et al. 1992), fungi (Levine et al. 1970), and others (Ebert 1998). They have also been applied for vaccine development (e.g., yellow fever and polio which used attenuated strains of the parasite (Kew et al. 1981)). The advantage of SPEs is that alterations in parasite genotype, phenotype and often virulence can be tracked in real time (Ebert 1998). During *in vitro* experiments parasites are propagated by transfer to a new host or to a new artificial environment (such as culture medium) at a specific point in time or life phase (Ebert 1998, Ford et al. 2002). It is commonly observed that parasites maintained in culture become “attenuated” (i.e., become less virulent in hosts). It is usually thought that this occurs because as they become adapted to growth in culture, their ability to grow within hosts decreases (Ebert 1998, Ford et al. 2002). Evidence suggests that the degree of attenuation in culture is influenced by the timing of passage in the parasite’s life history and maintenance practices (da Silva & Sacks 1987, Wozencraft & Blackwell 1987, Rey et al. 1990) and attenuated parasite strains can rapidly revert to higher virulent forms when re-exposed to naïve hosts (Cann et al. 1984, Macadam et al. 1989, Minor 1993,

Nielsen et al. 2001). This makes it clear that virulence can be altered by within-host evolutionary dynamics. In nature, it must also be affected by between-host evolutionary factors including (but not limited to) heterogeneous host populations (Regoes et al. 2000, Dobson 2004, Keesing et al. 2006), spatially (geographically) confined host adaptation (Boots 1999, Dybdahl & Storfer 2003, Galvani 2003) and various modes of parasite transmission (Day 2001).

In summary, virulence is an extremely complex product of the host, the pathogen and the environment that we are just beginning to understand. Although concepts regarding virulence have advanced significantly in recent decades, a comprehensive understanding of host-pathogen dynamics and "virulence management" remains a challenge for future research. It is now commonly accepted that virulence is a dynamic product of the interactions among hosts, pathogens and their environments and that evolution does not necessarily lead to a benign association between host and parasite. Implicit in all developing views of virulence is an appreciation for the complexities of host-parasite dynamics. It is clear that the level of virulence, from benign commensalism to high lethality, will greatly depend on the biology of the host and the parasite and on their shared environment. Current advances in the fields of evolutionary epidemiology, disease ecology, molecular biology and microbiology should enhance our understanding of virulence and disease.

Part II. Amphibian Chytridiomycosis

Worldwide amphibian declines

At the World Congress of Herpetology in 1989, herpetologists discussed unusual and alarming patterns of disappearances of amphibian populations in protected areas (Barinaga 1990, Wake & Morowitz 1991, Collins & Storfer 2003). Due to a lack of high-quality, long-term census data, it was difficult to determine if anecdotal observations reflected normal amphibian population fluctuations or true declines (Pechmann & Wilbur 1994). However, evidence of genuine declines continued to mount

and Conservation International's Center for Applied Biodiversity Science in completing a global synthesis of the declines concluded that amphibians are far more threatened than birds or mammals (Stuart et al. 2004). In their report Stuart et al. (2004) noted that many amphibian species were suffering declines due to typical causes such as habitat destruction and overexploitation (such as harvesting for human consumption). However, the report also acknowledged that many dramatic population declines were taking place in pristine locations where no obvious cause was apparent (La Marca & Reinthaler 1991, Tyler 1991, Crump et al. 1992, Carey 1993, Fellers & Drost 1993, Ingram & McDonald 1993, Richards et al. 1993, Vial et al. 1993, Blaustein et al. 1994, Pounds & Crump 1994, Stebbins & Cohen 1995, Drost & Fellers 1996, Laurance et al. 1996, Lips 1998, 1999, Alford et al. 2001, Young et al. 2001, La Marca et al. 2005)

The various hypotheses on the causes of amphibian declines were collated and included: overexploitation, land use change, UV radiation, contaminants, global climate change, and emerging infectious diseases (Alford & Richards 1999, Collins & Storfer 2003). Although it was clear that many amphibian declines are related to factors such as habitat loss, it was suggested that many dramatic declines and disappearances in apparently undisturbed habitats were due to disease (Carey 1993, Laurance et al. 1996, 1997, Berger et al. 1998, Berger et al. 1999a, Daszak et al. 1999, Daszak et al. 2003). However, there was no general consensus on the most plausible cause of frog declines in protected areas and these losses were termed "enigmatic" by Stuart et al. (2004).

Amphibian declines and disease

In Australia, where amphibian population disappearances were reported for multiple species in the 1990s (Tyler 1991, Ingram & McDonald 1993, Richards et al. 1993) researchers proposed that an introduced pathogen may be responsible for the declines (Laurance et al. 1996). This suggestion was met with scepticism mostly because the available data were considered insufficient to support the hypothesis (Alford & Richards 1997, Hero & Gillespie 1997, McCallum 2005) and also because traditionally disease

was not viewed as capable of causing permanent declines or extinction events (Scott 1988, Anderson & May 1991, Hudson et al. 2002).

Proving causation in wild amphibian population declines was a challenge (Carey & Bryant 1995, Carey et al. 2001, Daszak et al. 2003). Declines that were detected occurred rapidly (Laurance et al. 1996, Lips 1998, 1999, Briggs et al. 2005, Lips et al. 2006, Woodhams et al. 2008b) and often there was little available information on populations prior to declines (Alford & Richards 1997, Lips et al. 2008). In order to definitively link diseases with amphibian declines, Daszak et al. (2003) identified four critical requirements for demonstrating the causal relationship. First, Koch's postulates needed to be fulfilled for the putative pathogenic agent. Koch's postulates, named after Dr. Robert Koch, are multi-step assessments that involve isolating and purifying a pathogen from a diseased animal, exposing otherwise healthy hosts, reproducing the disease and re-isolating the same pathogen (Koch 1891). Koch's postulates, formulated in the 19th century, cannot be applied in all host-pathogen systems (Fredricks & Relman 1996) and have since been refined and updated (Evans 1976) but they remain the basis for principles in identifying causative agents of disease. Second, the pathogenic agent needed to be associated with mortality events that led to an overall decline in the population. Third, an explanation for the cause of death, or the mechanism of pathogenesis, needed to be provided and supported by pathological evidence. Fourth, the evidence that the mortalities caused population declines needed to be unequivocal. Daszak et al. (2003) proposed that these criteria should be met in order to sufficiently demonstrate a link between disease and amphibian declines.

Batrachochytrium dendrobatidis and chytridiomycosis

In 1990's, researchers identified an organism associated with wild frog mass-mortality events in Australia and Central America (Berger et al. 1998) and in captive frogs in North America (Pessier et al. 1999, Longcore et al. 1999). A fungus, *Batrachochytrium dendrobatidis (Bd)*, was identified in histological sections taken from the skin of dead

and dying frogs (Berger et al. 1998, Berger 2001), satisfying the second criterion of Daszak et al. (2003). The taxonomy of the organism was initially unknown and required the creation of a monotypic genus in Chytridiomycota, a phylum of fungi not previously known as pathogens of vertebrates (Longcore et al. 1999). This microbe appeared to cause disease in otherwise healthy amphibians when exposed to infected pieces of shed skin and the biology of the aquatic pathogen seemed to explain the patterns of declines (Berger et al. 1998, Berger 2001). Furthermore, once isolated from the skin of an infected frog, *Bd* was pathogenic and lethal to amphibians in subsequent exposures (Longcore et al. 1999, Nichols et al. 2001), demonstrating the ability of the organism to cause disease, fulfilling Koch's postulates and the first criterion proposed by Daszak et al. (2003). Following this discovery, reports associating *Bd* and amphibian declines came from nearly every continent where amphibians occur (Berger et al. 1998, Muths et al. 2002, Briggs & Burgin 2003, Briggs et al. 2005, Weldon 2005, Bosch & Martínez-Solano 2006, Lips et al. 2006, Kusrini et al. 2008). Retrospective studies of museum specimens showed *Bd* was present during past amphibian die-offs (Aplin & Kirkpatrick 2000, Ouellet et al. 2005, Puschendorf et al. 2006b). Although the patterns of *Bd* emergence were not always clear (Ouellet et al. 2005, Puschendorf et al. 2006b) and infections have been detected with limited or no evidence of amphibian die-offs (Beard & O'Neill 2005, Longcore et al. 2007, Frías-Alvarez et al. 2008), additional proof to meet the second and forth criteria came from more recent, well-documented declines (Briggs et al. 2005, Lips et al. 2006, Woodhams et al. 2008b).

Amphibian declines due to *Bd* continue to be reported (Muths et al. 2003, Woodhams et al. 2008b, Kusrini et al. 2008). Today *Bd* is widely recognised for its to ability to spread rapidly though amphibian populations, infect numerous, phylogenetically distant species, cause high mortality and persist even at low host densities (Berger et al. 1998, Retallick et al. 2004, Woodhams & Alford 2005, Lips et al. 2006, Skerratt et al. 2007). These disease characteristics render population recovery from chytridiomycosis especially difficult and present strong empirical evidence for disease-induced extinctions (Schloegel et al. 2006, Mitchell et al. 2008). However, the third criterion of Daszak et al. (2003) is still unfulfilled. A fundamental question remains unanswered: how does *Bd* kill

amphibians? Resolving the mechanism of pathogenesis is an imperative for explaining how a superficial skin fungus could be responsible for the loss of amphibian biodiversity worldwide.

Hypotheses regarding pathogenesis

Even with a growing body of literature on chytridiomycosis, the mechanism of mortality remains an important unanswered question (Gascon et al. 2007, Mitchell et al. 2008, Rosenblum et al. 2008, Wake & Vredenburg 2008). This gap in our understanding of chytridiomycosis exists for several reasons. First, cutaneous fungal infections are not commonly fatal to terrestrial vertebrates unless acting opportunistically with other predisposing factors, so there were no similar systems to which chytridiomycosis could be compared. Second, the location of the fungus in the most superficial layers of skin (Berger et al. 1998, Berger et al. 1999b, Berger 2001, Berger et al. 2005c, Puschendorf & Bolaños 2006, North & Alford 2008) and the minimal host reaction to infection (Berger et al. 2005c, Woodhams et al. 2007a) are unusual for fatal infections in vertebrates. Third, because there are no consistent pathological changes in internal organs of diseased amphibians detectable at the level of light microscopy (Berger et al. 1998, Berger 2001), traditional methods used to understand pathogenesis have proved ineffective.

Two hypotheses for the cause of mortality in amphibians infected with *Bd* have been suggested. The first is that *Bd* disrupts osmoregulation in the skin of infected amphibians (Berger et al. 1998, Berger 2001). This hypothesis was proposed because 1) the skin of amphibians is a physiological organ that tightly regulates the exchange of respiratory gases, water and electrolytes (Jorgensen et al. 1954, Alvarado & Kirschner 1963, Deyrup 1964, Moore & Lofts 1964, Mullen & Alvarado 1976, Fischbarg & Whittembury 1977, Shoemaker 1977, Larsen et al. 1987, Boutilier et al. 1992, Erspamer et al. 1994, Jorgensen 1994a, b, 1997, Wright et al. 2001, Word & Hillman 2005) and 2) *Bd* is predominantly found in the ventral integument which is particularly important to physiological processes (Berger et al. 1998, Berger et al. 1999b, Berger 2001, Berger et

al. 2005c, Puschendorf & Bolaños 2006a, North & Alford 2008). The second hypothesis concerning pathogenesis is that *Bd* produces a toxin that affects organs (Berger et al. 1998, Blaustein et al. 2005). The two hypotheses are not necessarily mutually exclusive and to date the available evidence is insufficient to determine if either or both are correct.

Host characteristics- amphibian epidermis

Anatomy of amphibian epidermis— The epidermis, which is superficial to the dermis, consists of multiple (5-7) layers of epithelial cells and specialized cell types (Farquhar & Palade 1965, Erspamer et al. 1994). Epidermal layers include (from deep to superficial): *stratum germinitivum, stratum spinosum, stratum granulosum,* and *stratum corneum*. The *stratum germinativum* is the basal layer and is composed of cuboidal or columnar cells (Farquhar & Palade 1965). Cellular division at this layer generates new cells that migrate superficially (Erspamer et al. 1994) and differentiate into various cell types. The cells of the *stratum corneum* are differentiated as keratinocytes, meaning they contain the cytoskeletal protein keratin (Farquhar & Palade 1965, Erspamer et al. 1994), and specialized cells including horny beak cells, goblet cells, mitochondria-rich cells, flask cells, Merkel cells, Leydig cells, Langerhans cells, cement gland cells, melanocytes and others (Erspamer et al. 1994). The dermis contains multiple types of glands (Lillywhite 1971, Blaylock et al. 1976, Erspamer et al. 1994, Clarke 1997, Bowie et al. 1999, Lenzi-Mattos et al. 2005), which are reviewed in Chapter four.

Physiology of amphibian epidermis— Amphibian skin is unique among terrestrial vertebrates because it is highly physiologically active; the skin is permeable to water and a site of regulated transport for ions (electrolytes) and respiratory gases (Jorgensen et al. 1954, Alvarado & Kirschner 1963, Deyrup 1964, Moore & Lofts 1964, Fischbarg & Whittembury 1977, Shoemaker 1977, Larsen et al. 1987, Boutilier et al. 1992, Erspamer et al. 1994, Jorgensen 1994a, b, 1997, Wright et al. 2001, Word & Hillman 2005). In order to maintain osmotic balance, amphibians must maintain a hyperosmotic internal environment relative to the hypoosmotic external environment. This is accomplished by

active regulation of transport of multiple electrolytes (including sodium, magnesium, potassium, chloride) across the surface of the skin (Deyrup 1964, Moore & Lofts 1964, Boutilier et al. 1992, Erspamer et al. 1994, Jorgensen 1997, Wright et al. 2001). A steady inward flux of sodium must be maintained despite its movement against an electrochemical gradient. This is accomplished via a cyclic AMP regulated pathway, the sodium potassium pump, which exchanges potassium ions for sodium ions, thereby regulating intracellular and extracellular concentrations. Water flow results when an osmotic gradient is established by electrical currents, induced by an exchange of these solutes (Larsen 1970, Kirschner 1983, Jorgensen 1997).

The selective barrier properties of frog skin are primarily determined by electrolyte transport in the flask-shaped mitochondrial-rich cells (MR cells) of *stratum granulosum* (Masoni & Garcia-Romeu 1979, Brown et al. 1981, Larsen et al. 1987, Larsen et al. 1996, Ehrenfeld 1997). However, all epidermal cells "work" together. As a multi-layered composition of many cell types, amphibian epidermal cells probably act as "functional syncytium" to maintain required concentrations of electrolytes and water balance (Larsen 1991). The permeability of frog skin varies over the body surface of an individual and also among species (Moore & Lofts 1964, Erspamer et al. 1994). In some species, for example in bufonids, osmotic permeability is greatest in an area of ventral integument commonly referred to as the pelvic patch (Czopek 1965, Baldwin 1974, Word & Hillman 2005), where there is dense cutaneous vasculature (Czopek 1965). Despite some variation in permeability, amphibian skin, across all species, is a central organ in maintaining water and electrolyte equilibrium.

Healthy amphibians regularly moult by shedding their outer keratinised layer, as a replacement *stratum corneum* forms (Ewer 1951, Jorgensen & Larsen 1961, Budtz & Larsen 1973, 1975, Larsen 1976, Budtz 1977, Masoni & Garcia-Romeu 1979, Budtz 1985b, a, 1988, Jorgensen 1988). The process is dependent on physiology and behaviour (Bouwer et al. 1953, Castanho & De Luca 2001). The frequency of moulting varies by species and can vary from a few days to several weeks depending on many factors including temperature, age, size and sex (Larsen 1976). Alterations in water permeability

and electrolyte transport occur during a normal moulting event (Jorgensen 1949, Larsen 1970). A temporary increase in sodium permeability as well as an increase in sodium excretion lead to a net sodium loss (Jorgensen 1949).

The unique properties of amphibian skin have made it a model for studying epithelial transport (Ussing & Zerahn 1951, Koefoed-Johnsen et al. 1952, Kirschner 1983), which is now the foundation for a wide range of medical research (Larsen 2002). However, the importance of these cutaneous functions in maintaining homeostasis makes amphibians especially vulnerable to the effects of epidermal infections (Wright et al. 2001).

Epidermal pathology of chytridiomycosis— In infected amphibians *Bd* sporangia are found in cells of the *statum corneum* and the *stratum granulosum* of the epidermis (Berger et al. 1998, Berger et al. 1999b, Pessier et al. 1999, Berger 2001, Berger et al. 2005a, Berger et al. 2005c). Primary pathological abnormalities include cell hyperplasia and hyperkeratosis or "thickening" of the *stratum corneum* (Berger et al. 1998, Berger et al. 1999b, Pessier et al. 1999, Berger 2001, Berger et al. 2005a, Berger et al. 2005c). Other pathological changes in the epidermis include cytoplasmic degeneration and vacuolation in scattered cells in the *stratum granulosum* (Berger 2001, Berger et al 2005a). These cytopathic changes are not severe in most cases, but can result in sloughing of the *stratum corneum* and erosions (Berger 2001). Almost all subsequent reports of amphibian mortality in association with *Bd* have consistently described the same epidermal clinical signs of infection, especially irregular skin sloughing, epidermal hyperplasia and hyperkeratosis (Nichols et al. 2001, Mazzoni et al. 2003, Daszak et al. 2004, Carey et al. 2006, Puschendorf & Bolaños 2006, Kriger et al. 2007).

The significance of irregular skin sloughing is unclear. In one study, salamanders (*Ambystoma tigrinum*) sustained *Bd* infections more than 60 days after experimental exposure, seemingly without adverse affects, but with a notable increase in the sloughing of skin (Davidson et al. 2003). Because *Bd* was found in sloughed skin, it was hypothesised that the increase in sloughing might assist amphibians in shedding the infection (Davidson et al. 2003). In support of this hypothesis Berger et al. (2004)

suggested that increased moult frequency may be a reason frogs can clear infection at higher temperatures (Berger et al. 2004). One study monitored moult patterns of toads (*Bufo boreas*) by noting the disappearance of a lipstick mark on the dorsal integument (Bendsen 1956) and quantifying skin shedding before and after *Bd* infection (Voyles et al. 2005). Toads were randomly assigned to exposure and control groups, inoculated by immersion in *Bd* and control solutions, and molt patterns were tracked over the course of infection. Control toads typically had a 2-6 day intermoult cycle, producing 1-2 large pieces of sloughed skin. In contrast, infected frogs that developed severe clinical signs of disease did not have a full body moult, but continually shed more than 100 small, tattered pieces of skin. It was suggested that the change in moult pattern is a maladaptive response to *Bd* infection in this species (Voyles et al. 2005).

Parasite Characteristics- *Batrachochytrium dendrobatidis*

Morphology, growth and development— Species in the phylum Chytridiomycota are identified by ultrastructural morphology of the zoospore, especially the flagellar apparatus (Mueller et al. 2004, James 2007). *Batrachochytrium dendrobatidis* was originally isolated from, and named for, a blue poison dart frog (*Dendrobates azureus*) (Longcore et al. 1999). Multiple *Bd* isolates from various amphibian species have been isolated into pure culture (personal communication, Joyce Longcore). Once in culture, *Bd* develops through multiple life stages. The earliest life stage is the infectious, motile zoospore, utilising a posterior flagellum (Berger et al. 1998, Longcore et al. 1999, Berger 2001, Berger et al. 2005a). The zoospore encysts, absorbs the flagellum and develops rhizoids (Berger et al. 2005a). The maturing thallus develops a zoosporangium (i.e. container for zoospores) in which the contents cleave and develop into flagellated zoospores. In this mature life stage, a discharge tube forms and the zoospores are released into the external environment to continue the life cycle (Berger et al. 1998, Longcore et al. 1999, Berger 2001, Berger et al. 2005a).

To date our understanding of *Bd* nutrient utilization is incomplete (Longcore et al. 1999, James 2007, Piotrowski et al. 2004, Berger et al. 2005a, Rosenblum et al. 2008, Symonds et al. 2008). This is a critical question for two reasons. First, it is unclear whether *Bd* can persist in the environment as a saprobe, utilizing non-amphibian organic materials, which has important implications for the evolution of virulence and the biology of chytridiomycosis (James 2007, Mitchell et al. 2008). Second, determining what amphibian nutrient sources are utilised by *Bd* may explain a key step in pathogenesis (Rosenblum et al. 2008, Symonds et al. 2008).

It is a misconception that *Bd* only uses keratin as a nutrient source (Altig 2007). Although *Bd* is found in keratinising epidermal cells and tadpole mouthparts (Berger et al. 1998, Longcore et al. 1999, Berger 2001, Fellers et al. 2001, Rachowicz & Vredenburg 2004, Berger et al. 2005a, Blaustein et al. 2005, Woodhams & Alford 2005, Knapp & Morgan 2006, Smith et al. 2007), *Bd* is also found in cells containing prekeratin (Berger et al. 2005a) and it does not grow in pure keratin cultures (Piotrowski 2004, Rosenblum et al. 2008). *In vitro Bd* grows on various agar preparations. Mixtures of various concentrations have included peptonized milk, typtone, gelatin hydrolysate, lactose, glucose, asparagine, yeast extract, malt extract, peptone, sucrose, maltose, sorbitol, glycerol, sterilized snakeskin, ground feather meal, sloughed amphibian skin (Longcore et al. 1999, Piotrowski et al. 2004, Symonds et al. 2008) and aquatic insects (Cashins et al. in prep). Growth and development rates in culture can vary among nutrient conditions. Even within cultures with standardised nutrients, growth rates can be inconsistent (Symonds et al. 2008, Cashins et al. in prep). Currently *Bd* isolates are most commonly cultured on tryptone/gelatin hydrolysate/lactose (TGhL) media (Longcore et al. 1999). Serial culturing influences growth and development (and infectivity and virulence) of many pathogens and some researchers have expressed concern regarding selective pressures on *Bd* in culture (Retallick & Miera 2007, Symonds et al. 2008).

Rates of growth and development of *Bd* are also influenced by temperature. The optimal temperature range appears to be approximately 17-23ºC, though *Bd* can grow at lower temperatures (Berger 2001, Piotrowski et al. 2004, Woodhams et al. 2008a). Cultures of

Bd incubated at 30ºC and higher died (Piotrowski 2004). Woodhams et al. (2008a) observed and modeled how reproductive life-history traits were adjusted depending on temperature. At lower temperatures (7-10ºC) motile *Bd* zoospores took longer to encyst, mature and produce propagules than *Bd* zoospores maintained in warmer (17-23ºC) temperatures. It was concluded that this mechanism enables *Bd* to maintain a relatively high long-term growth rate across a range of temperatures (Woodhams et al. 2008a). These results may help to explain how pathogenicity is maintained at lower temperatures (Carey et al. 2006) and the population level effects of *Bd* in low temperature environments (Woodhams et al. 2003, Berger et al. 2004, Woodhams & Alford 2005, Pounds et al. 2006, Kriger & Hero 2007b, a, 2008, Muths et al. 2008, Skerratt et al. 2008).

Batrachochytrium dendrobatidis *and disease*— The reproductive life cycle of *Bd* is presumed to be the same in frog skin where the thalli live and mature within the *stratum granulosum* and *stratum corneum* (Longcore et al. 1999, Pessier et al. 1999, Berger 2001, Berger et al. 2005a). Zoospores colonise cells deeper in the epidermis and develop endogenously (i.e. within the epidermal cells) (Longcore et al. 1999, Berger et al. 2005a). Although it is unclear how cell entry is achieved, Longcore et al. (1999) hypothesised that a zoospore might encyst on the exterior surface of an epidermal cell and insert *Bd* nucleic material using a germ tube. Following cell entry, *Bd* sporangia develop within the epidermal cells, completely filling the cells, and move from deep to more superficial, coinciding with the normal directional movement of epidermal cells. Berger et al. (2005a) observed that the rate of development of *Bd* was timed such that the final stages of development, formation of discharge tube, occurred when infected epidermal cells were most superficial. Also, discharge tubes were oriented toward the external environment, suggesting that *Bd* is well adapted to frog skin (Berger et al. 2005a).

Some evidence suggests that *Bd* enzymatic activity directly influences pathogenesis. The initial penetration of *Bd* into amphibian epidermal cells likely requires digestive enzymes and dissolution of cellular cytoplasm has been observed (Berger et al. 2005a). In culture *Bd* secretes extracellular proteases that degrade casein and gelatin (Piotrowski et al.

2004) and proteolytic enzyme activity has been detected for multiple *Bd* isolates (Piotrowski et al. 2004, Symonds et al. 2008, Fisher et al. 2009). At the molecular level, genomic research into *Bd* is revealing differential expression patterns in genes such as serine protease and fungalysin metallopeptidase, two enzymes involved in pathogenesis in multiple fungal pathogens (Rosenblum et al. 2008). These enzymes may have toxic effects on amphibians. In a test of susceptibility of multiple amphibian species at the larval stage, Blaustein et al. (2005) observed rapid mortality and suggested the possibility that *Bd* produced a "toxic substance" that killed the tadpoles.

In addition to enzymatic activity, the reproductive biology of *Bd* is likely to be an important determinant of pathogenicity. When maintained in *in vitro* high-nutrient conditions, exponential growth and a peak in zoospore production is followed by a decrease in zoospore production and activity, presumably due to exhaustion of nutrient resources (Woodhams et al. 2008a) or possibly a buildup of inhibitory metabolites. In frog skin, however, growth limitations due to nutrient exhaustion are unlikely (Woodhams et al. 2008a), and local re-infection on an individual host (Berger et al. 2005a) probably leads to an exponential increase in *Bd* load and thus, development of severe infections and mortality (Carey et al. 2006). In infection experiments on susceptible frogs, clinical signs of disease develop in amphibians with the highest numbers of zoospore equivalents, suggesting that reproduction and zoospore production and/or intensity of infection are important in pathogenesis.

The influence of temperature on *Bd* growth and development is probably one of the most important determinants of chytridiomycosis outbreaks in wild amphibian populations (Bradley et al. 2002, Woodhams et al. 2003, Berger et al. 2004, Retallick et al. 2004, Woodhams & Alford 2005, Carey et al. 2006, Drew et al. 2006, Kriger & Hero 2006, Pounds et al. 2006, Rachowicz et al. 2006, Kriger & Hero 2007a, b, Rowley & Alford 2007, Kriger & Hero 2008, Muths et al. 2008, Skerratt et al. 2008). Severe amphibian declines attributed to chytridiomycosis occur in upland sites in tropical areas where temperatures are generally cool (Berger et al. 2004, Lips et al. 2006, Skerratt et al. 2007, Brem & Lips 2008, Skerratt et al. 2008) and low temperature is significantly related to

high prevalence (Woodhams & Alford 2005, Drew et al. 2006, Kriger & Hero 2007a, b, Muths et al. 2008). Furthermore, niche modelling of *Bd* distributions predicts that *Bd* should occur where temperatures are <27ºC (Ron 2005, Skerratt et al. 2008, Puschendorf et al. in press). Recognition of the importance of temperature led to the hypothesis that human-induced global climate change could be driving outbreaks of chytridiomycosis if changing climatic conditions create a thermal optimum for *Bd* (Pounds et al. 2006). This hypothesis is controversial (Alexander & Eischeid 2001, Alford et al. 2007, Bosch et al. 2007, Di Rosa et al. 2007, Fisher 2007, Skerratt et al. 2007, Laurance 2008, Lips et al. 2008) and largely untested, but it highlights the need to better understand the role of temperature in the impact of chytridiomycosis and the potential influence of changing climatic conditions.

To date genetic work has shown that little variation exists among isolates worldwide and suggests that *Bd* is a pandemic clone (Morehouse et al. 2003, Morgan et al. 2007). Experimental evidence suggests that *Bd* virulence may differ among isolates (Berger et al. 2005b, Retallick & Miera 2007, Fisher et al. 2009), but the underlying reasons for the variation are unknown. Reduced mortality has been observed in amphibian populations after surviving the initial emergence of chytridiomycosis (Morgan et al. 2007), and different populations, even within species, can exhibit different outcomes to infection (Briggs et al. 2005), suggesting the possibility of evolving *Bd* virulence. Differential virulence among isolates is a topic that remains to be explored and perhaps more importantly, offers promise of insight into putative co-evolution between *Bd* and amphibians (Retallick et al. 2004).

Conclusions and direction

Despite a decade of research and increased attention on chytridiomycosis, critical gaps remain in our understanding of the disease (Skerratt et al. 2007, Gascon et al. 2007, Wake & Vredenburg 2008). This study investigated the virulence and pathogenicity of chytridiomycosis because these are some of the most important and least understood

aspects of the disease. First, even within a single host species, virulence of *Bd* isolates appears to vary (Berger et al. 2005b, Briggs et al. 2005, Morgan et al. 2007, Retallick & Miera 2007, Fisher et al. 2009). The cause of this variability has not been determined. To characterise differences among isolates this study examined growth and development *in vitro* for three *Bd* isolates and manipulated nutrient and temperature conditions for a single isolate over multiple generations (Chapters four and five). Finally, it is unknown why and how amphibians die from chytridiomycosis. In order to investigate the mechanisms of mortality, this study focused on the epidermal disruption that occurs with infection and monitored other physiological changes that coincide with severe disease and death (Chapters six and seven).

CHAPTER THREE

General materials and methods

Background information

The common green tree frog (*Litoria caerulea*) was selected as the best species for infection experiments for several reasons. First, *L. caerulea* is not currently threatened and is listed as a "species of least concern" according to the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species (IUCN 2008). Second, *L. caerulea* is found in the city of Townsville and the immediate surrounding residential areas in large numbers. We confirmed that the frogs were negative for *Bd* prior to experiments using PCR analysis (described below), but we also made the assumption these local populations have been *Bd*-free in the past. There are no records of infections or reports of mass-mortality events in the Townsville area and climatic models predict that the area is too hot and dry to support *Bd* (Retallick 2002). However, It was necessary to use wild caught animals because there was no reliable source of captive bred adult *L. caerulea* in Australia. Third, *L. caerulea* are known to be highly susceptible to *Bd* infection from previous exposure experiments with one study reporting mortality rates of 100% (Berger et al. 2005b). Fourth, *L. caerulea* typically have a body mass large enough to enable collection of a volume of blood sufficient for laboratory analyses via cardiac or venpuncture.

Permits— These methods were performed with James Cook University and University of Sydney Animal Ethics Committee approval (Permit numbers JCU- A1085, A593, US-K22/6-2007/3/4652). Animals were collected and moved interstate with approval from Queensland Parks and Wildlife Service (Scientific Purposes Permit numbers: WISP03866106, WISP04143907, Movement Permit number WIWM04381507) and New South Wales National Parks and Wildlife Service (Import License Number IE0705693).

Figure 3.1 A common green tree frog (*Litoria caerulea*) found in Townsville, Australia.

Frog collection

Adult common green tree frogs (*L. caerulea*; *n* = 66, mean mass: 62.46 gms ± 2.4 s.e.m) were collected in January and February 2007 and 2008 from residential areas of Townsville, Queensland. Each frog was collected by hand using a new plastic bag and then transferred to an individual plastic container (200x240x330 mm^3). Frogs were held in temperature (18-23ºC) and light (12L/12D) controlled facilities at James Cook University, Townsville, Australia.

Animal husbandry

Frogs were fed vitamin-dusted crickets (medium-sized, Pisces Enterprises, Kenmore, Australia) *ad libitum* twice per week. Tap water (250 ml) was changed twice per week until experimental exposures began then tap water was replaced by 20% Holtfretter's solution (250 ml, (in mMol) 6 NaCl, 0.06 KCL, 0.09, CaCl2, 0.24, NaCO3, pH 6.5).

Containers were maintained in a level position so water covered the bottom but frogs were able to climb up the dry walls. The same procedures were followed during infection experiments except that the size of the containers varied by experiment.

Diagnosis of *Batrachochytrium dendrobatidis* infection

Quantitative polymerase chain reaction— To confirm frogs were not infected with *Bd* prior to inoculation, samples for quantitative polymerase chain reaction (qPCR) were obtained by rubbing a sterile cotton swab over the ventral surfaces and digits (Hyatt et al. 2007). The ventral surface was swabbed 10 times while the digits were swabbed 5 times on each hand and foot. Following exposure to Bd, frogs were swabbed every 10 d. Diagnosis of *Bd* infection status was performed by Taqman real-time PCR assay according to Boyle et al. (2004). All samples were analyzed in triplicate and compared with CSIRO-Australian Animal Health Laboratory (AAHL) zoospore standards to determine zoospore equivalents (Hyatt et al. 2007).

Histological techniques— Tissue samples collected following frog deaths were fixed in 10% buffered neutral formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin using routine histological techniques (Humason 1967, Berger et al. 2002, Olsen et al. 2004). Skin tissues were examined for the presence of *Bd* zoosporangia and for epidermal lesions such as spongiosis, irregular epidermal layers and hyperkeratosis.

Maintenance of *Batrachochytrium dendrobatidis* culture

Isolates GibboRiver-L.lesueuri-00-LB, TullyRiver-L.rheocola-07-LB and Rockhampton-L.caerulea-99-LB, were originally obtained from diseased *Litoria lesueuri*, *Litoria rheocola* and *Litoria caerulea* respectively. All isolates were purified and cultured on tryptone/gelatin hydrolysate/lactose (TGhL) agar with antibiotics (Longcore et al. 1999),

and then passaged into liquid TGhL broth (tryptone, gelatin hydrolysate, lactose) (Longcore et al. 1999) into 25-cm^2 cell culture flasks. Cultures were maintained at James Cook University in TGhL broth at 4°C and passaged every 2 to 3 months.

Inoculation

Frogs were randomly (using a random number generator) assigned to exposure and control groups. Exposure solutions consisted of *Bd* zoospores in a dilute salt solution and 20 % Holtfretter's solution (Wright et al. 2001). The zoospores were harvested either by washing agar plates of *Bd* culture (Berger 2001), or by filtering liquid cultures to remove sporangia using sterile filter paper (Whatman, 3). Zoospore concentrations were determined using a haemocytometer (Improved Neubauer Bright-line) and the concentration was adjusted as needed by addition of dilute salt solution (in mMol: KH_2PO_4 1, $CaCl_2.H_2O$ 0.2, $MgCl_2$.2H2O 0.1). Frogs were exposed to *Bd* zoospores in plastic containers via shallow immersion in a bath of the exposure solution. The inoculating dose of *Bd* zoospores varied among experiments. Uninfected control frogs were held in a bath of Holtfretter's solution with equal quantities of added dilute salt solution collected from agar plates or from sterile TGhL without *Bd*. After 24 h frogs were moved to fresh containers with 20% Holtfretter's solution (pH 6.5).

Disease assessment

Following inoculations frogs were monitored daily for changes in behaviour and clinical signs of chytridiomycosis. Behaviours were recorded daily including calling, physical activity and contact with water. During twice weekly feedings, number of crickets uneaten and type of skin sloughings were recorded for each animal. Clinical signs of disease included lethargy, inappetence, cutaneous erythema, irregular skin sloughing, abnormal posture (hind legs abducted or splayed out) and loss of righting reflex.

Righting reflex tests involved manually positioning frogs in dorsal recumbency and noting the time until they reoriented to a normal position.

A scoring system was developed to quantify clinical signs and to assess the severity of disease. Frogs with no clinical signs of infection scored a 0 and were designated as aclinical. Frogs scoring 1, 2, or 3 were designated as clinically diseased according to severity of clinical signs (1: lethargy and inappetence. 2: lethargy, inappetence, cutaneous erythema, and irregular skin sloughing. 3: lethargy, inappetence, cutaneous erythema, irregular skin sloughing, abnormal posture and loss of righting reflex).

Clinical Signs of Chytridiomycosis			
Aclinical	**Diseased**	**Diseased**	**Severely Diseased**
0	**1**	**2**	**3**
No clinical signs	**Lethargy**	**Lethargy**	**Lethargy**
	Inappetance	**Inappetance**	**Inappetance**
		Cutaneous erythema	**Cutaneous erythema**
		Irregular skin sloughing	**Irregular skin sloughing**
			Abnormal posture
			Loss of righting reflex

Table 3.1 Scoring for clinical signs of chytridiomycosis. Scores reflect the number and severity of clinical signs.

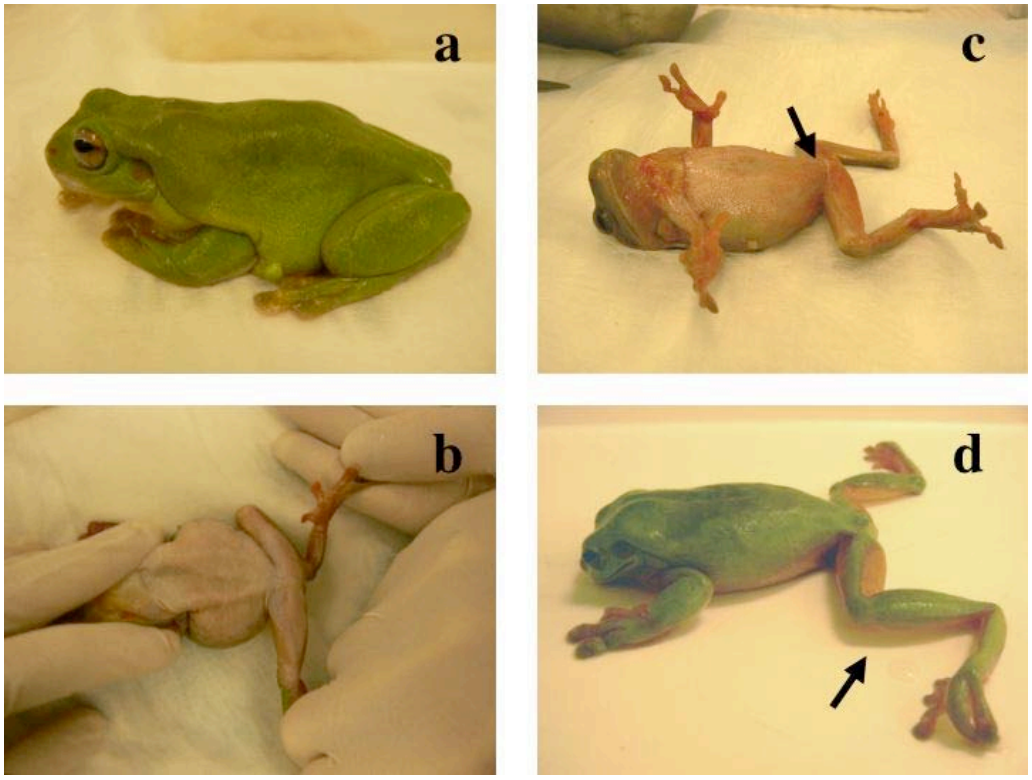

Figure 3.3 Healthy (a, b) and severely diseased (c, d) *Litoria caerulea* in infection experiments. The frog in images c and d show most of the clinical signs of chytridiomycosis including cutaneous erythema (arrow in image c) and abnormal posture with hind legs abducted (arrow in d), and loss of righting reflex (c).

Post mortem examinations

Full post mortem examinations were performed on dead frogs (Berger 2001). Frogs were handled with disposable gloves and examined for any obvious external lesions. Each frog was weighed and its snout-to-vent length was recorded. Frogs were then swabbed for detection of *Bd* with qPCR analysis. A skin and abdominal musculature incision was made along the ventral abdominal midline, from pelvis to the xiphoid (posterior end of the sternum). Samples were collected from each of the following organs: fat bodies, heart, intestines, kidney, liver, lung, ventral skin, dorsal skin and skeletal muscle. These samples were placed in 10% buffered neutral formalin for histopathology. One small (1-2 mm^2) skin biopsy was collected from the ventral

abdominal region (2-3 cm lateral to midline), placed in sterile 2 ml plastic screw top tubes and frozen in liquid nitrogen.

Additional methods

Chapters four and five contain methods for serials passage experiments that occurred in different thermal and nutrient conditions. Chapter seven contains methods for the following experiments:

- Plasma biochemistry (blood sample collections, preservation, analyses)
- Urine biochemistry (blood sample collections, preservation, analyses)
- Histology of skin biopsies
- Ussing chamber (electrolyte transport) tests
- Cardiac implant surgical procedures and electrical functioning
- Electrolyte supplementation
- Plasma biochemistry of free-living *Litoria caerulea*
- Plasma biochemistry in *Litoria caerulea* with other diseases or lethal conditions.

CHAPTER FOUR

Life history adjustments of an amphibian pathogen: Differential growth of *Batrachochytrium dendrobatidis*

Chapter overview

This chapter describes studies to investigate how an isolate of *Bd* responds to different environmental conditions and whether these responses persist when conditions are changed. The chapter is presented as a manuscript for publication, listing the authors involved, although it has not yet been submitted. The work presented in this chapter is my own with intellectual and technical input from other authors.

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Abstract

Life-history adjustments have been investigated in a wide variety of organisms but the application of life history theory to host-pathogen systems is relatively new. Evolutionary pressures act on both the host and the pathogen to optimize fitness. Pathogen life-history evolution is important because microbial characteristics, such as rates of maturation or reproduction, often play a central role in disease development. The fungal pathogen *Batrachochytrium dendrobatidis (Bd)* causes the lethal amphibian disease chytridiomycosis and although *Bd* virulence is not well understood, disease development appears to be closely linked to pathogen load. We investigated life-history adjustments of a single isolate of *Bd* by quantifying infectious zoospores in different temperature and nutrient conditions for multiple generations. Cultures maintained at 4ºC for 17 passages had an earlier release of zoospores and longer period of high zoospore densities than cultures maintained at 23ºC for the same number of passages, suggesting

an adaptive response to lower temperatures. Cultures passaged in 0.2% tryptone TGhL broth for 24 passages had earlier zoospore release and a faster life cycle turnover, but lower zoospore densities, than cultures serially passaged in 1.6% tryptone TGhL. This pattern was reversed when cultures maintained in 0.2% tryptone TGhL were passed back into 1.6% tryptone TGhL, demonstrating phenotypic plasticity in response to nutrient concentrations after 24 passages. These findings may help to explain why the impact of chytridiomycosis has been severe in wild amphibian populations in a wide range of environments. Investigating the response of *Bd* to biotic and abiotic factors, as well as the possible evolution of this lethal pathogen in response to selective pressures, may be critical for understanding virulence. A better understanding of these factors also provides the potential to predict future outbreaks and target management actions to minimise impacts.

Introduction

Evolution of life histories, and variation in life-history adjustments, contributes to the enormous complexity and diversity of the natural world (Stearns 1976, Roff 1992). A basic assumption of life-history theory is fitness (i.e., success at passing genetic information on to future generations) is maximised within a set of constraints by attaining an optimal balance of the costs and benefits of life-history trade-offs (Roff 1992). For example, reproduction comes with a cost for growth and survival. Trade-offs between fecundity and rate of maturation have been investigated in a wide range of plants and animals. Parasite evolution operates under the same principles of natural selection as it does in any other organism (Bull 1994, Nowak & May 1994, Ebert & Bull 2003, Ewald 2004). Parasite fitness is maximised by striking an optimal balance between reproduction and persistence (Day 2003, Dybdahl & Storfer 2003, Galvani 2003) and epidemiological models predict that parasite life-history strategies are selected to maximise lifetime transmission (Galvani 2003). Within this framework, life-history adjustments that balance within-host reproduction and between host transmission will likely influence the incidence of disease (Day 2003, Woodhams et al. 2008a), but a wide range of factors,

including environmental conditions such as temperature and nutrient availability, could alter the balance of the host-pathogen dynamic.

One host-pathogen system that has been used to investigate these theoretical principles is a fungal disease of amphibians called chytridiomycosis. The pathogen that causes chytridiomycosis, *Batrachochytrium dendrobatidis* (*Bd*), is lethal to many species of amphibians in laboratory experiments (Berger et al. 1998, 2005b, Longcore et al. 1999, Berger 2001, Nichols et al. 2001, Daszak et al. 2004, Woodhams et al. 2004, Carey et al. 2006, Lips et al. 2006). Outbreaks of chytridiomycosis have dramatically reduced the abundance of amphibian species in naïve populations (Berger et al. 1998, Lips 1998, 1999, Lips et al. 2006, Schloegel et al. 2006, Skerratt et al. 2007) but *Bd* is endemic in surviving amphibian populations (Woodhams & Alford 2003, Retallick et al. 2004, Kriger & Hero 2006, 2007a, b, 2008, Brem & Lips 2008). The presence of *Bd* in extant populations offers an opportunity to examine differential selection pressures on *Bd* in a variety of environments. Adaptations of either the host or the pathogen that affect the rate of reproduction of *Bd* may influence the virulence of *Bd* and probability of chytridiomycosis outbreaks.

In culture, *Bd* develops through multiple life stages (Longcore et al. 1999, Berger et al 2005a). The earliest life stage is the infectious motile zoospore, with a posteriorly located flagellum (Longcore et al. 1999). Zoospores encyst, absorb the flagellum and develop a thallus with rhizoids. The maturing thallus produces zoosporangium in which the contents cleave and develop into flagellated zoospores (Longcore et al. 1999, Berger et al 2005a). At maturity, the zoosporangium forms a discharge tube and the zoospores are released into the environment (or possibly within skin layers of a host) to continue the life cycle. When maintained in high-nutrient conditions *in vitro*, exponential growth and a peak in zoospore production is followed by a decrease in zoospore production and activity, presumably due to exhaustion of nutrient resources (Woodhams et al. 2008) or the build up of inhibitory metabolites. In frog skin, however, growth limitations due to nutrient exhaustion are unlikely (Woodhams et al. 2008a). Local re-infection on an individual host can lead to an exponential increase in *Bd* load and thus development of

severe infections (Carey et al. 2006). It was also suggested that infections above a species-specific threshold may lead to mortality (Carey et al. 2006). Build-up of infection could be slowed in some amphibians by shedding and cell turnover (Berger 2001). In infection experiments on susceptible frogs, clinical signs of disease develop in amphibians with the highest numbers of zoospore equivalents (Chapters six and seven), supporting the suggestion that rate of reproduction and zoospore densities are important in pathogenesis. In contrast resistant amphibian species manage to suppress their sporangial burden, and can remain infected but otherwise healthy (Daszak et al. 2004, Berger et al. 2009).

Temperature and *Batrachochytrium dendrobatidis*

Patterns of chytridiomycosis outbreaks and subsequent endemic prevalence in wild amphibian populations suggest that environmental conditions such as temperature (Bradley et al. 2002, Woodhams et al. 2003, Berger et al. 2004, Retallick et al. 2004, Woodhams & Alford 2005, Carey et al. 2006, Drew et al. 2006, Kriger & Hero 2006, Pounds et al. 2006, Rachowicz et al. 2006, Kriger & Hero 2007a, b, Rowley & Alford 2007, Kriger & Hero 2008, Muths et al. 2008, Skerratt et al. 2008), and humidity (Puschendorf et al. 2009) are important determinants of disease. Amphibian declines in tropical areas that are attributed to chytridiomycosis have mainly occurred in upland sites with lower temperatures (McDonald and Alford 1999, Lips et al. 2006, Skerratt et al. 2007) and prevalence and mortality rates are greater at these locations during cooler months (Berger et al. 2004, Drew et al. 2006, Kriger & Hero 2007a, b). Niche modelling predicts *Bd* occurrence where temperatures are < 27ºC (Ron 2005, Skerratt et al. 2008, Puschendorf et al. 2009). The link between disease and temperature is further supported by laboratory experiments. Maximal growth of an isolate of *Bd* in culture (Piotrowski et al 2004) occurred within a temperature range of approximately 17-25ºC and high mortality of experimentally inoculated frogs occurs at < 25ºC (Berger 2001, Woodhams et al. 2003, Berger et al. 2004, Carey et al 2006). Furthermore, the mounting evidence of the importance of temperature led to the hypothesis that human-induced global climate

change could be driving outbreaks of chytridiomycosis if changing climatic conditions create a thermal optimum for *Bd* (Pounds et al. 2006). This hypothesis is controversial (Alexander & Eischeid 2001, Alford et al. 2007, Bosch et al. 2007, Di Rosa et al. 2007, Fisher 2007, Skerratt et al. 2007, Laurance 2008, Lips et al. 2008), but it highlights the need to better understand the role of temperature in controlling the impact of chytridiomycosis.

Nutrient availability and *Batrachochytrium dendrobatidis*

In addition to temperature, the ability to respond to nutrient availability may be a critical factor in disease development (James 2007). Some evidence suggests *Bd* may persist independent of amphibian hosts either in an alternative life stage (Di Rosa et al. 2007) and/or by saprobic growth (Johnson & Speare 2003, Mitchell et al. 2008) using non-amphibian organic materials as nutrient resources. Saprobic growth may help to explain the devastating impact of the disease because models demonstrate that amphibian-independent persistence of *Bd* should amplify the impact on populations (Mitchell et al. 2008). There is limited evidence of *Bd* in the environment; it has not been isolated but DNA has been detected in water bodies (Kirshtein et al. 2007, Walker et al. 2007) and on rocks (Lips et al 2006). However, *Bd* can be cultured on various sterile natural substrates and on simple artificial media, and therefore does not appear to be an obligate intracellular parasite. In the laboratory *Bd* can alter its growth and reproduction in response to differences in media concentration (James 2007), on substrates such as snakeskin (Symonds et al. 2008, Cashins et al. in prep) and aquatic insects (Cashins et al. in prep). These changes have all been characterised during short-term experiments; no experiments to date have investigated long-term responses of *Bd* isolates to differing environmental conditions. Long-term experiments on *Bd* are important to determine phenotypically plastic and/or adaptive responses of *Bd* to environmental conditions. The presence of phenotypic plasticity in response to variation in environmental conditions might decrease the effects of natural selection, if the plasticity is such that a near-optimal response is produced in all environments encountered. However, it has also been

suggested that plasticity itself may have various limits and costs (DeWitt et al. 1998, Relyea 2002). To determine whether *Bd* shows responses to differences in environmental conditions in the long-term, and the nature of any response, we investigated whether *Bd* adapts to differences in temperature and nutrient conditions over multiple generations.

Methods

Experiment 1- Temperature

Culture of Batrachochytrium dendrobatidis— The isolate, GibboRiver-L.lesueuri-00-LB-1, was originally obtained from a diseased juvenile *Litoria lesueuri*, cultured on tryptone/gelatin hydrolysate/lactose (TGhL) agar with antibiotics (Longcore et al. 1999) and then cryoarchived (Boyle et al. 2003). An aliquot of the cryoarchived culture was revived (Boyle et al. 2003) and then passaged into liquid TGhL broth (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose, 1000 ml distilled water) (Longcore et al. 1999, Boyle et al. 2003). Two 25-cm^2 cell culture flasks containing "Low Temperature History" culture (LTH) were moved into 4°C and passaged every 2 to 3 months by transferring 2ml of actively growing culture into 8 ml of new TGhL liquid medium. After 38 months, a second aliquot of the same cryoarchived isolate, GibboRiver-L.lesueuri-00-LB-1, was revived and passaged into liquid TGhL broth using identical procedures. These "High Temperature History" cultures (HTH) were maintained at 23ºC and passaged every 4-6 days, when zoospore density was near maximum levels (determined by visual inspection). When LTH and HTH cultures were matched in passage history (17 passages), a series of plate experiments were conducted.

Preparation of inocula— LTH and HTH cultures were filtered to remove sporangia using a sterile filter paper (Whatman filters, Number 3). Cultures were centrifuged (500 g x 10 minutes), removing the supernatant and resuspending the zoospores in sterile dilute salt solution (in mMol: KH_2PO_4 (1), $CaCl_2.H_2O$ (0.2), $MgCl_2.2H2O$ (0.1)). Zoospore concentrations were determined using a haemocytometer (Improved Neubauer Bright-line) and adjusted as needed by addition of dilute salt solution to a concentration of 120 x

10^4 zoospores per ml. The LTH and HTH zoospore inocula (50 µL) were each pipetted into 25 wells of six sterile 96 well plates (see below). Two aliquots of the LTH and HTH zoospores were heat killed by holding in a 90ºC water bath for 20 minutes and then each were pipetted into 5 wells for a negative control. Six plates were organised with two sections (for LTH and HTH inocula) of wells each containing 50 µL TGhL medium, and a perimeter of 36 wells with 100 µL sterile water to avoid evaporation of the wells containing *Bd* inocula.

Reciprocal transplant experiment— Six identically organised plates were created as outlined above, each containing both LTH and HTH cultures. Three plates were incubated at 23ºC and three plates at 4ºC to allow examination of the responses of the cultures to two thermal conditions. Plates were inspected by light microscopy to monitor zoospore encystment and maturation of the zoosporangia. Once the maturing zoosporangia released the first zoospores, zoospore densities were quantified by randomly selecting 10 wells from the LTH and HTH sections of each plate, drawing off 30 µL of supernatant and counting zoospore numbers using a haemocytometer. This procedure was repeated daily (10 wells per day) for plates at 23ºC because they contained faster-growing cultures. The procedure was repeated every 7 d (10 wells per day) for slower-growing cultures held at 4ºC.

Experiment 2- Nutrient availability

An aliquot of the same cryopreserved isolate, GibboRiver-L.lesueuri-00-LB-1, was revived and passaged into flasks containing 1.6% tryptone TGhL liquid broth with "High nutrient history" (HNH) and 0.2% tryptone TGhL "Low nutrient history" (LNH). These cultures were incubated at 23ºC and were passaged every 4-6 days, which was the time required to complete the *Bd* lifecycle and reach peak zoospore densities in the higher concentration cultures. After 24 passages in these two media, inocula of each culture were prepared as described above, however, the plates were organized into two sections for each of the inocula (HNH and LNH). In one section the 50 µL of the media (1.6%

and 0.2% tryptone TGhL broths) in which the cultures had been passaged were added to the wells. In the second section, 1.6% TGhL was added to wells. With this plate design we could quantify zoospore densities of HNH and LNH in their original media and could also determine whether cultures maintained in lower media concentrations (LNH) would lose the ability to respond to higher media concentrations. Plates were incubated at 23ºC, monitored daily and zoospore densities were quantified in 5 wells per culture per day as described above.

Statistics— All data were analysed using R, version 2.7.1. We quantified days to zoospore release and zoospore densities on the first day of release and day of maximum zoospore densities. Zoospore densities are reported as mean ± s.d. per ml ($x10^4$).

Results

Experiment 1. Temperature

Encystment and maturation—Rate of encystment was not quantified, but zoospores maintained at 23ºC had all encysted within 24 h when examined microscopically. In contrast, most zoospores maintained at 4ºC were still active (although moving slowly) up to 4 d after plate inoculations. Encysted zoospores maintained at 23ºC matured and produced new zoospores within 3 d whereas, at 4ºC, new zoospores were not observed until 24 d after encystment (Figure 4.1c).

Zoospore densities— There was a highly significant group effect for zoospore densities of HTH cultures and LTH cultures at 23ºC (repeated measures ANOVA, $P < 0.001$; Figure 4.1a) and at 4ºC (repeated measures ANOVA, $P < 0.001$; Figure 4.1b). Cultures maintained at 23ºC had greater numbers of zoospores on the first day of zoospore release than cultures maintained at 4ºC (HTH (at 23ºC day 4): 80.5 ± 28.59, (4ºC day 35): 5.6 ± 4.55; Student's T test, $P < 0.001$; LTH (at 23ºC day 3): 125 ± 43.95, (at 4ºC day 27): 7.6 ± 6.02; Student's T test, $P < 0.001$: Figure 4.1c). The period of time that zoospores were active was longer at 4ºC (HTH: 43 d, LTH: 49 d) than at 23ºC (HTH: 6 LTH: 7 d). It is

unclear if cultures maintained at 4ºC had a greater total number of zoospores (as measured by the cumulative number of active zoospores per day), or if the protracted period of high zoospore density occurred because sporangia matured more slowly and individual zoospores were active for longer periods of time. Regardless, the period of high zoospore density was substantially greater in cultures held at 4ºC (Figure 4.1c).

Differential response between HTH and LTH cultures—Cultures maintained in high temperatures (23ºC) and low temperatures (4ºC) for multiple generations differed in their response to the temperature treatments. Both cultures had similar numbers of zoospores on the day of maximal zoospore density at 23ºC (HTH: 165.2 ± 34.96, LTH: 168 ± 59.51; Student's T test, $P = 0.89$; Figure 4.1a), and also at 4ºC (HTH: 64.1 ± 25.21, LTH: 54.2 ± 26.8; Student's T test, $P = 0.45$; Figure 4.1b), but the timing of zoospore release and the densities of zoospores on the first day of release were different. At 23ºC LTH cultures released zoospores on day 3 whereas HTH cultures did not release zoospores until day 4 (Figure 4.1a). On their respective first days of zoospore release, zoospore densities were significantly higher in LTH cultures at 23ºC (LTH day 3: 125 ± 43.95, HTH day 4: 80.5 ± 28.59; Student's T Test, $P = 0.016$, Figure 4.1a). At 4ºC LTH cultures released zoospores on day 27 whereas HTH cultures released zoospores on day 35 (Figure 4.1b). In both temperature conditions, LTH cultures had a longer period of high zoospore densities. It is not clear whether LTH cultures produced a greater total number of zoospores or if the zoospores they produced were simply active for a longer period of time. This is more clearly seen in a composite image of the results from each culture history at each temperature (Figure 4.1c).

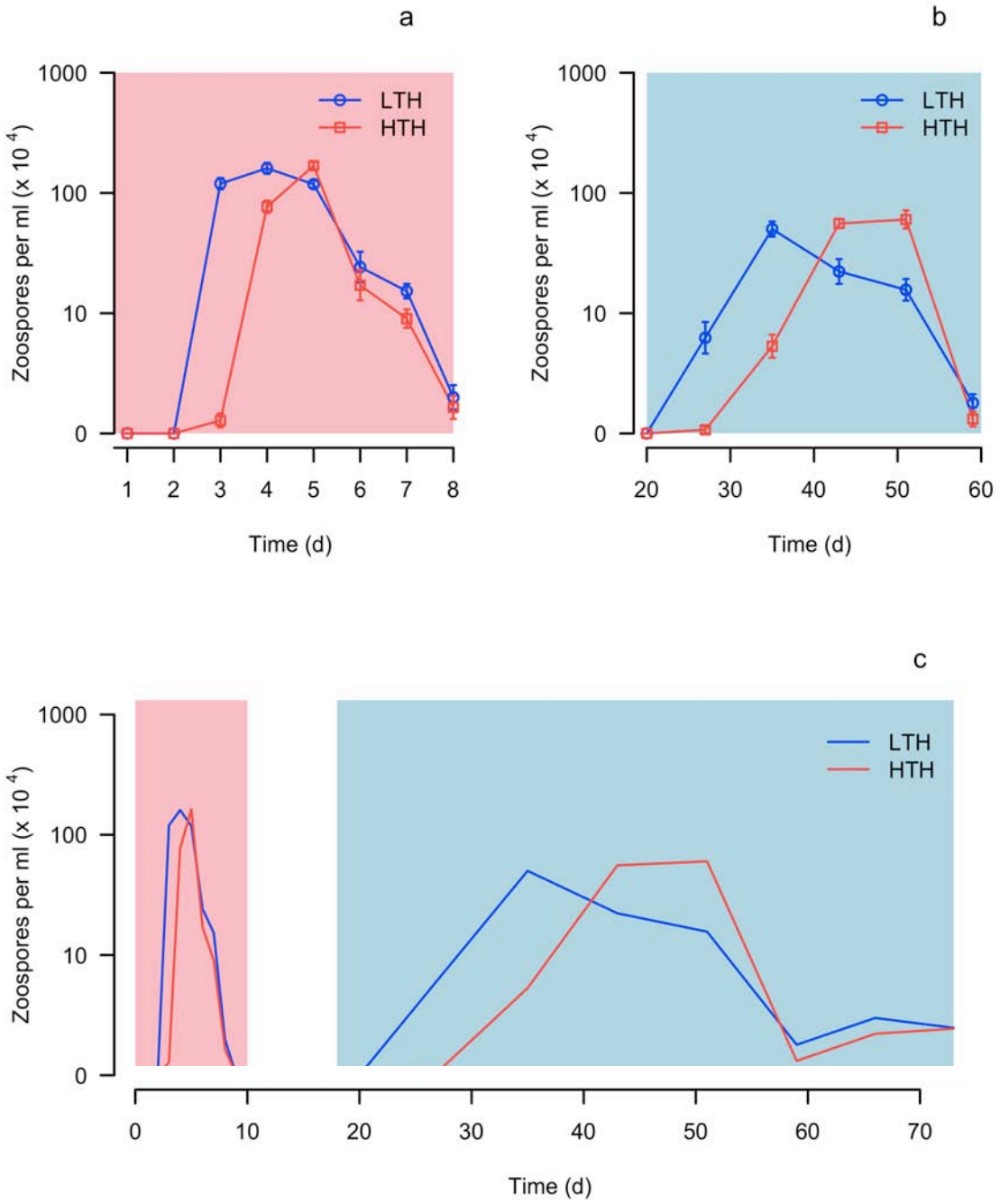

Figure 4.1 Density of zoospores at (a) 23ºC, (b) 4 ºC and (c) both 23ºC and 4ºC (on same time scale) in cultures of an isolate of *Batrachochytrium dendrobatidis* with two thermal histories. Red line indicates cultures previously maintained at 23ºC, "High Temperature History" (HTH). Blue line indicates cultures previously maintained at 4ºC, "Low Temperature History" (LTH).

Experiment 2. Nutrient availability

Encystment and maturation— Cultures in low nutrient concentrations (0.2% tryptone TGhL) encysted, matured and released zoospores sooner than cultures in high nutrient conditions (1.6% tryptone TGhL) (Figure 4.2a). Rate of encystment was not quantified, but cultures in 0.2% tryptone TGhL encysted within 24 h whereas most zoospores from cultures in 1.6% tryptone TGhL were still motile up to day 2 when viewed by microscopic examination. Cultures in 0.2% tryptone TGhL matured faster than those in 1.6% tryptone TGhL and had new zoospores within 2 d of inoculation. In contrast, zoospores were not observed in high nutrient cultures until day 3 (Figure 4.2a).

Zoospore densities— There was a highly significant group effect for zoospore densities when LNH cultures were in 0.2% tryptone TGhL and HNH cultures were in 1.6% tryptone TGhL (repeated measures ANOVA, $P < 0.001$; Figure 4.2a). Cultures previously maintained in 1.6% tryptone TGhL had a greater number of zoospores than cultures previously maintained at low nutrient concentrations on the first day of zoospore release (LNH in 0.2% day 2: 1.2 ± 0.84, HNH in 1.6% day 3, 48.2 ± 14.34; Student's T test, $P = 0.002$; Figure 4.2a) and on the day of maximal zoospore density (LNH in 0.2% day 3: 20 ± 7.03, HNH in 1.6% day 4: 406.2 ± 58.68; Student's T test, $P < 0.001$; Figure 4.2a). The period of zoospore activity was shorter in low nutrient conditions with no motile zoospores after day 7 (Figure 4.2a).

There was also a significant group effect for zoospore densities when LNH cultures and HNH cultures were in 1.6% tryptone TGhL (repeated measures ANOVA, $P < 0.001$; Figure 4.2b). On the first day of zoospore release LNH cultures had significantly higher densities of zoospores in 1.6% typtone TGhL than in 0.2% tryptone TGhL (LNH in 0.2% day 2: 1.2 ± 0.84, LNH in 1.6% day 3: 45.38 ± 11.67; Student's T test, $P < 0.001$) but still lower densities than HNH cultures (LNH in 1.6% day 3: 45.38 ± 11.67, HNH in 1.6% day 3: 76.75 ± 27.63; Student's T test, $P = 0.015$; Figure 4.2b).

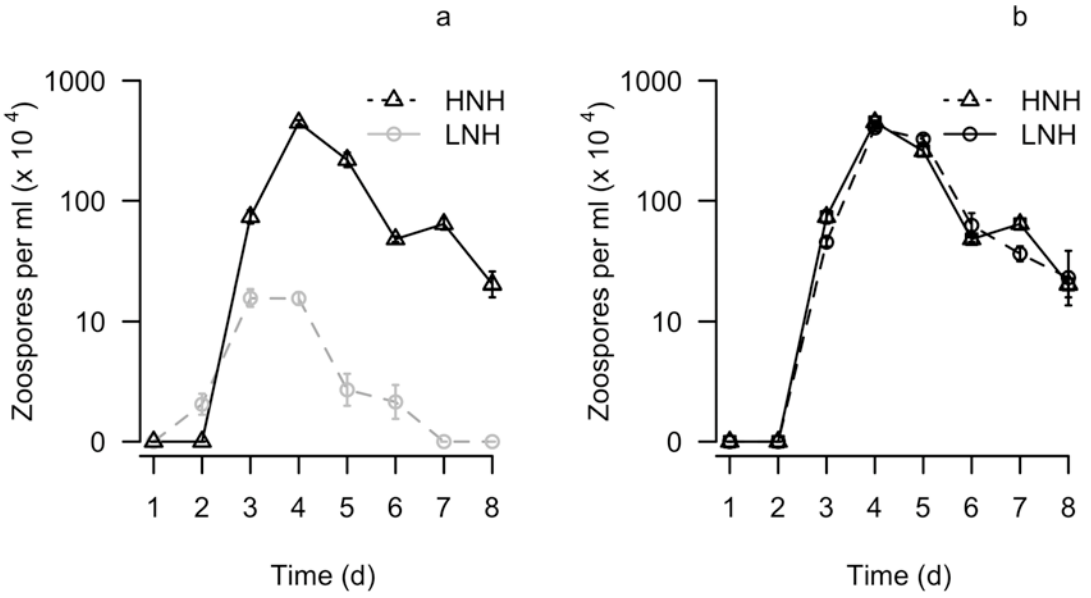

Figure 4.2 Zoospore densities of *Batrachochytrium dendrobatidis* from a single isolate previously maintained in 1.6% and 0.2% tryptone broth nutrient concentrations for 24 passages when cultured in (a) media they had been maintained in and (b) 1.6% TGhL. The grey dashed line (a) indicates "Low Nutrient History" (LNH) cultures in low (0.2% tryptone broth) nutrient conditions. The black dashed line (b) indicates "Low Nutrient History" (LNH) in 1.6% TGhL (b). The solid black line (a and b) indicates "High Nutrient History" (HNH) cultures in 1.6% TGhL.

Discussion

Life-history adjustments should maximise fitness for all organisms (Stearns 1976, Roff 1992), including pathogens (Day et al. 2003). Changes in pathogen rates of growth, maturation and reproduction may influence the incidence of disease (Day et al. 2003, Woodhams et al. 2008a). We used *in vitro* serial passage experiments to investigate rates of growth and development of *Bd* in two temperatures and two nutrient concentrations over multiple generations in culture. Changes in the patterns of zoospore densities observed through time indicate that *Bd* responds to changes in biotic and abiotic factors in ways that should lead to relatively high fitness across a wide range of environmental conditions. Our results demonstrate that variability may exist within a single *Bd* isolate and characteristics may be selected for (*in vitro*) after a relatively small number of life

cycles. *Bd* could potentially respond to long-term culturing in particular environments and therefore influence virulence and the impact of chytridiomycosis.

In Experiment 1 *Bd* cultures had longer periods of high zoospore densities in colder temperatures. These results agree with the findings of Woodhams et al. (2008a) who suggested that *Bd* may trade increased fecundity for extended zoospore activity in colder temperatures and thus influence the transmission dynamics of chytridiomycosis. By maintaining *Bd* at 4ºC for multiple generations we aimed to examine the response of *Bd* to low temperatures for a longer period of time. However, due to the response of *Bd* to the cold temperatures, it was difficult to determine the point of maximum zoospore densities for passaging. We cannot rule out the possibility that the results reflect timing of passage practices if passaging occurred after a peak in zoospore densities. Our results suggest, however, that *Bd* cultures maintained at 4ºC over multiple generations may have had an adaptive response to temperature. Adaptations to shift zoospore release earlier in the life history under cold conditions may have also led to earlier zoospore release in warmer temperatures: "Low Temperature History" (LTH) cultures had zoospore densities for a longer period than "High Temperature History" (HTH) cultures. This shift in the norm of reaction was seen at both 23°C and at 4°C (LTH cultures had longer periods of high zoospore densities than HTH cultures), but at 4°C peaks in zoospore densities differed by 16 days compared with 1 day at 23°C.

Although *Bd* growth rate decreases at low temperatures, adjustments to the *Bd* lifecycle, such as prolonged period of high zoospore densities, may lead to enhanced transmission. If long-term exposure to low temperatures alters the response of *Bd* so that zoospore densities are high for longer periods when introduced to warmer temperatures, amphibians may face a greater risk the end of cold periods or when the pathogen is introduced from cooler regions into warmer ones. Such an adaptive response could lead to higher infection rates and a greater impact on amphibian populations. If this hypothesis is true, selection for *Bd* growth characteristics due to different climates is likely to occur as *Bd* becomes endemic to host populations or spreads into new regions. Additional research is needed to further understand the adaptive response of *Bd* in a wide

variety of temperature conditions. Future experiments should focus on the response of *Bd* at a range of temperatures experienced by amphibians in the wild. Also, under the chytrid-thermal-optimum hypothesis Pounds et al. (2006) proposed that *Bd* should "flourish" in areas with less variable temperature conditions, where cloudiness makes microhabitats more homeothermic. By manipulating the temperature regimes for more and less variable thermal conditions *in vitro*, their hypothesis could be explicitly tested to better understand *Bd*'s response to varying climate conditions.

In Experiment 2 *Bd* maintained in low nutrient conditions released zoospores earlier than *Bd* in high nutrient conditions. This agrees with the results of James (2007). In low nutrient conditions developing sporangia matured faster and released new zoospores earlier than those in high nutrient conditions, suggesting *Bd* can be a virulent pathogen in a wide range of nutrient environments. The *Bd* cultures in low nutrient conditions appeared to have faster life cycle turn over with no motile zoospores after day 7. This may have been due to higher zoospore mortality and/or zoospores encysting more rapidly. When zoospores with a "Low Nutrient History" were passed back into high nutrient conditions the timing of maturation and development closely matched that of cultures maintained in high nutrient conditions, although zoospore densities were statistically different. These results suggest *Bd* is phenotypically plastic in its response to changes in nutrient conditions but there may be little adaptive variation available as the norms of reaction remained fixed under a wide range of nutrient regimes.

It is important to note that these experiments examined *Bd* growth and development but *Bd* virulence was not tested. It is therefore difficult to say if differential zoospore densities would have corresponded with differential virulence. An exposure experiment on a susceptible frog species testing tests two cultures passaged under different nutrient and/or temperature regimes could resolve this question. Our results have implications for *Bd* maintenance following isolation as they indicate very strongly that new isolates should always be cryo-archived so they can later be studied without the possible effects of long-term evolution in culture. Small variations in laboratory practices may have important effects on *Bd*, which has relatively short generation times. By passaging *Bd* at

periods near maximal zoospore densities (approximately day 4) we may have selected for *Bd* with an earlier release of zoospores and greater zoospore densities but these results might have been drastically different if *Bd* cultures were passaged after peak zoospore densities (approximately day 7-9). An additional experiment could test this possibility by reviving aliquots of the same isolate and using serial passage experiments for both cultures: (at 23ºC) one culture could be passaged at peak zoospore densities (days 4-6), the other at a point past peak zoospore densities (days 7-9). Any change in the rates of zoospore production could also be measured by quantifying zoospore densities in the two cultures in a time series (for example, at passage 10, 20, 30 and so on).

There have been several key studies on the physiology of *Bd* (Piotrowski et al. 2004, Symonds et al. 2008), but background information about isolate origins or passage history is sometimes incomplete. The same is true for laboratory infection experiments aimed at understanding host-pathogen dynamics. This information could be extremely important to the interpretation of experimental outcomes and should be carefully considered by researchers working with *Bd*. Furthermore, the importance of cryo-archiving isolates for future virulence research cannot be understated. By sourcing cryo-archived isolates to compare with more recently isolated strains, our methods could be used to characterise a possible evolution in *Bd* virulence and may be important for long-term amphibian conservation efforts (see Chapter eight).

CHAPTER FIVE

Differential growth and virulence of *Batrachochytrium dendrobatidis*

Chapter overview

This chapter describes studies to investigate differences among three *Bd* isolates and if passage history influences virulence of *Bd*. The chapter is presented as a manuscript for publication, listing the authors involved, although it has not yet been submitted. The work presented in this chapter is my own with intellectual and technical input from other authors.

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Abstract

Virulence of infectious parasites can be unstable and can evolve rapidly depending on the evolutionary dynamics of the organism. One potential consequence of altered parasite virulence is disease outbreaks that can have devastating effects on host populations. Serial passage experiments (SPEs) aim to characterise parasite biology and dynamics, often with the underlying objective of understanding virulence. We used SPEs to investigate differential growth and virulence of *Batrachochytrium dendrobatidis*, a fungal pathogen of amphibians that causes a highly lethal disease known as chytridiomycosis. Zoospore densities among three isolates of *Bd* are significantly different. Two cultures that were originally derived from the same cryo-archived isolate were passaged 50 and 10 times near the point of peak zoospore densities. These two cultures ("P50" and "P10") had significantly different zoospore densities on the first day of zoospore release and on

the day of maximum zoospore densities. These patterns of zoospore densities *in vitro* corresponded with differences in prevalence and intensities of infection in exposed *Litoria caerulea*. However, the differences in response variables *in vivo* (prevalence and intensities of infection in exposed *L. caerulea*) were not significant and no mortality occurred in any experimental group. These results demonstrate that variation can exist within a single isolate of *Bd* and have important implications for practical laboratory maintenance of *Bd* isolates as well as for understanding *Bd* virulence.

Introduction

Infectious diseases can alter host population densities, community dynamics and potentially entire ecosystems (Scott 1988, Daszak et al. 2000, Hudson et al. 2002, de Castro & Bolker 2005, Whiles et al. 2006). In many cases the biology, life histories and evolutionary dynamics of the pathogens are not well understood despite their significant threats to wildlife diversity and human health (Daszak et al. 2003). Insight into parasite evolution should provide a better understanding of the evolution of virulence and the mechanisms driving disease epidemics (Bull 1994, Ebert 1998, Ebert & Bull 2003). The evolutionary dynamics of parasites and by extension, virulence, have been investigated using serial passage experiments in which parasites are propagated under constant environmental conditions (Ebert 1998). Serial passage experiments (SPEs) have been used for *in vitro* and *in vivo* investigations of a wide variety of parasites including viruses (Sabin & Schlesinger 1945, Zuckerman et al. 1994), bacteria (Cushion & Walzer 1984, Maisnier-Patin et al. 2002, Somerville et al. 2002), protozoa (Lecompte et al. 1992), fungi (Levin et al. 1970), and others (Ebert 1998). The advantage of SPEs is that alterations in parasite genotype, phenotype and often virulence can be tracked in real time (Ebert 1998). During SPEs parasites are propagated by transfer to a naïve host or to a new artificial environment (such as culture media) at a specific point in time or life phase of the parasite (Ebert 1998, Ford et al. 2002).

Although it is commonly thought that parasite virulence attenuates in culture (Ebert 1998, Ford et al. 2002), evidence suggests shifts in virulence are influenced by passage timing, the point of the parasite's life cycle at which it is propagated (da Silva & Sacks 1987, Wozencraft & Blackwell 1987, Rey et al. 1990). Attenuated parasite strains often can rapidly revert to a virulent form when re-exposed to a naïve host (Cann et al. 1984, Macadam et al. 1989, Minor 1993, Nielsen et al. 2001). Thus it is increasingly clear that virulence can be greatly affected by how parasites are maintained in culture. Understanding how parasites evolve in culture, including the evolution of attenuation, may aid in understanding the evolution of virulence, and may also have practical applications. For example, the ability to reliably infect hosts in controlled conditions is critical to the study of disease (Ford et al. 2002). Because *in vitro* serial culturing puts selective pressures on parasites that may lead to attenuation, the culture history of a parasite may distort the outcome of experiments that are aimed at understanding host-pathogen interactions in nature, where hosts encounter strains of the pathogen with very different histories. This makes it necessary to understand how laboratory culture practices can influence parasite growth, development and virulence.

We used SPEs to investigate growth and virulence of a lethal amphibian pathogen. The fungus *Batrachochytrium dendrobatidis* (*Bd*) causes the disease chytridiomycosis and is highly virulent to multiple species of amphibians in laboratory infection experiments (Berger et al. 1998, 2005b, Woodhams et al. 2003, Daszak et al. 2004, Carey et al. 2006, Lips et al. 2006). However, not all species of amphibians succumb to disease in exposure experiments (Berger 2001, Daszak et al. 2004, Retallick & Miera 2007, Carey et al. in prep). In wild amphibian populations outbreaks of chytridiomycosis have dramatically reduced the abundance of amphibian species with local and global extinctions reported (Lips et al. 2006, Schloegel et al. 2006, Woodhams et al. 2008b) but some species and populations of species that have survived initial declines now persist in the wild with a wide range of levels of infection (Retallick et al. 2004, Woodhams & Alford 2005, Kriger & Hero 2007a,b). These apparent changes in the effects of *Bd* infection on individuals and populations is likely due to environmental conditions influencing disease dynamics but also, it suggests the possibility of differences in host

susceptibility, differences in parasite virulence or both. Although many studies have focused on the roles of innate immunity (Rollins-Smith et al. 2002a, Rollins-Smith et al. 2002b, Woodhams et al. 2004, Rollins-Smith et al. 2005, Woodhams et al. 2006a, Woodhams et al. 2006b, Woodhams et al. 2007b) and behaviour (Rowley 2006, Rowley & Alford 2007) in host susceptibility, the possibility of differential virulence has not been thoroughly examined (Retallick & Meira 2007, Fisher et al. 2009).

Many investigators have observed that growth patterns differ among *Bd* isolates in culture (Symonds et al. 2008, Fisher et al. 2009, James et al. in prep, personal communication R. James, R. Webb, S. Bell, N. Kenyon, E. Davidson and D. Woodhams) and preliminary data suggests that *Bd* virulence may differ among isolates when susceptible species are exposed to them in infection experiments (Berger et al. 2005, Retallick & Miera, 2007, Fisher et al. 2009, personal communication D. Woodhams, C. Briggs, N. Kenyon). Reduced mortality has been observed in populations after surviving initial emergence (Morgan et al. 2007) and different populations, even within species, can experience a range of the effects following initial outbreaks (Woodhams & Alford 2005, Briggs et al. 2005). Temperature has been consistently shown to be an important factor in determining the population effects of chytridiomycosis (Berger 2001, Bradley et al. 2002, Woodhams et al. 2003, Berger et al. 2004, Retallick et al. 2004, Woodhams & Alford 2005, Carey et al. 2006, Drew et al. 2006, Kriger & Hero 2006, Pounds et al. 2006, Rachowicz et al. 2006, Kriger & Hero 2007a, b, Rowley & Alford 2007, Kriger & Hero 2008, Muths et al. 2008, Skerratt et al. 2008), however, the other determinants of this variation within species are unknown. Because amphibians often become morbid and die from chytridiomycosis when intensities of infection are high, the rate production of infectious *Bd* zoospores is likely to be an important factor in pathogenesis and *Bd* virulence (Carey et al. 2006). Therefore, we quantified zoospore production among *Bd* isolates and within replicates of a single isolate with differing passage histories. We also measured the virulence of *Bd* cultures that had experienced different passage histories using a frog exposure experiment.

Methods

Experiment 1- Comparing three *Batrachochytrium dendrobatidis* isolates

Isolates GibboRiver-L.lesueuri-00-LB, TullyRiver-L.rheocola-07-LB and Rockhampton-L.caerulea-99-LB, were originally obtained from diseased *Litoria lesueuri*, *Litoria rheocola* and *Litoria caerulea* respectively. All isolates were purified and cultured on tryptone/gelatin hydrolysate/lactose (TGhL) agar with antibiotics (Longcore et al. 1999) and then passaged into liquid TGhL broth (tryptone, gelatin hydrolysate, lactose) (Longcore et al. 1999) into 25-cm^2 cell culture flasks. Cultures were maintained at James Cook University in TGhL broth at 4°C and passaged every 2 to 3 months.

The *Bd* cultures were filtered to remove sporangia using sterile filter paper (Whatman, 3). Cultures were washed by centrifuging (500 g for 10 minutes), after removing the supernatant the zoospores were resuspended in sterile dilute salt solution (in mMol: KH_2PO_4 (1), $CaCl_2.H_2O$ (0.2), $MgCl_2.2H2O$ (0.1). Zoospore concentrations were determined using a haemocytometer (Improved Neubauer Bright-line) and adjusted as needed by addition of dilute salt solution to a concentration of 90 x 10^3 zoospores ml^{-1}. Experiments were done using sterile 96 well plates (Tissue culture test plates-96, TPP) that were organized with three sections for each isolate. Zoospore inocula (50 µL) were each pipetted into 20 wells containing 50 µL TGhL media. The plate had a perimeter of 36 wells with 100 µL sterile water to avoid evaporation. Plates were inspected microscopically each day to monitor zoospore encystment, development and maturation of the zoosporangia. Once the maturing zoosporangia produced the first zoospores, zoospore density was quantified daily by randomly selecting 10 wells containing each of the three isolates, drawing off 30 µL of supernatant and counting zoospore numbers using a haemocytometer.

Experiment 2- Multiple passages with one *Batrachochytrium dendrobatidis* isolate

The isolate, GibboRiver-L.lesueuri-00-LB-1, was originally obtained from a diseased juvenile *L. lesueuri*, cultured on tryptone/gelatin hydrolysate/lactose (TGhL) agar with antibiotics (Longcore et al. 1999) and then cryoarchived (Boyle et al. 2003). An aliquot of the cryoarchived culture was revived (Boyle et al. 2003) and then passaged into liquid TGhL broth (Longcore et al. 1999) in 25-cm^2 cell culture flasks. Flasks were visually inspected daily to monitor zoospore encystment and maturation of the zoosporangia. Cultures were passaged into new media when zoospore density was near its maximum, for 50 passages. This culture with a higher number of passages will be referred to as "P50". A second aliquot of the same isolate was revived 250 d later and was treated identically for 10 passages. This culture with a lower number of passages will be referred to as "P10". Zoospore concentrations were determined using a haemocytometer (Improved Neubauer Bright-line) and adjusted as needed by addition of dilute salt solution to a concentration of 93 x 10^4 zoospores ml^{-1}. The two isolates were inoculated into 96-well plates and zoospore production was quantified daily (10 wells per culture per day) as described above.

Experiment 3- Experimental exposures of *Litoria caerulea*

Adult common green tree frogs (*Litoria caerulea*; n = 30, mean mass: 21.34 ± 5.64 s.d.) were collected in January and February, 2008 from residential areas of Townsville, Queensland. Each frog was collected using a new plastic bag and then transferred to an individual plastic container (200x240x330 mm^3) that was maintained in temperature (18-23ºC) and light (12L/12D) controlled facilities at James Cook University, Townsville, Australia. Frogs were fed vitamin-dusted crickets (medium-sized, Pisces Inc.) *ad libitum* twice per week. Tap water (250 ml) was changed twice a week until experimental exposures began and was then replaced by 20% Holtfretter's solution (in mMol: NaCl (6), KCL (0.06), $CaCl_2$ (0.09), $NaCO_3$ (0.24), pH 6.5, 250 ml). Containers were maintained in a level position so water covered the bottom but frogs were able to climb

up the dry walls. To confirm that frogs were not infected with *Bd* prior to inoculation, samples for testing for *Bd* were obtained by rubbing a sterile cotton swab over the ventral surfaces and digits (Hyatt et al. 2007). Diagnosis of *Bd* infection status was performed using a Taqman real-time polymerase chain reaction (PCR) assay (Boyle et al. 2004). All samples were analyzed in triplicate and compared with Australian Animal Health Laboratory zoospore standards to determine zoospore equivalents.

Zoospores from isolates with high (P50) and low (P10) passage histories were filtered to remove sporangia (described above). Zoospore concentrations were determined using a haemocytometer (Improved Neubauer Bright-line) and adjusted as needed to reach 93 x 10^4 zoospores ml^{-1} by diluting with dilute salt solution. Frogs were randomly assigned to exposure (P50 and P10 isolate treatments) and control groups. Frogs were exposed to *Bd* in plastic containers via shallow immersion in a bath of Holtfretter's solution containing zoospores. Uninfected control frogs were held in a bath of with added the same volume of TGhL but without zoospores. After 24 h frogs were moved to fresh containers with 20% Holtfretter's solution (pH 6.5).

Frogs were randomly assigned to exposure (P50 and P10 isolate treatments) and control groups. Exposure solutions (P50 and P10 isolate treatments) consisted of *Bd* zoospores in a dilute salt solution and 20 % Holtfretter's solution (Wright et al. 2001). Frogs were exposed to *Bd* zoospores in plastic containers via shallow immersion in a bath of exposure solutions. Uninfected control frogs were held in a bath containing equal volumes (equal to volumes in exposure solutions) of sterile TGhL with no *Bd* zoospores and Holtfretter's solution. After 24 h frogs were moved to fresh containers with 20% Holtfretter's solution (pH 6.5). Following exposure to *Bd*, frogs were swabbed again at 21, 58 and 108 d post exposure.

Results

Experiment 1- Comparing three *Batrachochytrium dendrobatidis* isolates

We hypothesized that zoospore densities might differ among isolates. To test this hypothesis we recorded time to zoospore release (number of days to first observed zoospores) and quantified zoospore densities on the first day of zoospore release and on the day of maximum zoospore density of three *Bd* isolates. Microscopic inspection of these cultures suggested that their rates of maturation and morphological characteristics were not substantially different (Figure 5.1), but zoospore densities among the three isolates were significantly different (repeated measures ANOVA, $P < 0.001$; Figure 5.2). New zoospores were visible and active on day 3 for all three isolates. Zoospore densities in isolates GibboRiver-L.lesueuri-00-LB (155.3 ± 50.82) and TullyRiver-L.rheocola-07-LB (171.5 ± 25.17) did not differ on the first day of zoospore release (ANOVA with Tukey HSD test $P = 0.545$) but were significantly different than zoospore densities in Rockhampton-L.caerulea-99-LB (5.25 ± 2.71: ANOVA with Tukey HSD test $P < 0.001$; Figure 5.2). On the respective days of peak zoospore densities, the GibboRiver-L.lesueuri-00-LB isolate (day 4: 213.6 ± 33.87) had significantly more zoospores than TullyRiver-L.rheocola-07-LB (day 3: 171.5 ± 25.18) and Rockhampton-L.caerulea-99-LB (day 4: 38.13 ± 8.20 ANOVA $P < 0.001$). For all three isolates the period of maximum zoospore densities was completed by day six but zoospores were active to ten days following initial inoculations.

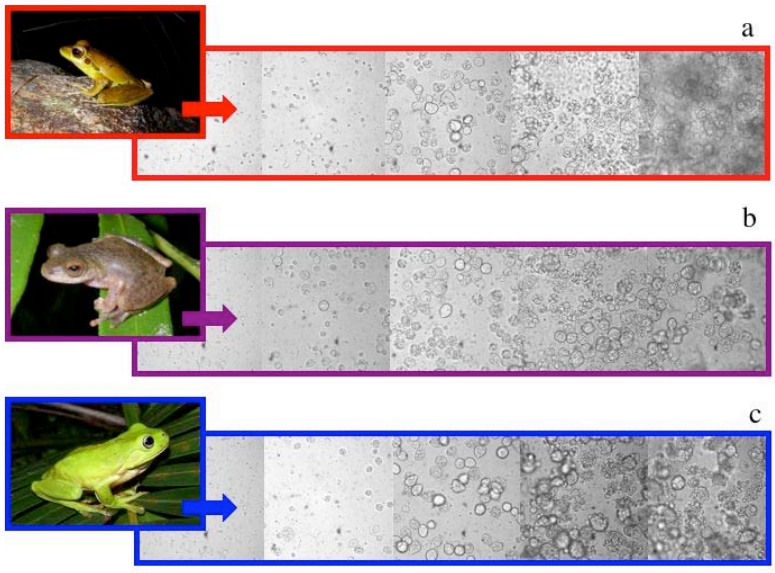

Figure 5.1 Light microscopy images of three isolates of *Batrachochytrium dendrobatidis*: (a) GibboRiver-L.lesueuri-00-LB, (b) TullyRiver-L.rheocola-07-LB, (c) Rockhampton-L.caerulea-99-LB. Images show successive developmental stages of *Bd* from active zoospores (far left) to mature sporangia (far right). The images of frogs (left side) are representative images of the frog species from which each isolate was originally cultured. The colors match the lines in Figure 5.2 showing zoospore production for each isolate.

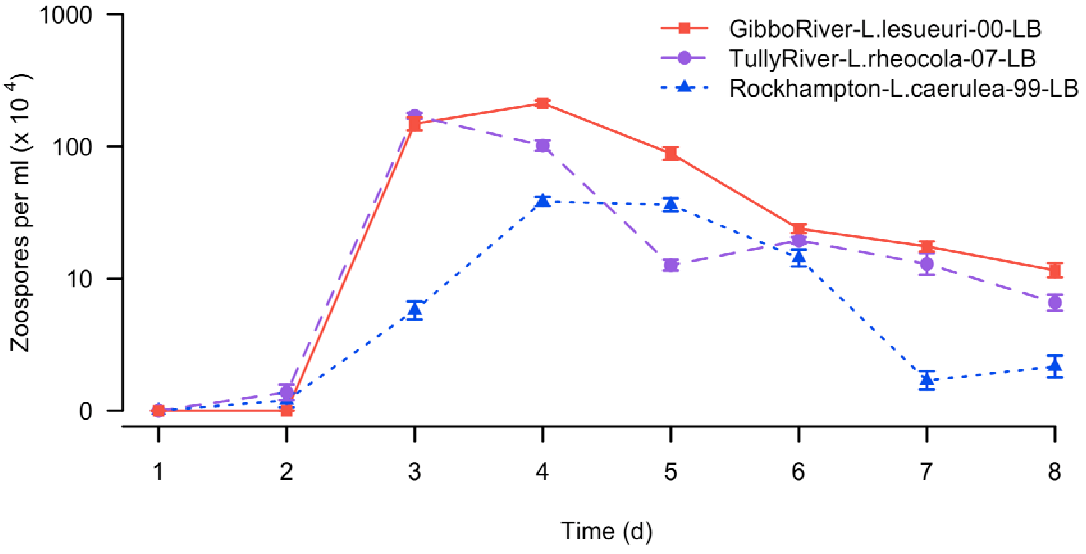

Figure 5.2 Zoospore density over time in three isolates of *Batrachochytrium dendrobatidis*, red: GibboRiver-L.lesueuri-00-LB-P24, purple: TullyRiver-L.rheocola-07-LB-P23, blue: Rockhampton-L.caerulea-99-LB-P24.

Experiment 2- Multiple passages with one *Batrachochytrium dendrobatidis* isolate

By serially passaging the GibboRiver-L.lesueuri-00-LB isolate at the peak of its zoospore density, we eliminated the contribution of zoospores produced after this peak in each generation. This produced strong selection for early zoospore release and maximum zoospore densities. We predicted that 50 passages with this selective pressure should produce a greater response than 10 passages. We found significant differences between the high and low passage isolates patterns of zoospore density over time (repeated measures ANOVA, $P < 0.001$). New zoospores were observed in cultures from both histories three days after inoculations. The culture with the higher number of passages (P50), contained significantly more zoospores (127.2 ± 33.38) on the first day of zoospore release, day 4, than the culture with a lower number of passages (P10) (1.8 ± 1.2: Student's T test, $P < 0.001$; Figure 5.3). The P50 culture also had higher zoospore densities on the day of maximum zoospore densities than the P10 culture (P50, day 6, 58.8 ± 9.38, P10, day 5, 135.1 ± 36.06; Student's T test, $P < 0.001$; Figure 5.3). In both cultures the period of maximum zoospore density was completed by day 7 however, active zoospores were seen up to 14 days following initial inoculations.

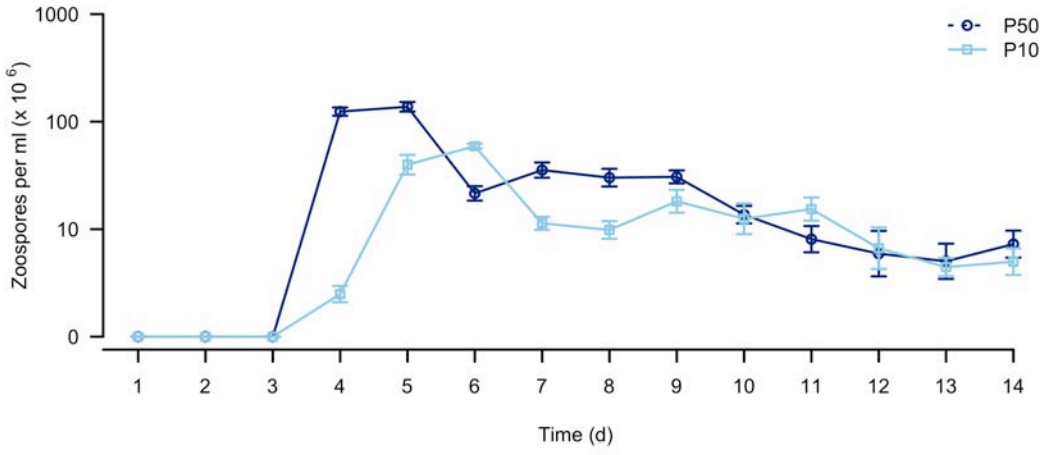

Figure 5.3 Zoospore densities over 14 days in culture of an isolate of *Batrachochytrium dendrobatidis*, GibboRiver-L.lesueuri-00-LB, that had two different passage histories. The dark blue line shows zoospores produced in an isolate that had been passaged 50 times (P50). The light blue line shows zoospores produced in an isolate that had been passaged 10 times (P10).

Experiment 3- Experimental exposures of *Litoria caerulea*

The same cultures as in experiment 2, P50 and P10, were used in an exposure experiment on adult frogs of *Litoria caerulea*. Although the results of experiment 2 indicated that selection had led to earlier zoospore release and higher zoospore densities in the P50 culture, this might not have produced increased virulence in infected frogs; if it reflected adaptation to conditions *in vitro*, virulence could have decreased. Unexpectedly, there was no mortality in any group. Mild clinical signs of infection including lethargy, inappetence and slight discoloration were recorded for two frogs in the P50 group and 2 frogs in the P10 group. More severe clinical signs of infection did not develop in any group. Frogs with mild clinical signs seemed to recover, regaining normal color, activity and appetite, by the termination of the experiment. There was a significant decrease in mass (paired T test, $P = 0.003$) in the P50 group whereas there was no significant change in the P10 group (paired T test, $P = 0.6641$). However, the control group also lost weight (paired T test, $P = 0.03$). The change in mass (final minus initial weight) was greater for the P50 group. However this is not significantly different from the P10 and control groups (ANOVA, $P = 0.48$ Figure 5.4).

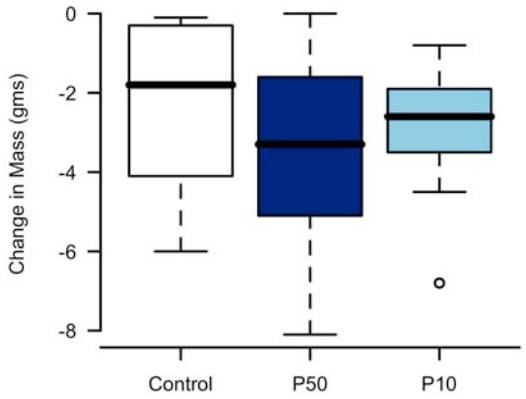

Figure 5.4 Change in mass (final weight minus initial weight) in *Litoria caerulea* experimentally exposed to two cultures (dark blue bar: P50 and light blue bar: P10) of the GibboRiver-L.lesueuri-00-LB isolate with different passage histories and in control frogs exposed to sterile medium.

Using PCR analysis on swab samples, we found that there was no significant difference in prevalence (Fisher's exact test, $P = 0.63$) in *L. caerulea* exposed to the P50 zoospores, 80% (8/10) became infected during the experiment. In comparison, 60% (6/10) frogs became infected in the group that was exposed to P10 zoospores. The frogs that became infected with the P50 zoospores had slightly higher intensities of infection than the frogs exposed to P10 zoospores, but there was no significant group effect (repeated measures ANOVA, $P = 0.156$; Figure 5.6).

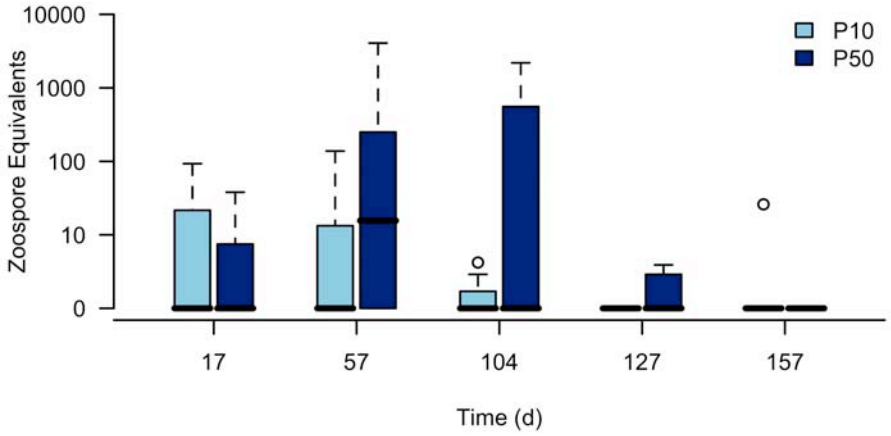

Figure 5.6 Intensity of infection in *Litoria caerulea* infected with one isolate of *Batrachochytrium dendrobatidis* with 2 different passage histories. The frogs exposed to zoospores from a higher passage culture (P50- dark blue) had greater intensities of infection than frogs exposed to zoospores from a lower passage culture (P10-light blue).

Discussion

Differential growth and virulence of *Batrachochytrium dendrobatidis* has been reported in several studies. However, it is unclear why this variation exists (Berger et al. 2005, Retallick & Miera 2007, Fisher et al. 2009). We quantified zoospore densities among three isolates originally cultured from different amphibian species and found that they had significantly different zoospore densities. Two of the isolates tested were known to be differentially virulent to *Litoria caerulea* in controlled infection experiments (Berger et al. 2005b). However, long-term maintenance practices for these isolates were not

identical and it is therefore impossible to know whether it was their different origins or differences in their passage histories that affected their virulence.

By reviving two aliquots of a single isolate of *Bd*, GibboRiver-L.lesueuri-00-LB, and subjecting them to different passage histories, we demonstrated significant effects of passage history on zoospore densities. Zoospore densities of the P50 culture was not quantified early after revival, so the differences we observed could conceivably have been caused by differences in the aliquots that were revived to start the P50 and P10 cultures. However, both were samples from the same culture, and the P10 was frozen in liquid nitrogen until it was revived, so sample differences or effects of history during preservation seem very unlikely. Our results in experiment 2 were consistent with our predictions; by passaging at the point of peak zoospore density, we were selecting for earlier reproduction and because passaging involves subsampling, we were also selecting for the production of greater number of zoospores. The P50 culture reproduced earlier and produced more zoospores than the P10 culture with a shorter history of selection. Thus serial culturing practice appears to have affected the rate and temporal pattern of zoospore production. The mechanisms underlying these effects are unknown. However, this study demonstrates that patterns of zoospore production can differ within a single isolate of *Bd*. These suggest that *Bd* may rapidly adjust and adapt to changes in conditions, and that the phenotypes of serially maintained cultures in the laboratory may diverge from those of *Bd* in nature.

Although quantifying patterns of zoospore density in culture is valuable for understanding rates of population growth of *Bd*, the importance of zoospore numbers for *Bd* virulence is less clear. In experiment 3 we found that the P50 culture that produced more zoospores and we predicted that the P50 might be more virulent. The patterns observed in our measured response variables, prevalence and intensity of infection, support this possibility. However, these differences were not significant and furthermore, there was no mortality in any group of *Litoria caerulea* exposed to P50 and P10 cultures. This result was unexpected because this isolate, GibboRiver-L.lesueuri-00-LB, was originally chosen for this investigation because it was highly virulent in other infection

experiments. This isolate caused 90% mortality of *Litoria caerulea* in a separate experiment using virtually identical methods (see Chapter 7). Differences between the other experiment and the present study include the length of time frogs were held in captivity, the seasonal timing of exposure, and isolate maintenance practices (cultures for the previous infection experiment had comparable passage history, but were held at 4°C rather than 23°C). Therefore, it is difficult to explain the lack of mortality, and additional exposure experiments with similar methods are needed to resolve this question.

Our results do demonstrate that the P50 culture had earlier and higher zoospore densities and suggest that it may have been more virulent following serial culturing practices. This contradicts the general assumption that pathogens tend to attenuate in culture. Attenuation is presumably the result of pathogens becoming adapted to the conditions of culture and losing some of their ability to exploit hosts as resources. However, evidence from other systems suggests that attenuation should be highly dependent on the biology of the pathogen and the culturing practices (Rey et al. 1990, Wozencraft et al. 1987, DaSilva & Sacks 1987). Our results support that hypothesis; our culture system and medium may have been similar enough to host tissues in features that are important to the growth and proliferation of *Bd* that becoming adapted to rapid reproduction in culture also increased the reproductive rate of *Bd* in infected amphibian hosts.

Due to short generation times and high proliferation rates, pathogens can evolve rapidly (Cann et al. 1984, Macadam et al. 1989, Minor 1993, Ebert 1998, Nielsen et al. 2001). For disease research, stability of virulence properties presents a challenge (Michel & Garcia 2003) as virulence can evolve rapidly in both directions (Ebert & Bull 2003, Stearns & Koella 2007). However, changes in virulence also represent an opportunity to better understand the mechanisms of disease. For example, evolution of virulence that is manipulated towards attenuation but then reverts to high virulence may reveal factors that determine the optimal virulence for a particular host-pathogen dynamic. We found significant differences in zoospore densities, lifespan of zoospores and period of zoospore activity in *Bd* isolates maintained in cold (4°C) conditions for multiple generations (Chapter four). We also found that timing of pathogen propagation may influence growth

and zoospore production of *Bd*. If zoospore production plays a role in pathogenesis of chytridiomycosis, then serial culturing practices may impact the evolution of *Bd* virulence.

Understanding mechanisms of virulence and factors driving its evolution are important given the threat of infectious disease to wildlife and human health. Understanding the factors that influence pathogenicity will lead to a greater understanding of the disease and thus, direct more effective conservation action for threatened amphibians. With advancements in basic virulence and disease research we may be able to distinguish novel pathogens from pathogens that have increased in virulence (Daszak et al., 2000, Rachowicz et al., 2005), assess potential disease risk (Kuiken et al. 2005), track pathogen movements (Kuiken et al. 2005, Kang et al. 2006), use attenuated strains as vaccines and incorporate disease resistance into breeding programs (Schlaepfer 2007). Management of infectious diseases will remain a challenge for continuing research until we can define the mechanisms of virulence and understand the evolutionary dynamics of infectious pathogens.

CHAPTER SIX

Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis

Chapter overview

This chapter describes presents preliminary findings on pathogenesis of chytridiomycosis. It is presented with the same format as previous chapters and a published version can be found in the appendices.

Published work: Voyles J, Berger L, Webb R, Young S, Speare R, Werner J, Rudd D, Campbell R, Skerratt LF (2007). Electrolyte disruption and osmotic imbalance in amphibians with chytridiomycosis. Diseases of Aquatic Organisms 77, 113-118.

Abstract

Mounting evidence implicates the disease chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), in global amphibian declines and extinctions. While the virulence of this disease has been clearly demonstrated, there is, as yet, no mechanistic explanation for how *Bd* kills amphibians. To investigate the pathology of chytridiomycosis blood samples were collected from uninfected, aclinically infected and clinically diseased amphibians and analyzed for a wide range of biochemical and hematological parameters. Here we show that green tree frogs (*Litoria caerulea*) with severe chytridiomycosis had reduced plasma osmolality, sodium, potassium, magnesium and chloride concentrations. Stable plasma albumin, haematocrit and urea levels indicated that hydration status was unaffected, signifying depletion of electrolytes from circulation rather than dilution due to increased water uptake. These results suggest that *Bd* kills amphibians by disrupting normal epidermal functioning leading to osmotic imbalance through loss of electrolytes. Determining how *Bd* kills amphibians is

fundamental to understanding the host-pathogen relationship and thus the population declines attributed to *Bd*. Understanding the mechanisms of mortality may also explain interspecific variation in susceptibility to chytridiomycosis.

Introduction

Amphibians are currently undergoing the fastest rate of extinction of any vertebrate group (Stuart et al. 2004). While habitat destruction or overexploitation are obvious primary causes in some declines, determining why amphibians are experiencing catastrophic declines in protected areas has been more challenging. Amphibian mass-mortalities and declines have coincided with the appearance of the fungal pathogen *Batrachochytrium dendrobatidis* in wild amphibian communities (Briggs et al. 2005, Lips et al. 2006, Woodhams et al. 2008b). This fungus is lethal to many species of amphibians (Longcore et al. 1999, Nichols et al. 2001, Daszak et al. 2004, Lips et al. 2006, Berger et al. 1998, 2005b, Carey et al. 2006), yet the mechanism by which it causes death is unknown (Gascon et al. 2007, Mitchell et al. 2008, Rosenblum et al. 2008, Wake & Vredenburg 2008). *Batrachochytrium dendrobatidis* is confined to the superficial layers of the epidermis and causes no consistent pathological changes in internal organs (Berger et al. 1998, Pessier et al. 1999). Two hypotheses as to the cause of mortality in amphibians infected with *Bd* have been suggested. The first is that *Bd* disrupts osmoregulation in the skin of infected amphibians and the second is that *Bd* produces a toxin that affects organs (Berger et al. 1998, Berger 2001, Blaustein et al. 2005). These hypotheses are not necessarily mutually exclusive. However, no study has provided data to determine if either or both hypotheses are correct.

Amphibian skin is well studied due to its unique functions. The integument is a site of regulated transport for water, ions (electrolytes) and respiratory gases (Jorgensen et al. 1954, Alvarado & Kirschner 1963, Deyrup 1964, Moore & Lofts 1964, Fischbarg & Whittembury 1977, Shoemaker 1977, Larsen et al. 1987, Boutilier et al. 1992, Erspamer et al. 1994, Jorgensen 1994a, b, 1997, Wright et al. 2001, Word & Hillman 2005).

Permeability of frog skin varies over the body surface of an individual and also among species (Deyrup 1964, Heatwole & Barthalmus 1994). In some species osmotic permeability is greatest in an area of ventral integument commonly referred to as the pelvic patch (Baldwin 1974, Czopek 1965, Word et al. 2005), where there is dense cutaneous vasculature (Czopek 1965). Concomitantly, *Bd* occurs more commonly and at higher density in the ventral integument of infected frogs (Berger et al. 2005c, Puschendorf & Bolaños 2006). *Batrachochytrium dendrobatidis* grows within the keratinized cells of the superficial epidermis and causes irregular skin sloughing, hyperplasia and hyperkeratosis (Berger et al. 1998, 1999, 2005 a,b,c Pessier et al. 1999). Other pathological changes including cytoplasmic degeneration and vacuolation in scattered cells have been observed by light and electron microscopy, but these changes are not usually severe (Berger 2001). Thus it is unclear how a superficial skin infection kills frogs.

The aim of this research was to investigate pathogenesis in amphibians with chytridiomycosis. We evaluated changes in physiological parameters after infection with *Bd* in the common green tree frog (*Litoria caerulea*), a species known to be susceptible to infection (Berger et al. 2005b). We found severely diseased frogs had reduced blood plasma osmolality and electrolyte concentrations indicating osmotic imbalance. Other biochemical and hematological parameters including plasma proteins, tissue enzymes and haematocrit were measured as markers of general health and did not vary significantly.

Methods

Blood samples were collected twice over the course of infection during an outbreak in captive-bred *Litoria caerulea.* This outbreak was monitored by swabbing frogs every ten days for 73 days for *Bd* zoospore equivalents, determined by real-time PCR analysis (Boyle et al. 2004). Together with clinical signs of disease, most of which are apparent only in the few days before death (Berger 2001), these PCR results were used as

indicators of severity of infection (Figure 4.1). Blood was collected for haematology and plasma biochemistry before and after frogs became diseased.

Frogs were housed individually at 18-23ºC and fed vitamin-dusted crickets. Ten *L. caerulea* became infected and two were uninfected. Within 16 days after suspected exposure, blood (< 1% frog weight) was collected with a heparinized syringe and needle via the ventral abdominal vein or heart after anesthesia by shallow immersion in a solution bath of 0.1% MS222 (tricaine methanesulfonate, Sigma Chemical). MS222 does not kill *Bd* (Webb et al. 2005). A sample of whole blood was analyzed (Bayer 865 rapidlab blood gas analyzer) for electrolyte concentrations, pH and carbon dioxide. Haemoglobin was measured using the modified Drabkin's method. Total red cells were counted using a modified Neubauer haemocytometer with Nat-Herrick's solution as the diluent. Plasma osmolality was measured using a Knauer automatic osmometer with 400 mMol l^{-1} standards.

Litoria caerulea were monitored daily for clinical signs of chytridiomycosis including lethargy, inappetence, decreased respiration rate, cutaneous erythema, irregular skin sloughing and abnormal posture (legs splayed out posteriorly). A second blood sample was collected immediately before euthanasia for seven infected *L. caerulea* when they showed obvious clinical signs between 53 and 73 days post exposure (days 53, 56, 59. 59, 59, 60, 72) and for three infected and two uninfected frogs that were clinically normal (days 72 to 74). Samples were tested by blood gas analysis as previously described. A large range of blood biochemical parameters was measured to evaluate functioning of, or damage to, organs including liver, kidney muscle and pancreas. For this, plasma was analyzed for 16 biochemical parameters (amylase, lactate, aspartate, aminotransferase, creatine kinase, glutamate dehydrogenase, hemoglobin, platelets, haematocrit, albumin, bile acids, cholesterol, glucose, globulin, total protein and urea; Olympus AU400 at 37C at IDEXX Laboratories, Brisbane).

These methods were performed with James Cook University Animal Ethics Committee approval (Permit No. A593). All data were analyzed using SPSS statistics, version 12.0.

Data sets were tested for normality and homogeneity of variance (homoscedasticity) and non-parametric instead of parametric tests were used when violations of assumptions occurred. Asterisks (*) in graphs and table indicate significance when $p < 0.05$.

Results

Skin swab samples collected on the date of death in diseased frogs ($n = 7$) and at termination of the experiment in aclinically-infected amphibians ($n = 3$), indicated *Bd* zoospore equivalents were greater in severely diseased amphibians (Mann-Whitney Test, $P = 0.017$; Figure 6.1). Swabs from uninfected amphibians ($n = 2$) were negative.

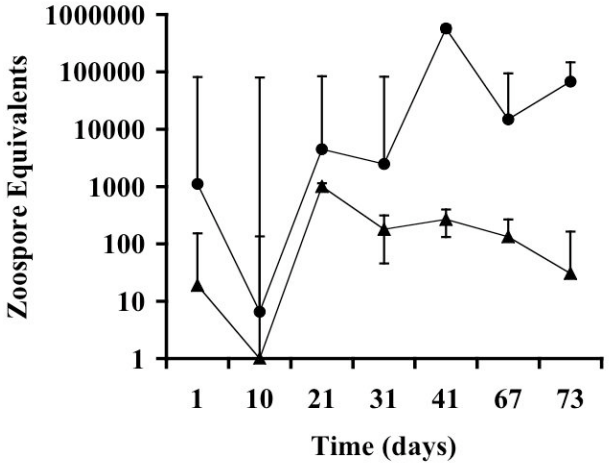

Figure 6.1. Zoospore equivalents over time in severely diseased (circles: $n = 7$) and aclinical (triangles: $n = 3$) *Litoria caerulea* infected with *Batrachochytrium dendrobatidis*. Uninfected amphibians (with zero zoospores) are not shown. Results are from real-time PCR on skin swabs.

There were no significant differences in blood test results between frogs that were uninfected and frogs that were infected but aclinical. However, amphibians with severe clinical signs of disease had significantly reduced plasma osmolality compared with both uninfected and aclinical groups combined (Table 6.1). Further, severely diseased frogs had significant reductions in plasma sodium, potassium, chloride and magnesium

concentrations (Table 6.1). None of the other 16 biochemical parameters (listed in Methods) varied significantly (Table 6.1).

Infection Status	**Uninfectd/Aclinical**			**Clinical**			
Measured Variables	**Mean**	**S.D.**	**N**	**Mean**	**S.D.**	**N**	**p-value**
Mass (gms)	50.52	16.1	5	35.7	5.3	7	p = 0.109
Electrolyte Concentrations							
Osmolarity (mMol l^{-1})	217.2	28.5	5	153.5	46.6	6	p = 0.026 *
Sodium (mMol l^{-1})	102.8	7.9	5	74.4	9.5	7	p < 0.001 *
Potassium (mMol l^{-1})	2.74	0.3	5	1.5	0.8	7	p = 0.016 *
Magnesium (mMol l^{-1})	0.98	0.4	5	0.3	0.2	7	p = 0.027 *
Chloride(mMol l^{-1})	79	7.5	5	47.3	9.4	7	p < 0.001 *
Sodium: Potassium Ratio	32.52	3.5	5	30.2	6.6	6	p = 0.491
Blood Gases							
CO2 (pCO2 mmHg)	35.54	12.2	5	25.3	9.7	6	p = 0.153
pH	7.31	0.1	5	7.2	0.2	7	p = 0.369
Enzymes/Markers							
Amylase (IU l^{-1})	3100.6	948	5	3482.4	1517.5	5	p = 0.646
AST (IU l^{-1})	368	194.5	5	1003.3	895.2	7	p = 0.154
GLDH (IU l^{-1})	12	4.8	5	31.7	30	7	p = 0.181
Creatine Kinase (IU l^{-1})	1627.8	895.6	5	2782.4	2524.1	7	p = 0.356
Hematology							
RBC (x 10^{12} l^{-1})	0.42	0.96	5	0.52	0.17	6	p = 0.291
Hemoglobin (gms l^{-1})	0.163	0	5	0.2	0.1	6	p = 0.704
Platelets	8680.2	5220.2	5	6523	3158.703	6	p = 0.418
PCV (%)	22.8	5.6	5	26.5	7.1	6	p = 0.371
Other Blood Constituents							
Albumin (gms l^{-1})	26.6	5.4	5	30.1	3.9	7	p = 0.212
Bile Acids (µMol l^{-1})	7	8.5	5	35.7	41.1	7	p = 0.160
Cholesterol (mMol l^{-1})	1.4	0.9	5	2	2.3	5	p = 0.630
Glucose (mMol l^{-1})	3.62	1.1	5	3.6	2.1	3	p = 0.964
Globulin (gms l^{-1})	17.2	3.7	5	26.7	9.8	7	p = 0.068
Total Protein (gms l^{-1})	43.8	8.7	5	56.9	12.9	7	p = 0.079
Urea (mMol l^{-1})	9.02	2.4	5	11.5	8.1	7	p = 0.531

Table 6.1 Biochemical and hematological parameters in *Litoria caerulea* infected with *Batrachochytrium dendrobatidis*. Designation of aclinically infected/uninfected or severely infected was determined by clinical signs and by measuring the number of zoospore equivalents by real-time PCR. Uninfected frogs were grouped with the aclinical frogs because there were no significant differences between the two groups.

In addition to comparing results among groups of amphibians with different severities of disease, we evaluated the changes in a range of biochemical parameters within individual *L. caerulea* as infections progressed. Significant biochemical changes occurred solely in amphibians that developed severe disease. Plasma concentrations of sodium, potassium, chloride and blood pH in severely diseased amphibians were significantly decreased in final blood samples compared with initial blood samples (Figure 6.2). None of these

changes were detected in aclinically infected or uninfected amphibians (Figure 6.2). Furthermore, no other measured parameter (carbon dioxide, glucose, red cell count, haematocrit and hemoglobin) varied significantly in any group over time.

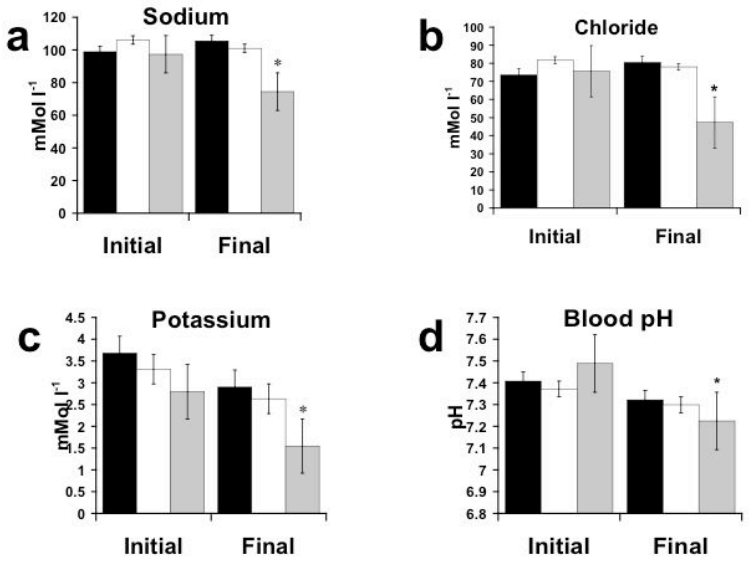

Figure 6.2 Changes in electrolyte concentrations and blood pH between initial and final blood samples in *Litoria caerulea* infected with *Batrachochytrium dendrobatidis*. The bar graphs show mean values (± s.e.m.). For this graph uninfected individuals are separated from aclinically-infected individuals. a, Plasma sodium concentrations were reduced in final blood samples from severely diseased *Litoria caerulea* (grey bars, $n = 7$, Paired T test, $P < 0.001$) but not in aclinically infected (white bars, $n = 3$) or uninfected (black bars, $n = 2$). b, Plasma chloride concentrations were reduced in final blood samples from the same severely diseased *Litoria caerulea* (Paired t test, $P < 0.001$) but not in aclinically infected or uninfected amphibians. c, Plasma potassium concentrations were reduced in final blood samples from the same severely diseased *Litoria caerulea* (Paired T test, $P = 0.036$) but not in aclinically infected or uninfected amphibians. Blood pH was reduced in final blood samples from the same severely diseased *Litoria caerulea* (Paired T test, $P = 0.006$) but not in aclinically infected or uninfected amphibians.

Discussion

We provide evidence that severe chytridiomycosis causes decreases in blood pH and plasma osmolality sodium, potassium, magnesium and chloride concentrations. Reduction in electrolyte concentrations could occur if electrolytes were diluted due to

increased water uptake or if there was an overall loss from the blood. We found no significant change from initial to final measurements in body mass, haematocrit, albumin or urea, indicating that hydration status is probably unaffected in severely diseased frogs. Therefore, it appears that the reductions in electrolyte concentrations were due to depletion from circulation rather than water uptake. The disproportionate loss of electrolytes compared with water signifies an imbalance in osmotic homeostasis.

Osmotic balance in amphibians is complex because there are multiple sites of regulation (Deyrup 1964, Heatwole & Barthalmus 1994, Jorgensen 1997). Amphibian skin is one site of regulation and is critical to water and electrolyte homeostasis (Deyrup 1964, Heatwole & Barthalmus 1994, Jorgensen 1997). Water flow results when an osmotic gradient is established by an exchange of electrolytes across the integument (Ussing & Zerahn 1951, Deyrup 1964, Erspamer et al. 1994, Jorgensen 1997). This tightly regulated transport is influenced not only by the salinity of the external aquatic environment, but also by plasma osmolality (Parsons et al. 1990) and skin circulation (Erspamer et al. 1994). Thus cutaneous osmoregulation and plasma osmolality are linked. Further, damage to amphibian skin can lead to fatal electrolyte imbalances (Wright et al. 2001). We suggest that *Bd* disrupts normal cutaneous transport, possibly through the alteration of electrolyte channels, leading to electrolyte loss and osmotic imbalance in diseased amphibians.

Pathogens are known to compromise function in other epithelia such as mammalian trachea and intestine (Kunzelmann et al. 2000, Berkes et al. 2003). Electrolyte transport is disrupted by pathogen adhesion to protein-based receptors (Cameron & Douglas 1996, Kunzelmann et al. 2000, Berkes et al. 2003) or by pathogen-secreted toxins that alter cell function (Kunzelmann et al. 2000, Berkes et al. 2003). For example, sodium transport and fluid balance were disrupted when a viral pathogen was introduced to mouse tracheal epithelium (Kunzelmann et al. 2000). This resulted from a down-regulation of amiloride-sensitive sodium channels in the apical membranes of the epithelium (Kunzelmann et al. 2000). It is possible that *Bd* disrupts sodium channels by a comparable mechanism. The selective barrier properties of frog skin are primarily determined by electrolyte transport

in the mitochondrial-rich cells of stratum granulosum (Masoni & Garcia-Romeu 1979, Brown et al. 1981, Larsen et al. 1987, Larsen et al. 1996, Ehrenfeld 1997), where *Bd* is found in severe infections (Berger et al. 1998, Berger et al. 1999b, Berger 2001, Berger et al. 2005c, Puschendorf & Bolanos 2006, North & Alford 2008). This hypothesis is further supported by Ussing chamber tests (Ussing & Zerahn 1951) using skin samples from infected *Bufo woodhousii,* which showed that active sodium transport was reduced in diseased toads under short-circuit conditions (Voyles et al. 2005). However, these results did not clarify if the loss of sodium and other electrolytes could occur via the skin or other excretion pathways. More specific tests investigating disruption of electrolyte transport are underway.

Although our results do not detail the exact mechanism by which *Bd* disrupts epidermal functioning, the severe reduction in plasma electrolytes is a plausible cause of mortality. Reduced plasma osmolality and reduced plasma electrolyte concentrations, particularly hyponatremia (low sodium) and hypokalemia (low potassium), are potentially life-threatening conditions because these electrolytes are crucial in cell membrane function. In addition, sodium and potassium facilitate action potential conduction in smooth and cardiac muscle and are important in multiple physiological processes.

The three *L. caerulea* that appeared healthy despite infection had no significant changes in plasma osmolality or electrolyte concentrations. These results suggest that electrolyte reductions occur only in terminal stages of infection and may account for the neurological signs such as muscle tetany that precede death (Berger 2001). While most amphibians can tolerate changes in plasma electrolyte levels (Deyrup 1964), the observed decrease of approximately 30% plasma sodium and 50% plasma potassium concentrations in diseased frogs may be too extreme. Additional research is needed to resolve whether these conditions lead to death through cardiac arrhythmia, myocardial failure, organ failure, or a combination of these and other factors.

Determining how *Bd* kills amphibians is essential to understanding the biology of chytridiomycosis. Additionally, research on the mechanisms of pathogenesis may

explain why there is interspecific variation in susceptibility to the disease, a key question for amphibian conservation. Resolving the pathophysiological effects will provide crucial information for researchers, wildlife managers and veterinary clinicians in treating captive frogs and facilitating the recovery of wild frog populations that are currently affected by the disease.

CHAPTER SEVEN

Pathogenesis of amphibian chytridiomycosis: a pathological perfect storm?

Chapter overview

This chapter describes multiple experiments aimed at understanding pathogenesis of chytridiomycosis using pathophysiological techniques. The chapter is presented as a manuscript for publication, listing the authors involved, although it has not yet been submitted. The majority of the work presented in this chapter is my own. The intellectual and technical contributions from other authors are outlined in the preface.

Authors: Jamie Voyles, Sam Young, Lee Berger, Craig Campbell, Wyatt F. Voyles, Anuwat Dinudom, David Cook, Rebecca Webb, Ross A. Alford, Lee F. Skerratt, Virginia Boone, Rick Speare

Abstract

Fungi are rarely highly virulent as primary pathogens in terrestrial vertebrates and yet the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), which causes the skin disease chytridiomycosis, is implicated in global amphibian declines and extinctions. How a superficial skin fungus could lead to catastrophic extirpations worldwide is perplexing. With few examples of disease-induced extinctions, chytridiomycosis is the subject of intensive investigations aimed at understanding disease emergence and its threat to global biodiversity. Central to these investigations is resolving the mechanism by which *Bd* causes death. Here we show a sequential alignment of pathophysiological changes associated with chytridiomycosis which are initially localized to the skin but progressively alter systemic functioning, leading to mortality. Experimentally infected green tree frogs (*Litoria caerulea*) had inhibited electrolyte transport in the skin and

reduced plasma electrolyte concentrations with asystolic cardiac arrest as the terminal event. The unique importance of the skin in maintaining amphibian homeostasis, and the ability of *Bd* to disrupt critical cutaneous functions are two key factors that make a life-compromising pathophysiology and explain how mortality can occur in a wide range of amphibian species. The disease characteristics of chytridiomycosis, taken separately, might not be so devastating, but together create a "perfect storm" of concomitant variables that threaten amphibians worldwide. That these factors have converged, resulting in a global pandemic, provides insight into infectious diseases and the associated risks for the loss of biodiversity.

Introduction

The phrase “perfect storm” has been used to describe the convergence of multiple variables creating favourable conditions for unusual, but not unexplainable, events. The phenomenon of worldwide amphibian declines due to chytridiomycosis may well illustrate such a scenario. Initially there was a reluctance to accept disease as a direct driver of declines and extinctions (Alford & Richards 1997, Hero & Gillespie 1997, McCallum 2005). However, *Bd* is now recognized for its ability to spread rapidly though amphibian populations, infect numerous, phylogenetically distant species, cause high mortality and persist even at low host densities (Berger et al. 1998, Retallick et al. 2004, Woodhams & Alford 2005, Lips et al. 2006, Skerratt et al. 2007). These disease characteristics not only render population recovery from chytridiomycosis especially difficult, but also present strong empirical evidence for disease-induced extinctions (De Castro & Bolker 2005, Mitchell et al. 2008). A “perfect storm” of severe disease can best be explained if all components, and their spatiotemporal alignment, are considered. In the case of chytridiomycosis, a gap remains in our understanding of all variables and a fundamental question remains unanswered: how does *Bd* kill amphibians? Resolving the mechanism of pathogenesis is imperative to demonstrate how a superficial skin fungus can cause devastating declines (Daszak et al 2003).

The details of the pahtogenesis of chytridiomycosis have evaded scientists for several reasons. First, cutaneous fungal infections are not commonly fatal without other predisposing factors (Schaechter et al. 1993, Mueller et al. 2004); so there were no similar systems for comparison. Second, the taxonomy of the organism was initially unknown and required creation of a monotypic genus in the Chytridiomycota, a phylum of fungi not previously known as pathogens of vertebrates (Longcore et al. 1999). Third, the location of the fungus in the most superficial layers of skin (Berger et al. 1998, Berger et al. 1999b 2005c) and the minimal host reaction to infection (Berger et al. 2005c, Woodhams et al. 2007) are unusual for fatal infections in vertebrates. Finally, there are no consistent pathological changes in internal organs of diseased amphibians detectable using light microscopy (Berger et al. 1998), rendering traditional methods used to understand pathogenesis ineffective. At the molecular level, differential expression of life-stage specific peptidase genes suggests *Bd* pathogenicity factors (Rosenblum et al. 2008). However, as in most diseases, determining the proximate cause of death in an infected host is inherently challenging because multiple physiological systems shut down prior to death. Given the cryptic mechanisms of disease development in chytridiomycosis, it was necessary to investigate the functional response to infection using pathophysiological techniques.

Amphibian skin is unique among terrestrial vertebrates because it is actively involved in the exchange of respiratory gases, water and electrolytes (Jorgensen et al. 1954, Alvarado & Kirschner 1963, Deyrup 1964, Moore & Lofts 1964, Fischbarg & Whittembury 1977, Shoemaker 1977, Larsen et al. 1987, Boutilier et al. 1992, Erspamer et al. 1994, Jorgensen 1994a, b, 1997, Wright et al. 2001, Word & Hillman 2005). In infected frogs, *Bd* is confined to the outer layers of the epidermis with higher intensities of infection in the ventral areas and the digits (Berger et al. 1998, Pessier et al. 1999, Puschendorf & Bolanos 2006). Due to the role of amphibian skin in maintaining osmotic balance, other studies have suggested that *Bd* might disrupt cutaneous osmoregulatory functioning (Berger et al. 1998, Voyles et al. 2005). To investigate this possibility, we performed multiple, linked infection experiments with green tree frogs (*Litoria caerulea*), which are susceptible to chytridiomycosis in laboratory infection experiments (Berger et al. 2005b).

Methods: General

While tracking the development of infections, we measured epidermal electrolyte transport in isolated skin preparations with Ussing chambers, measured biochemical parameters in blood and urine, assessed changes in epidermal condition from biopsy samples, and followed cardiac electrical activity using implanted biotransmitters. To determine if the changes in biochemical parameters are specific to chytridiomycosis, an additional infection experiment was performed in which an electrolyte-enriched solution was administered to frogs that became severely diseased. Also, the laboratory results from severely diseased *L. caerulea* were compared with blood values from free-living, healthy *L. caerulea* and *L. caerulea* with other lethal conditions.

Animal care— Adult common green tree frogs (*Litoria caerulea*; n = 66, mean mass ± s.e.m: 62.46 ± 2.4 g) were collected in January and February 2007 and 2008 from residential areas of Townsville, Queensland. Each frog was collected with a new plastic bag and then transferred to individual plastic containers (200x240x330 mm^3) in temperature- (18-23ºC) and light- (12L/12D) controlled facilities at James Cook University, Townsville, Australia. Frogs were fed vitamin-dusted crickets (medium-sized, Pisces Inc., Kenmore, Australia) *ad libitum* twice per week. Tap water (250 ml) was changed twice a week until experimental exposures began then tap water was replaced by 20% Holtfretter's solution (in mM: NaCl (6), KCL (0.06), $CaCl_2$ (0.09), $NaCO_3$ (0.24), pH 6.5, 250 ml). Containers were maintained in a level position so water covered the bottom but frogs were able to climb up the dry walls.

Diagnosis of Batrachochytrium dendrobatidis *infections*— To confirm frogs were not infected with *Bd* prior to inoculation, swabs samples were collected from each individual frog by rubbing a sterile cotton swab over the ventral surfaces and digits (Hyatt et al. 2007) and polymerase chain reaction (PCR) for *Bd* was performed (Taqman real-time PCR assay, Boyle et al. 2004). Following exposure to *Bd*, frogs were swabbed at intervals of 6 to 12 days. All swab samples were analysed in triplicate and compared

with Australian Animal Health Laboratory zoospore standards to determine zoospore equivalents.

Culture of Batrachochytrium dendrobatidis— Isolates were originally obtained from diseased frogs, cultured on tryptone/gelatin hydrolysate/lactose (TGhL) agar with antibiotics (Longcore et al. 1999) and then maintained in TGhL broth at 4°C and passaged every 2 to 3 months. The *Bd* isolate used for parts 1-5 was cultured from a wild adult *Litoria rheocola* from Tully River, Australia, identified as TullyRiver-L.rheocola-06-LB-1-p9 following the naming scheme proposed in Berger et al. (2005b). The isolate used for experiment 6 was from a juvenile *Litoria lesueuri*, GibboRiver-L.lesueuri-00-LB-1-p34. This isolate had been cryoarchived soon after isolation 7 years prior (Boyle et al. 2003). Zoospores for animal infections were harvested using dilute salt solution (in mMol: KH_2PO_4 (1), $CaCl_2.H_2O$ (0.2), $MgCl_2.2H2O$ (0.1)) after 4 d on agar plates maintained at 23°C and were counted in a haemocytometer (Boyle et al. 2004, Berger et al. 2005b).

Animal infections— Frogs were randomly assigned to exposure and control groups and exposed to *Bd* in plastic containers via shallow immersion in a bath of Holtfretter's solution containing zoospores. Dose varied by experiment (details below). Uninfected control frogs were held in a bath of Holtfretter's solution with added dilute salt solution collected from agar plates without *Bd* cultures. After 24 h, frogs were moved to fresh containers with 20% Holtfretter's solution (pH 6.5) that was changed twice weekly for the rest of the experiment.

Disease assessment— Frogs were monitored daily for clinical signs of chytridiomycosis including lethargy, inappetence, cutaneous erythema, irregular skin sloughing, abnormal posture (hind legs abducted) and loss of righting reflex. Righting reflex tests was done by placing the frogs in dorsal recumbency and recording the time taken to reorient to a normal position. Clinical signs were scored to provide a semi-quantitative assessment of the severity of disease. Frogs with no clinical signs of infection scored a 0 and were designated as aclinical. Frogs scoring 1, 2, or 3 were designated as clinically diseased

according to severity of clinical signs (1: lethargy and inappetence. 2: lethargy, inappetence, cutaneous erythema, and irregular skin sloughing. 3: lethargy, inappetence, cutaneous erythema, irregular skin sloughing, abnormal posture and loss of righting reflex).

Statistics— All data were analysed using R, version 2.7.1. Due to the high number of dependent variables, plasma and urine biochemical parameters were first analysed for dimension reduction using Principle Components Analysis. Four or more principle components were required to explain >75% of the data. Considering the high degree of independence among the variables, a general linear model was used with a Bonferroni correction technique: significance at $P = 0.004$ (plasma) and $P = 0.006$ (urine). A Bonferroni correction was also used for the Ussing chamber results, significance at $P = 0.012$. All data sets were tested for normality (Q-Q plots and Shapiro-Wilk's test), homogeneity of variance (Bartlett's test) and, where appropriate, sphericity (Maulchy's test). The results for PCR were log-transformed prior to statistical tests.

Methods: Exposure experiments

Blood, urine and skin biopsy samples were collected prior to exposure and over the course of infection to evaluate changes in physiological biochemistry and epidermal condition. Infected frogs reached terminal stages of infection asynchronously and therefore, to control for temporal sampling effects, we simultaneously tested a severely diseased frog with an uninfected frog and an infected but aclinical frog. Data were grouped by collection period: pre infection, post infection and late infection (following the first death, 60 d post infection). Due to the area of skin required for Ussing chamber (electrolyte transport) experiments, these tests could only be completed once, during late infection; sampling occurred when infected frogs showed clinical signs of severe disease.

For methods Parts 1-5, frogs in exposure groups were held in rectangular plastic containers (170x110x70 mm^3) that contained a 20% Holtfretter's bath solution (50 ml)

inoculated with 5 x 10^5 *Bd* zoospores. Frogs in control groups were held in a solution inoculated with an equivalent volume of dilute salt solution with no *Bd* zoospores.

Part 1. Plasma Biochemistry

We measured a large range of plasma biochemical parameters to assess functioning of, or damage to, organs including liver, kidney, muscle and pancreas. Blood samples were collected 20 d prior to exposure, 30 d post infection and at late stages of infection when infected frogs showed obvious clinical signs of disease. Blood samples (< 1% frog weight) were collected with a heparinised syringe and needle via cardiac puncture after anaesthesia by shallow immersion in 0.1% MS222 solution (tricaine methanesulfonate, Sigma Chemical) buffered with sodium bicarbonate (0.4%, Sigma Chemical), which does not kill *Bd* (Webb et al. 2005). Although anaesthesia for blood sampling can be stressful for amphibians, it does not alter blood electrolyte concentrations (Andersen & Wang 2002). Blood samples were collected into 0.6 ml lithium heparin tubes (Microtainer, Becton-Dickson), immediately centrifuged (10,000 g for 10 m) and the supernatant was decanted and frozen at -80°C. Plasma was later analysed for 12 biochemical parameters: calcium, chloride, potassium, sodium, aspartate aminotransferase, creatine kinase, albumin, bile acids, globulin, glucose, phosphorus and total protein (VetScan VS2 Chemistry Analyzer, Abaxis Inc., Union City, CA 94587).

Part 2. Urine Biochemistry

To avoid interfering with osmotic conditions, urine samples were collected opportunistically, in clean plastic containers while swabbing for PCR, rather than by forced expression. Urine samples were analysed for six biochemical parameters (osmolality, sodium, potassium, magnesium, chloride, calcium) at Gribbles Pathology (Melbourne, Australia). Seven additional biochemical parameters (glucose, bilirubin,

urobilinogen, ketones, protein, blood and pH were measured using Multistix Reagent Strips for Urinalysis (Bayer Diagnostics).

Part 3. Histology of skin biopsies

Small (1-2 mm^2) skin biopsies were collected from the ventral abdominal region (2-3 cm lateral to midline) of anaesthetized frogs following blood sample collections (20 d prior to exposure, 30 d post infection and in the late stages of infection). Biopsy incisions were closed with Vet Bond Tissue Adhesive (3M Animal Care Products). Skin samples were fixed in 10% buffered neutral formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin using routine histological techniques (Humason 1967, Berger et al. 2002, Olsen et al. 2004).

Part 4. Electrolyte transport in isolated skin samples

Following collection of final blood samples, frogs were transported to the University of Sydney (Sydney, Australia). Immediately after neural disruption, ventral skin samples (0.8 cm^2) were collected from the lower abdominal pelvic patch region and mounted in a modified Ussing chamber. Apical and basolateral surfaces of the monolayer were perfused with a modified frog Ringer's solution (in mM: NaCl (112), KCl (2.5), D-glucose (10), Na2HPO4 (2), CaCl2 (1), MgCl2 (1), Na+-HEPES (5), HEPES (5), pH 7.4) maintained at room temperature. The tissue was allowed to equilibrate prior to experimentation. All tests were carried out under open-circuit conditions (Ussing & Zerahn 1951, Kunzelmann et al. 2000). The transepithelial potential difference (V_{te}) was measured with reference to the blood side of the epithelium, and equivalent short-circuit current calculated according to Ohm's law using the Acquire and Analyse software (Physiologic Instruments, San Diego, CA). The rate of sodium absorption across the frog skin samples was assessed as the component of the short circuit current blocked by the addition of amiloride (10 µM, Sigma Chemical) to the apical solution. Amiloride is

known inhibitor of epithelial sodium channels (Benos 1979, Benos et al. 1981, Sariban-Sohraby & Benos 1986). We also examined the responsiveness of the skin samples to carbachol (100 µM, Sigma Chemical), a muscarinic agonist that activates chloride secretion in frog skin (Alvarado et al. 1975), and noradrenaline (10 µM, Sigma Chemical), an adrenergic agonist that activates sodium absorption and chloride secretion (Castillo & Orce 1997).

In a test of the toxin hypothesis, we also used a variety of techniques aimed at filtering a possible toxin from *Bd* cultures. The responsiveness of skin functioning from control *L. caerulea* skin samples were subjected to following treatments: 1) culture supernatant that was filtered to remove *Bd* sporangia and zoospores using a 0.2 micron filter, 2) solution of *Bd* that had been washed to remove media, resuspended and bead-beaten for protein extraction, 3) water collected from a container with a clinically diseased (terminal) *L. caerulea* and filtered to remove *Bd*, and 4. a control dilute salt solution (in mMol: KH_2PO_4 (1), $CaCl_2.H_2O$ (0.2), $MgCl_2.2H2O$ (0.1). These solutions were randomized and blindly introduced to the apical side of the epithelium following tissue equilibration.

Part 5. Cardiac biotelemetry

Approximately 30 d after collections, eight frogs were anesthetized by shallow immersion in 0.2% MS-222 (as above) to surgically implant cardiac biotransmitters. Procedures for ETA Surgical manual (Data Sciences International, 2001) for rats were followed with the following modifications.

Anaesthetised frogs were positioned in dorsal recumbency in a shallow oxygen- perfused pool of sterile 0.9% saline solution and were intermittently flushed with solution. A 35 mm skin and abdominal musculature incision was made along the ventral abdominal midline, caudal to the sternum. Implants (Chronic use TA10ETA-F20, Data Sciences International) were positioned in the abdominal cavity and leads were shortened to approximately 20 mm to accommodate the small body size. Biotransmitter leads were

secured in the left and right sternal musculature with single interrupted non-absorbable sutures (4-0 Prolene monofilament, Ethicon Inc.) and the overlying skin incision closed using the same suture pattern and reinforced with Vet Bond Tissue Adhesive. Biotransmitters were secured intracoelomically via incorporation into the abdominal musculature suture line; both abdominal musculature and skin incisions were closed using single interrupted non-absorbable sutures (4-0 Prolene monofilament, Ethicon Inc.). Frogs were monitored in a shallow pool of perfused saline for anaesthetic recovery. Antibiotics (50 mg/ml, 10 mg/kg enrofloxacin, Bayer Animal Health Australia Ltd.) and analgesics (1mg/ml, 1mg/kg, ketoprofen, Nature Vet Ltd.) were administered orally immediately following recovery from surgery. Frog patients were closely monitored for 24 hours and checked twice daily for one week following surgery. Enrofloxacin (5mg/kg) and ketoprofen (1 mg/kg) were applied topically on the dorsal skin surfaces daily for the following week.

Frogs were exposed to *Bd* four weeks following surgery. Frogs with cardiac biotransmitters were randomly assigned to control (n = 3) and exposure (n = 5) groups. Exposures to *Bd* occurred simultaneously with other frog groups and with identical procedures (described above). Cardiac electrograms were collected every 7 d and analysed for heart rate and signal amplitude. Once clinical signs became obvious in infected amphibians we continuously monitored and recorded all cardiac electrical activity.

Part 6. Electrolyte Supplementation

Frogs in exposure groups were held in round plastic containers (diameter- 12 cm x 6.5 cm) containing 35 ml of 20% Holtfretter's solution inoculated with 1 x 10^6 zoospores. Frogs used to control for infection were held in the same volume of solution with no *Bd* zoospores. Infected frogs that became diseased were assigned to a treatment group (receiving electrolyte supplementation) or an untreated group (no electrolyte supplementation).

An electrolyte treatment solution was based on recommended osmolality values for maintaining electrolyte balance in diseased amphibians (12% Whitaker-Wright solution in mM: NaCl (242), $MgSO_47H_20$ (4.3), $CaCl_2$ (2.85), KCl (2.85): Wright et al. 2001). Given the damage to electrolyte transport processes in amphibian skin and that the gut is able to preferentially absorb electrolytes (Policies et al. 2005, Teramoto et al. 2005), we opted to administer electrolyte solutions orally (via a crop tube) rather than by bath. Intravenous electrolyte supplementation is risky without continuous blood tests (Rainger & Dart 2006). Based on previous results showing losses of approximately 30% plasma sodium and 50% plasma potassium in amphibian chytridiomycosis (Chapter 6), a target concentration and dose per body weight was calculated based on formulae for treating hyponatremia (Adrogue & Madias 2000) and hypokalemia (Gennari 1998) in human patients with electrolyte imbalances.

Frogs in the treatment group (n = 6) received the oral supplement when they could no longer right themselves, or took longer than 10 s to reorient. Untreated frogs (n = 9) were handled identically, the crop tube was inserted as for treated frogs, but no electrolyte was administered. Following treatment, frogs were returned to their containers with fresh Holtfretter's solution so skin shedding could be assessed at each treatment period. Treatments were re-administered every 4-6 h. Time from initial loss of righting reflex to death (once no cardiac activity could be detected) was recorded in hours.

Part 7. Plasma biochemistry of free-living *Litoria caerulea*

Adult common green tree frogs (*Litoria caerulea*) were collected (as described above). Frogs were swabbed to confirm the animals were free of *Bd*, anaesthetised and blood samples were collected (as described above) within 48 hours of capture. Frogs were then released at the original collection point.

Part 8. Plasma biochemistry in *Litoria caerulea* with other lethal conditions

Blood was collected from five diseased wild adults of *L. caerulea* between January 2007 and May 2008 following anaesthesia (as described above). Two frogs presented with severe generalized trauma, one with bacterial septicaemia, one with severe sparganosis and thigh muscle abscessation, and one with progressive weight loss, anorexia and lethargy of unknown aetiology.

Results

All *L. caerulea* were confirmed to be negative for *Bd* with PCR analysis prior to exposures. Following exposure, swabs were collected from control frogs (n = 10), from infected but aclinical frogs (n = 8) and from frogs that became severely diseased (n = 15). These sample sizes combine frogs from the cardiac experiment and blood biochemistry experiment because the experiments occurred simultaneously. Zoospores equivalents were significantly higher in frogs that became severely diseased (repeated measures ANOVA, $P < 0.001$; Figure 7.1).

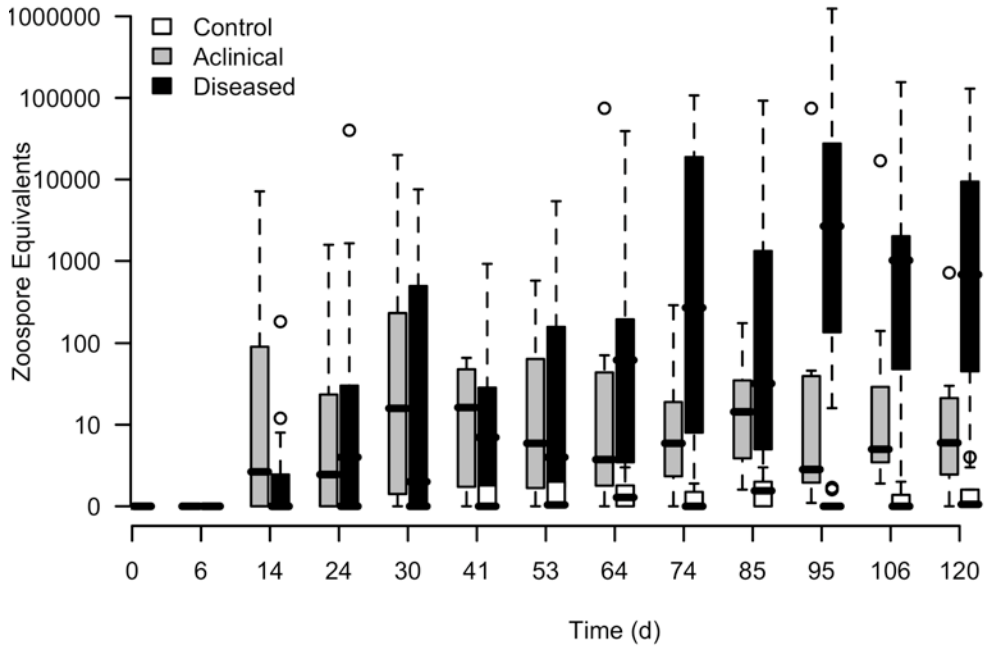

Figure 7.1 *Batrachochytrium dendrobatidis* load (zoospore equivalents) in experimentally infected *Litoria caerulea*. Results determined from real-time PCR on skin swabs collected over time (d). Frogs are grouped according to disease status: control (n = 10), aclinical (n = 8) and diseased (n = 15).

Part 1. Blood biochemistry

Blood samples were collected from control (n = 7), aclinical (n = 7) and diseased (n = 11) frogs, although sample sizes for each biochemical parameter varied depending on the analysis. Limited plasma volume restricted the number of tests that could be performed in some parameters, such as chloride. Plasma electrolyte concentrations were reduced in frogs with severe chytridiomycosis (Figure 7.2): plasma potassium (repeated measures ANOVA, P = 0.028) and plasma sodium (repeated measures ANOVA, P = 0.002*; Figure 7.2b). Of the 11 frogs that became diseased, all but one had plasma sodium concentrations that were below the detection limit of the available equipment (<100 mMol l^{-1}). For these frogs we used a conservative estimate of 99 mMol l^{-1} although plasma sodium concentrations were likely to be lower based on results from previous

work (Chapter six). Out of 10 additional blood biochemical parameters that were tested as markers of organ function and general health, none varied significantly (Table 7.1)

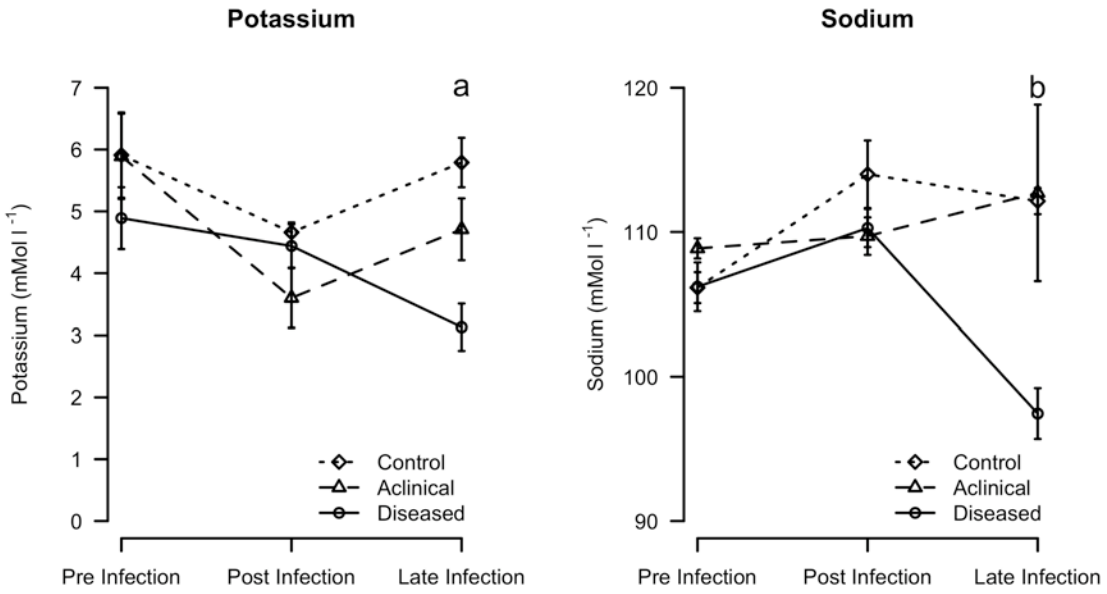

Figure 7.2 Blood plasma potassium and sodium concentrations in control and experimentally infected *Litoria caerulea* experimentally infected with *Batrachochytrium dendrobatidis* (dotted lines: control (n = 7), dashed lines: aclinical (n = 7), solid lines: diseased (n = 11). Lines show mean (± s.e.m) from three sample collections over the course of experimental infection.

Blood Biochemical Parameters						
	Pre Infection		**Mid Infection**		**Late Infection**	
	Mean ± s.e.m	**N**	**Mean ± s.e.m**	**N**	**Mean ± s.e.m**	**N**
CALCIUM (mMol l^{-1}): f = 0.5802, p = 0.679						
Control	2.34 ± 0.18	7	2.37 ± 0.14	7	2.43 ± 0.15	7
Aclinical	3.97 ± 1.09	7	4.08 ± 1.05	7	3.24 ± 0.13	7
Diseased	2.3 ± 0.16	10	2.33 ± 0.10	11	2.13 ± 0.15	10
CHLORIDE (mMol l^{-1}): f = 0.3189, p = 0.861						
Control	71.00 ± 1.23	6	86.25 ± 4.73	4	75.00 ± 4.26	4
Aclinical	73.00 ± 2.27	4	80.67 ± 6.25	6	77.40 ± 4.20	5
Diseased	70.10 ± 3.57	10	80.00 ± 3.01	6	67.00 ± 5.00	2
POTASSIUM (mMol l^{-1}): f = 3.0174, p = 0.028						
Control	5.91 ± 0.69	7	4.66 ± 0.16	7	5.79 ± 0.4	7
Aclinical	5.89 ± 0.69	7	3.6 ± 0.49	7	4.71 ± 0.50	7
Diseased	4.89 ± 0.50	10	4.44 ± 0.36	11	3.13 ± 0.39	10
SODIUM (mMol l^{-1}): f = 5.0167, p = 0.002						
Control	106.14 ± 1.08	7	114.85 ± 2.34	7	112.14 ± 0.91	7
Aclinical	108.86 ± 0.70	7	109.71 ± 1.30	7	112.71 ± 6.12	7
Diseased	106.20 ± 1.70	10	110.27 ± 1.32	11	97.45 ± 1.77	10
ASPARTATE AMINOTRANSFERASE (U l^{-1}): f = 0.4591, p = 0.7652						
Control	359.43 ± 50.37	7	292.66 ± 26.70	7	530.57 ± 169.83	7
Aclinical	218.87 ± 47.27	7	169.71 ± 31.56	7	494.57 ± 161.98	7
Diseased	275.1 ± 40.69	10	201.36 ± 22.92	11	637.8 ± 61.70	10
CREATINE KINASE (U l^{-1}): f = 0.3307, p = 0.856						
Control	2632.14 ± 734.87	7	2342.71 ± 399.71	7	2791.29 ± 447.9	7
Aclinical	1310.14 ± 218.56	7	1011.29 ± 259.10	7	2156.57 ± 428.48	7
Diseased	1778.9 ± 331.41	10	1416.09 ± 344.518	11	2834.38 ± 593.49	8
PHOSPHATES (mMol l^{-1}): f = 1.6238, p = 0.187						
Control	1.16 ± 0.15	6	1.23 ± 0.19	7	1.59 ± 0.11	7
Aclinical	1.41 ± 0.38	7	1.62 ± 0.2	7	1.55 ± 0.3	7
Diseased	0.84 ± 0.1	10	0.97 ± 0.07	11	1.72 ± 0.2	10
ALBUMIN (g l^{-1}), f-value= 0.9014, p = 0.472						
Control	19.29 ± 1.57	7	18.86 ± 1.78	7	22.57 ± 1.56	7
Aclinical	23.43 ± 1.88	7	21.57 ± 2.07	7	24.86 ± 2.77	7
Diseased	18.1 ± 2.29	10	18.27 ± 1.78	11	23.2 ± 1.55	10
URIC ACIDS (µMol l^{-1}): f =2.101, p = 0.098						
Control	5.33 ± 1.43	7	11.29 ± 4.00	7	16.29 ± 3.37	7
Aclinical	3.86 ± 1.34	7	8.0 ± 3.08	7	22.29 ± 6.09	7
Diseased	2.80 ± 1.76	10	8.18 ± 1.64	11	9.3 ± 2.08	10
GLUCOSE (mMol l^{-1}): f = 2.1930, p = 0.087						
Control	3.03 ± 0.31	7	2.5 ± 0.15	7	3.04 ± 0.22	7
Aclinical	2.63 ± 0.27	7	3.09 ± 0.27	7	3.11 ± 0.23	7
Diseased	2.58 ± 0.21	10	2.58 ± 0.13	10	2.18 ± 0.5	11
GLOBULIN (g l^{-1}): f = 0.2100, p = 0.1177						
Control	23.14 ± 2.04	7	22.4 ± 2.46	7	27.14 ± 1.7	7
Aclinical	28.71 ± 3.44	7	28.41 ± 2.41	7	37.1 ± 4.37	7
Diseased	26.7 ± 3.47	9	28.7 ± 3.6	9	38.4 ± 2.74	10
TOTAL PROTEIN (g l^{-1}): f = 1.843, p = 0.1412						
Control	42.57 ± 2	7	41.14 ± 3.84	7	49.71 ± 2	7
Aclinical	54 ± 4.09	7	53.57 ± 5.46	7	59 ± 6.67	7
Diseased	43.4 ± 4.9	10	45.73 ± 4.57	11	61.3 ± 3.85	10

Table 7.1 Plasma biochemical parameters from *Litoria caerulea* experimentally infected with *Batrachochytrium dendrobatidis*. Data sets tested with repeated measures ANOVA. Significant changes were detected in frogs that developed severe disease.

Hydration status in diseased frogs was not considerably altered. An increase of albumin and total protein with a decrease in body mass would indicate dehydration. In contrast overhydration is indicated by a decrease in total protein and albumin with an increase in body mass. Although there were slight increases in total protein and albumin, these changes occurred in all groups and were not statistically significant (Table 7.1). Body mass was slightly increased in the control group (in gms: Pre Infection: 46.32 ± 4.51, Post Infection: 45.25 ± 4.96, Late Infection 53.97 ± 5.53) but was unchanged in aclinical (in gms: Pre Infection: 42.21 ± 2.56, Post Infection: 40.1 ± 3.06, Late Infection: 43.7 ±1.56) and diseased groups (in gms: Pre Infection: 39.35 ± 3.75, Post Infection: 38.83 ± 2.59, Late Infection: 39.19 ± 3.29).

Part 2. Urine biochemistry

There were no significant differences in urine electrolytes, osmolality or pH among urine samples collected over the course of infection (Table 7.2). However, sample sizes were limited due to opportunistic collection of urine samples. Also, there was wide variation in urine parameters among individuals and the majority of data sets were not normally distributed. Therefore, these results should be interpreted with caution.

Urine Biochemical Parameters						
	Pre Infection		Mid Infection		Late Infection	
	Mean ± s.e.m	N	Mean ± s.e.m	N	Mean ± s.e.m	N
CALCIUM						
Control	1.84 ± NA	1	0.51 ± 0.15	4	0.14 ± 0.05	4
Aclinical	0.6 ± NA	1	0.08 ± NA	1	1.63 ± NA	1
Diseased	0.32 ± 0.16	2	0.13 ± 0.05	7	0.24 ± 0.1	4
CHLORIDE: f = 3.0757, p = 0.1204						
Control	17 ± NA	1	21 ± 1.7	4	13.5 ± 1.66	4
Aclinical	25.00 ± NA	1	NA ± NA	0	47.00 ± NA	1
Diseased	21.00 ± 5.00	2	17.57 ± 1.09	7	23.50 ± 3.80	4
POTASSIUM: f = 7.2897, p = 0.0425						
Control	3.3 ± NA	1	2.83 ± 0.53	4	2.93 ± 0.39	4
Aclinical	1.3 ± NA	1	1.70 ± NA	1	9.10 ± NA	1
Diseased	4.7 ± 3.4	2	2.03 ± 0.23	7	3.00 ± 0.41	4
MAGNESIUM: f = 0.3529, p = 0.7907						
Control	0.5 ± NA	1	0.28 ± 0.09	4	0.43 ± 0.14	4
Aclinical	0.20 ± NA	1	0.20 ± NA	1	0.50 ± NA	1
Diseased	0.10 ± 0.10	2	0.24 ± 0.37	7	0.23 ± 0.06	4
SODIUM: f = 3.0321, 0.1559						
Control	15 ± NA	1	23.25 ± 1.38	4	14.50 ± 2.50	4
Aclinical	29.00 ± NA	1	27.00 ± NA	1	32.00 ± NA	1
Diseased	23.50 ± 14.5	2	19.14 ± 2.27	7	24.25 ± 3.42	4
OSMOLALITY, f = 1.1418, p = 0.08						
Control	20 ± 0	2	57.83 ± 15.49	6	35.50 ± 8.45	4
Aclinical	NA ± NA	0	37.50 ± 10.50	2	102.33 ± 61.36	3
Diseased	96.50 ± 10.5	2	46.75 ± 4.51	11	58.11 ± 6.22	9
pH, f = 0.5575, p = 0.6982						
Control	7.5 ± 0.5	2	7.83 ± 0.13	6	7.58 ± 0.05	5
Aclinical	7.5 ± NA	1	6.85 ± 0.85	2	6.57 ± 0.56	3
Diseased	8 ± 0.5	2	7.51 ± 0.174	11	7.07 ± 0.27	10
HAEMOLYZED BLOOD, f = 0.8963, p = 0.4959						
Control	0 ± 0	2	0 ± 0	6	0 ± 0	5
Aclinical	0 ± NA	1	0 ± 0	2	80 ± 0	4
Diseased	0 ± 0	2	2.92 ± 2.17	11	96 ± 29.93	10

Table 7.2 Urine biochemical parameters from *Litoria caerulea* experimentally infected with *Batrachochytrium dendrobatidis*. Data sets tested with ANOVA repeated measures. There were no positive reactions for the following urine parameters: glucose, bilirubin, ketones, protein, urobilinogen (data not shown).

Although none of these parameters varied significantly, one urine parameter, haemolysed blood, suggested a treatment effect. There were only positive reactions on the reagent strips for haemolysed blood in the urine of aclinical and diseased frogs whereas no blood was detected in control frogs (Table 7.2, Figure 7.3). These changes were not statistically significant but the pattern suggested a group effect and warranted additional investigation (Figure 7.3).

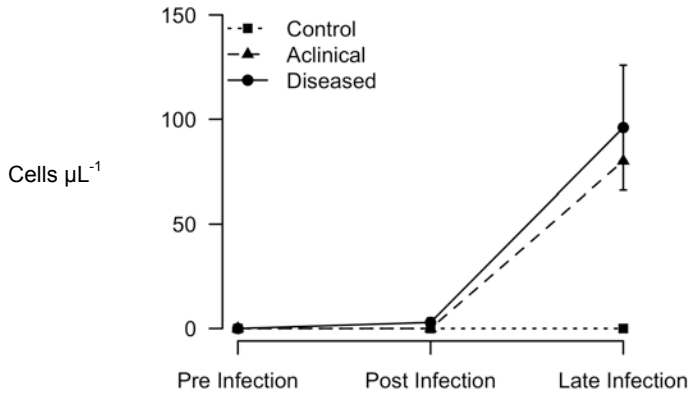

Figure 7.3 Haemolysed blood results in urine samples from control and experimentally infected *Litoria caerulea*. At late infection samples were collected from control (n = 5), aclinical (n = 4) and diseased (n = 10) frogs.

Negative results from commercial urinary sediment stains (Clay Adams Sedi-Stain, Becton Dickinson and Co.) in samples that tested positive for haemoglobin (n = 5) ruled out haematuria. In addition, kidney function appeared unaffected as there were no significant changes in plasma phosphorous levels (Table 7.1) and previous studies showed urea was not elevated in diseased frogs (Chapter six). Histological analysis of kidney sections suggested the possibility of haemoglobinuria (haemoglobin casts could be seen in the tubules), but after following up with a specific stain for haemoglobin, the histological results were inconclusive (personal communication Karrie Rose).

Because haemoglobinuria could have resulted from intravascular haemolysis of erythrocytes in hypoosmotic conditions, we tested osmotic fragility of cane toad (*Bufo marinus*) erythrocytes using routine techniques (Aldrich & Saunders 2001). A sample of packed toad erythrocytes (50 µL) was added to a range of concentrations of sodium chloride solution (prepared in distilled water at concentrations from 0.01 to 5% with distilled water as a positive control). The absorbance of the supernatants (read at 540 nm, Biorad spectrophotometer) indicated that erythrocyte haemolysis occurred when sodium chloride concentrations were 0.2% or less (<34 mM/L).

Part 3. Histology of skin biopsies

Sporangia of *Bd* were not seen on any pre infection samples and the epidermis appeared healthy. At 30 d post infection light, focal infections were seen in frogs that became diseased, associated with mild focal lesions such as erosions. Skin samples collected in late stages of infection had typical severe changes with spongiosis associated with hyperkeratosis, spongiosis of the *stratum granulosum*, irregular epidermal layers, erosions, and microscopic ulcerations where infected epidermis had sloughed.

Part 4. Electrolyte transport in isolated skin samples

Under resting conditions, the rate of net electrolyte transport across the skins, as assessed by the equivalent short circuit current, was significantly lower in infected (n = 7) skin samples (27.11 ± 8.12 µA per cm^2) than in control (n = 7) skin samples (65.44 ± 11.02 µA per cm^2; Student's T-test, P = 0.009; Figure 7.4a). This decrease in electrolyte transport rate was accompanied by a reduction in the transepithelial resistance (infected: 400.95 ± 86.37 ohms per cm^2, control: 939.94 ± 109.17 ohms per cm^2; Student's T-test, P = 0.0006; Figure 7.4b). This was due to marked inhibition of the rate of absorption of sodium (Figures 7.4 d and e), assessed as the component of the short circuit current that was blocked by amiloride (infected: 17.99 ± 9.80 µA per cm^2, control: 59.10 ± 14.72 µA per cm^2, Student's T-test, P = 0.038: Figure 7.4d). The short circuit current in the presence of amiloride did not differ between infected (9.12 ± 1.32 µA per cm^2) and control (6.34 ± 0.75 µA per cm^2) skin samples (Student's T-test, P = 0.079: Figure 7.4e). Additionally, we found that the responses to both carbachol (infected: 10.34 ± 5.22 µA per cm^2, uninfected: 44.84 ± 10.98 µA per cm^2, Student's T-test, P = 0.015: Figure 7.4f) and noradrenaline (infected: 4.l8 ± 2.45 µA per cm^2, uninfected: 61.48 ± 13.74 µA per cm^2, Student's T-test, P = 0.001: Figure 7.4g) were reduced in the skins of infected frogs. In our test of the toxin hypothesis, there was no detectable change in short-circuit current with any of the treatment solutions.

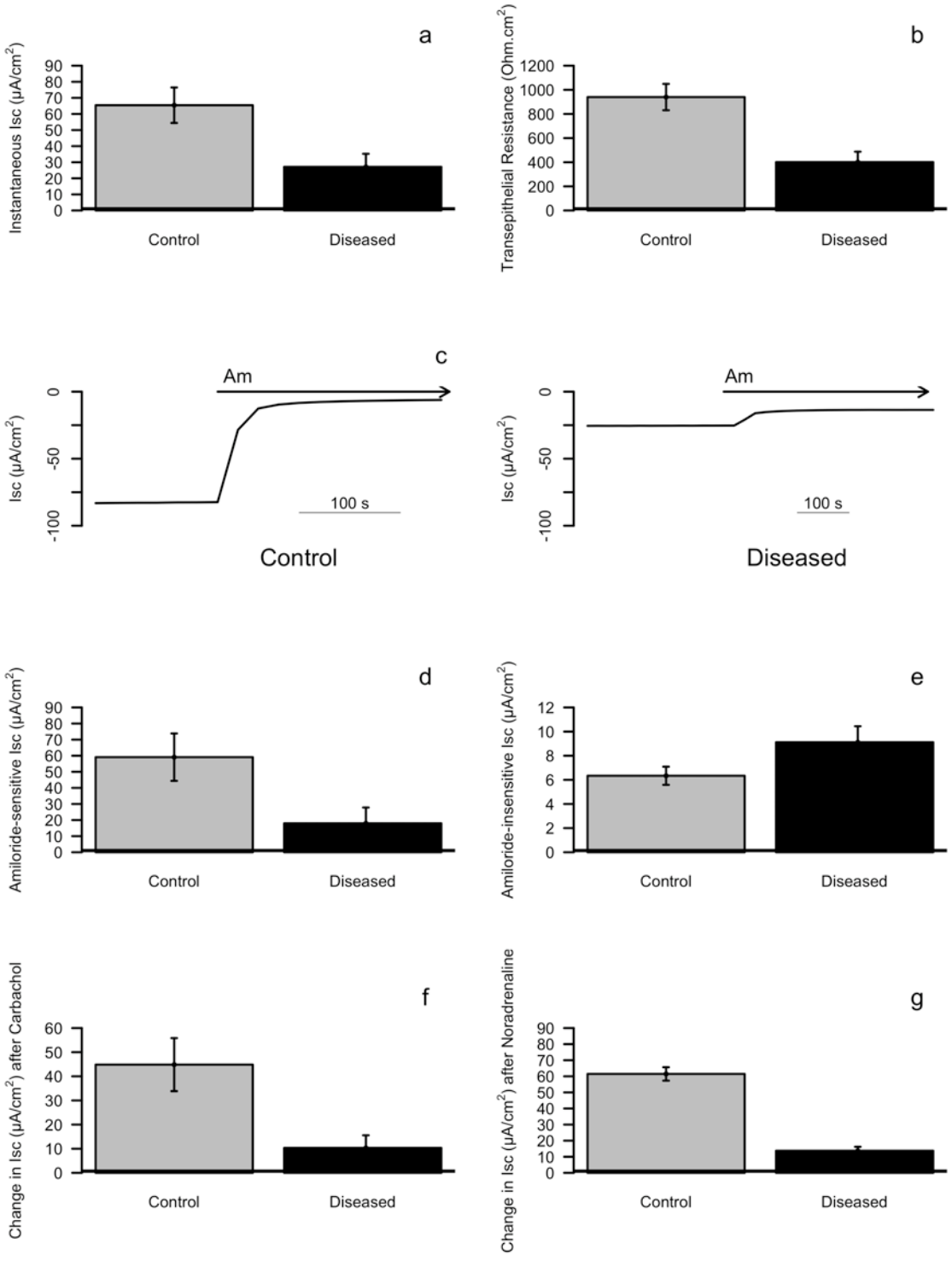

Figure 7.4 Electrolyte transport in ventral skin samples from *Litoria caerulea* experimentally infected with *Batrachochytrium dendrobatidis* in Ussing Chamber tests. Bar graphs show mean (± s.e.m) in skin samples from control (grey, $n = 7$) and diseased (black, $n = 7$) frogs in response to (d,e) amiloride, (f) carbachol and (g) noradrenaline. Tracings (c) show the response of control and infected skin samples to amiloride.

Part 5. Cardiac biotelemetry

Wet-field surgery was successful with no post-surgical complications. All eight frogs fully recovered within one hour after surgery and resumed normal behaviour. Cardiac implants were tested immediately following surgery. Ventricular depolarization was recorded but signal amplitudes were not sufficient to reliably determine other electrical activity such as depolarization of the atria. Electrical interference from non-cardiac muscles occurred in some frogs.

Of five frogs that became diseased and died, cardiac electrograms were collected from four individuals. Cardiac telemetry recordings demonstrated a consistent pattern of changes in each diseased frog. During the hours before death, heart rate slowed, ventricular depolarization amplitude decreased, and time of ventricular depolarization increased (Figure 7.5). These electrocardiographic changes progressed to cardiac standstill and asystolic arrest.

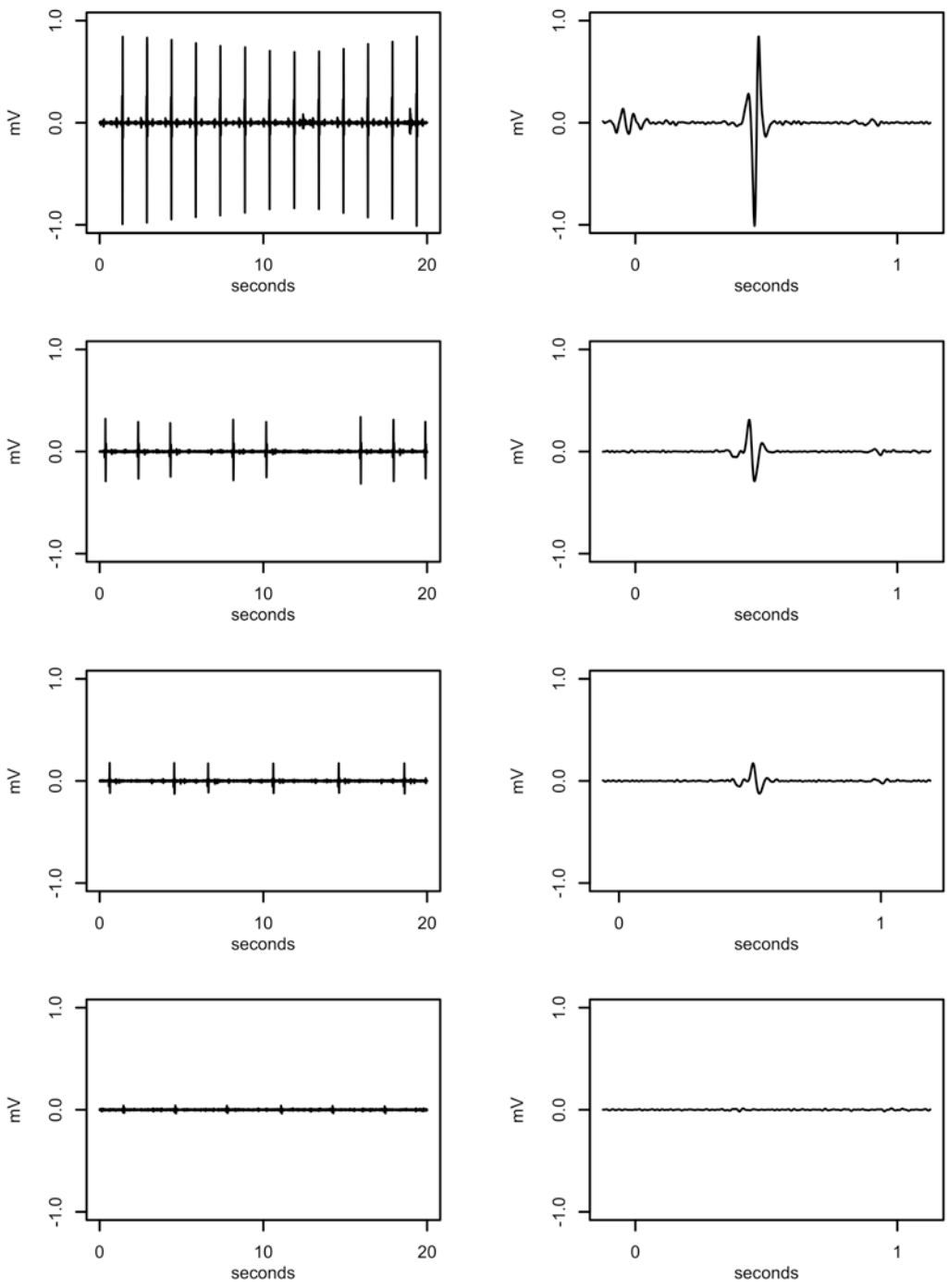

Figure 7.5 Original cardiac electrogram tracings from a *Litoria caerulea* with severe chytridiomycosis. Heart rate and signal amplitude were determined based on 20-second electrograms sweeps (left column). Cardiac electrical signal morphology was assessed using one-second sweeps (right column). Electrograms show cardiac electrical patterns consistent with asystolic cardiac arrest: slowing heart rate, decreasing ventricular depolarization amplitude and increasing time of ventricular depolarization from (first row) 18 to (second row) 3, (third row) 2 and (forth row) 0.5 hours prior to death.

Part 6. Electrolyte Supplementation

Of the 19 frogs exposed to *Bd*, 17 developed obvious clinical signs of disease between 25 and 58 d following exposure to *Bd*. Two frogs survived infection and were negative for *Bd* at the termination of the experiment and one frog died before treatment could be administered.

Following the initial supplementation, treatment frogs recovered normal posture and were considerably more active; one individual recovered sufficiently to climb out of the water on to the container walls and two individuals were able to jump to avoid capture. These signs of recovery were not seen in any untreated frogs. Treatment frogs ($n = 6$) lived more than 20 hours longer than untreated frogs ($n = 9$) (mean time post treatment: 32 ± 2.8 h, control: 10.7 ± 2.2 h, Student's T-test, $P < 0.001$). All diseased frogs continued to shed skin throughout the treatment period, regardless of supplementation. Although treatment continued every 4-6 hours and clinical signs were diminished in frogs with electrolyte supplementation, all frogs that became diseased eventually died.

Parts 7 and 8. Plasma biochemistry of free-living *Litoria caerulea* and in *Litoria caerulea* with other lethal conditions

We compared blood biochemical results from experimentally infected *L. caerulea* (in the late stages of infection) with those from free-living *L. caerulea* and *L. caerulea* with other lethal conditions. Sodium and potassium concentrations did not vary significantly among control, aclinical and free-living frog groups (Figure 7.6a and b). Plasma potassium concentrations in *L. caerulea* with severe chytridiomycosis are significantly different from those in control frogs (ANOVA Tukey post-hoc, $P = 0.009$*; Figure 7.6a) and those in frogs with other lethal conditions (ANOVA Tukey post-hoc, $P = 0.04$*; Figure 7.6a). Plasma sodium concentrations in *L. caerulea* with severe chytridiomycosis are significantly different from those in control frogs (ANOVA, Tukey Test, $p = 0.01$*; Figure 7.6b) and from those in free-living frogs (ANOVA, Tukey Test, $p = 0.003$*;

Figure 7.6b). Some frogs with other lethal conditions had reduced sodium concentrations (Figure 7.6b) but the variance was not homogenous (Levene's test for homogeneity of variance, $P = 0.197$)

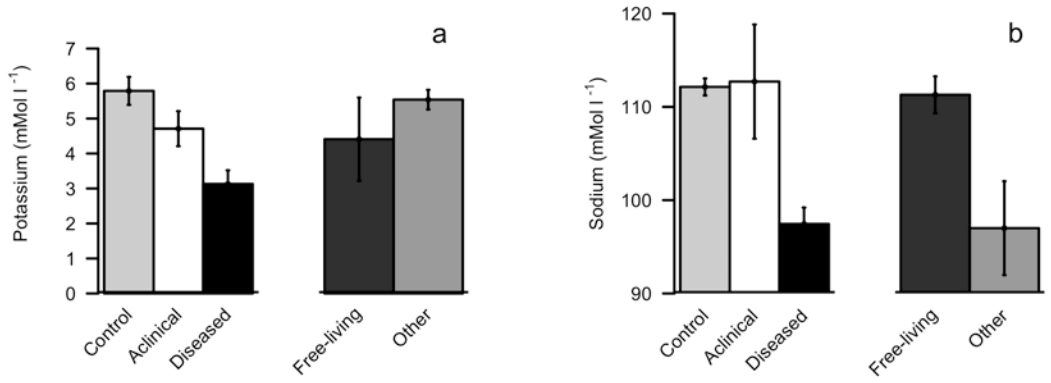

Figure 7.6 Blood plasma potassium (a) and sodium (b) concentrations in *Litoria caerulea* experimentally infected with *Batrachochytrium dendrobatidis*, free-living "normal" *L. caerulea* ($n = 5$) and *L. caerulea* ($n = 5$) with other lethal conditions. Results show means (± s.e.m).

Discussion

In this study severe clinical signs of disease appeared in *Litoria caerulea* with the highest burdens of *Bd*, concurrent with three critical changes in physiological systems: 1) inhibition of electrolyte transport across isolated ventral skin samples, 2) reductions in plasma electrolyte concentrations and 3) deterioration of cardiac electrical functioning. Our results show how disruption of normal osmoregulatory processes in amphibian skin and osmotic imbalance due to reduced electrolytes likely causes asystolic cardiac arrest in amphibians with severe chytridiomycosis.

First, the rate of net electrolyte transport and transepithelial resistance across the skin samples from infected frogs was significantly lower than those in uninfected skin samples (Figure 7.4), indicating that the effect of the infection was predominantly on sodium transport. These results demonstrate that *Bd* infection compromises electrolyte (sodium and chloride) transport and thus, osmoregulatory function in the skin of diseased frogs.

The exact cellular mechanisms are unknown and additional Ussing chamber research is needed to reveal further details. An experiment that incorporates chemical treatments could test the active transport system by isolating of specific cellular channels and pumps. For example, applying nystatin, which permeabolizes cell membranes, to the apical bath could test the functioning of the ATPase (sodium-potassium pump). The selective barrier properties of frog skin are primarily determined by electrolyte transport in the flask-shaped mitochondrial-rich cells (MR cells) of *stratum granulosum* (Masoni & Garcia-Romeu 1979, Brown et al. 1981, Larsen et al. 1987, Larsen et al. 1996, Ehrenfeld 1997). Follow up studies to histologically examine the MR cells and/or the ATPase pump (Choe et al. 2004) could further characterise the disrepair to the epidermal transport system, which appears to be a key factor in the pathogenesis of chytridiomycosis.

Second, plasma potassium and sodium concentrations were reduced in frogs with severe chytridiomycosis. An additional 10 blood and 13 urine parameters were tested as markers of organ function and general health; none of these blood parameters varied significantly. As hydration status of diseased frogs was unchanged, reductions in plasma potassium and sodium concentrations probably resulted from a loss of electrolytes from circulation rather than electrolyte dilution due to water uptake. These results agree with previous findings where electrolyte reductions were found in diseased frogs during an outbreak of chytridiomycosis in captive *L. caerulea* (Chapter six). Electrolyte imbalance, low plasma potassium (hypokalemia) and low plasma sodium (hyponatremia), could result from depletion via the epidermis or the kidney (Moore & Lofts 1964, Boutilier et al. 1992). Follow up tests on the urine of diseased frogs suggested the possibility of haemoglobinuria, which can result from intravascular haemolysis of erythrocytes in hypoosmotic conditions. Erythrocyte osmotic fragility is lower in amphibians than other vertebrates (Aldrich et al. 2006), and our tests indicate that *Bufo marinus* erythrocytes are relatively resistant to changes in concentrations of sodium chloride. The sodium chloride solutions probably did not adequately simulate the mixture of electrolytes normally present in the blood, and therefore additional work is needed to determine the importance of other plasma electrolytes (such as potassium and magnesium) in amphibian erythrocyte haemolysis. At this point we can draw no definitive conclusions regarding

intravascular haemolysis and although we found no indication of renal damage with plasma biochemistry, electrolyte loss via the kidney could not be ruled out. In contrast, the skin, which regulates bi-directional flux and overall balance of sodium and potassium (Larsen 1991, Boutilier et al. 1992), was severely damaged when assessed with histology and Ussing chamber tests. Thus the most parsimonious explanation for pathophysiology of chytridiomycosis is that the skin is the primary organ involved in electrolyte shifts and consequent osmotic imbalance.

Third, when severely diseased frogs were within hours of death, their cardiac electrical activity closely resembled agonal patterns associated with cardiac standstill, also known as asystolic or bradyasystolic cardiac arrest (Paradis et al. 1996), which can result from shifts in electrolyte balance. Once initiated by a triggering event such as an electrical conduction abnormality, this kind of cardiac death results from a cycle of myocyte ischemia, further conduction dysfunction and inadequate blood flow to the heart and extremities. Disruption of electrical conduction leads to mechanical dysfunction and eventual circulatory collapse. Several conditions can initiate this cycle: hypothermia, dehydration, hypovolemia, hypoxia, acidosis and electrolyte disturbances including shifts in calcium and potassium (Paradis et al. 1996). However, most of these conditions can be ruled out in this study. Hypothermia can be discounted because there were no changes in ambient temperatures. As body mass, total protein and albumin were unaffected, dehydration and hypovolemia are unlikely. Blood oxygen saturation was measured in each frog three times over the course of infection (data not presented) using a pulse oximeter (TuffSat, Datex-Ohmeda Inc.) on the thigh of the hind limb. Although pulse oximeters are not ideal for use in amphibians because readings are influenced by skin pigmentation, we detected a drop of approximately 20% in peripheral blood oxygen saturation in one individual *after* changes in electrical functioning were observed. This suggests that hypoxia was not the initiating event for cardiac electrical dysfunction. Therefore shifts in electrolytes, particularly potassium, and/or acidosis as the result of progressive acidosis are the most likely causes of bradycardia and eventual cardiac standstill in frogs with severe chytridiomycosis.

To determine whether the extent of electrolyte loss is specific to chytridiomycosis, we collected blood samples from uninfected, free-living *L. caerulea* and from frogs with other lethal conditions such as severe sparganosis, bacterial septicaemia and trauma. Lower limits of plasma electrolyte concentrations for *L. caerulea* are unknown but sodium and potassium concentrations did not vary significantly among control, aclinical and free-living frog groups, suggesting that electrolyte concentrations in our captive frogs were normal. In contrast, frogs that developed severe chytridiomycosis had significantly reduced sodium concentrations compared to free-living frogs. Although some frogs with other lethal conditions had reduced sodium concentrations, the variance was not homogenous, indicating that sodium reduction is not an obligate terminal change. Furthermore, plasma potassium levels in frogs with severe chytridiomycosis were significantly lower than concentrations in frogs with other lethal conditions.

In an additional infection experiment we administered an oral electrolyte supplement to *L. caerulea* in the terminal stages of infection. These frogs became more active and lived longer than diseased frogs that received no electrolyte treatment. Treated frogs continued to shed skin, a clinical sign of severe disease, and ultimately died. However, it is important to note that in this preliminary experiment the oral electrolyte supplement was administered in the terminal stages of infection, when the fatal cycle leading to asystolic cardiac arrest may have already been initiated. In understanding the mechanisms of death, our results support the hypothesis that electrolyte depletion is a crucial factor in pathogenesis, but additional work is needed to refine our understanding of the value of providing electrolytes as supportive care to amphibians with severe chytridiomycosis. We suggest multiple follow up experiments to build on these results: first, an experiment with a similar design, but provides electrolyte supplements earlier in the onset of clinical signs, second, an experiment including a range of dilutions of adjusted electrolyte solutions in the water baths (rather than an oral supplement) of infected amphibians and third, an experiment using electrolyte supplementation in combination with a fungicide known to be effective against *Bd* (such as itraconazole or chloramphenecol). In this last experiment, the elimination of *Bd* would be combined with the correction of electrolyte abnormalities. Experiments that identify the best way to treat amphibians with

chytridiomycosis will be important because although administering hypertonic solutions has inherent risks, it may be the best way to manage chytridiomycosis in a clinical setting. Although electrolyte treatment did not lead to full recovery, clinical improvement and delayed time to death indicate that electrolyte depletion is an important cause of morbidity and mortality.

Amphibians can tolerate greater electrolyte fluctuations than other terrestrial vertebrates (Deyrup 1964), but our results support the epidermal dysfunction hypothesis, which suggests that the disruption to cutaneous functioning and the extent of electrolyte imbalance that occurs in severe chytridiomycosis are sufficient to cause mortality. This does not disqualify the so-called "toxin hypothesis". Convincing evidence of a systemic toxin has not been provided, but *Bd* could produce a "toxin" that causes localized epidermal damage. Although enzymatic activity is not normally described as "toxic", the term "toxin" is defined as a poisonous substance formed as a part of cells or tissues as an extracellular product (Steadman 2000). Enzymes produced by *Bd* enzymes may fit that definition. We did not detect any epidermal transport changes with solutions treated to filter a possible toxin, but this could be a matter of the dilution of the solutions used. The discovery of differential expression patterns in genes such as serine protease and fungalysin metallopeptidase, two enzymes involved in pathogenesis in multiple fungal pathogens (Rosenblum et al. 2008), suggests that enzymatic activity could be involved in *Bd* pathogenesis. However, the functional role of these genes remains unclear and their importance for pathogenicity has not been conclusively demonstrated. This is due to the challenges of working with the molecular profile of *Bd*, which, at best, is only distantly related to organisms with moderately understood genomes. As the genomes of other fungi are investigated and the functional roles of their genetic repertoire are better understood, we may be able to generate a list of candidate proteins involved in chytridiomycosis pathogenesis (personal communication- Erica Rosenblum) and potentially establish the mechanism of destruction in key cell types of the amphibian epidermis.

The ability of *Bd* to compromise an essential organ is a critical attribute in a list of fatal pathogen characteristics that make *Bd* a formidable pathogen. Host-pathogen dynamics are driven by biotic and abiotic interactions at many levels, and the convergence of multiple variables (host susceptibility, pathogen virulence, population dynamics and environmental conditions) creates favourable conditions for disease outbreaks capable of causing population declines (Daszak et al. 2003, De Castro & Bolker 2005). Chytridiomycosis is an example of this possibility for several reasons. Compared to other terrestrial vertebrates, amphibians are particularly vulnerable to epidermal infections as their existence depends on physiological interactions of the skin with the external environment (Jorgensen et al. 1954, Alvarado & Kirschner 1963, Deyrup 1964, Moore & Lofts 1964, Fischbarg & Whittembury 1977, Shoemaker 1977, Larsen et al. 1987, Boutilier et al. 1992, Erspamer et al. 1994, Jorgensen 1994a, b, 1997, Wright et al. 2001, Word & Hillman 2005). Frog skin is a moist, nutritive substrate on which many microorganisms can flourish (Clark et al. 1997), but *Bd* has the added advantage of a temperature optimum for growth that matches the range of temperatures experienced by amphibian species (Piotrowski et al. 2004). Additionally, amphibian behavioural characteristics such as aggregating in retreat sites or during breeding season (Rowley & Alford 2007), and their association with water bodies (Lips et al. 2003, Skerratt et al. 2007, Brem & Lips 2008) increase opportunities for *Bd* transmission. The ability of *Bd* to persist in the environment, whether by an alternate life stage (Di Rosa at al. 2007) or by saprobic growth further amplifies the possibility of disease-induced extinctions (Mitchell et al. 2008), which had not previously been considered biologically possible (Anderson & May 1991, de Castro & Bolker 2005). Finally, anthropogenic spread of *Bd*, possibly via international frog trade, is a plausible explanation for its global emergence (Hanselmann et al. 2004). The *confluence* of all of these factors, including a life-compromising pathophysiology reported here, has created the disease equivalent of a "perfect storm" scenario such that amphibians are impacted over a broad host range and across the globe, resulting in an unparalleled pandemic

CHAPTER EIGHT

Discussion and future directions

Part I. Summary and discussion of results

Parasites that cause infectious diseases can regulate host population densities, alter community dynamics and impact entire ecosystems (Scott 1988, Daszak et al. 2000, Hudson et al. 2002, de Castro & Bolker 2005, Whiles et al. 2006). Disease has obvious consequences for animal populations that are already endangered (e.g., black footed ferrets (Thorne & Williams 1988) or Tasmanian devils (Lachish et al. 2007), but also, it can pose a significant threat in its own right (Scott 1988, Daszak et al. 2000, de Castro & Bolker 2005). Amphibians are experiencing unprecedented declines due, in part, to the disease chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). Although the end results of chytridiomycosis outbreaks may be unusual relative to other diseases, they should be explainable if certain facets of the disease are resolved. Yet many aspects of this disease are poorly understood. The experimental work presented in this thesis aimed to increase our understanding of chytridiomycosis by focusing on specific, testable hypothesis concerning 1) *Bd* growth, development and virulence, and 2) the mechanisms of pathogenesis. The data provide novel information about chytridiomycosis and, perhaps more importantly, reveal additional questions for future research.

Virulence of *Batrachochytrium dendrobatidis*

The data from Chapters four and five show that even within a single isolate *Bd* can vary in growth, development and zoospore densities. These differences were even more pronounced among isolates, and suggest that *Bd* isolate variability may be greater than is apparent from genetic studies (Morehouse et al. 2003, Morgan et al. 2007). A very recent study found phenotypic and genotypic differences among *Bd* isolates that suggested

natural selection may be acting on important fecundity-associated characters (size of sporangia) in different ecological zones (Fisher et al. 2009). My results agree with this suggestion and offer methods that may allow us to characterize *Bd* evolution. Due to the importance of such characters to pathogenicity, it is critical to better understand the evolutionary dynamics of *Bd* and especially how natural selection may be operating on *Bd* zoospore production. Furthermore, I suggest that these questions are not only important to answer pathogenesis-related questions, but they are also critical to other aspects of the host-pathogen dynamic, such as transmission. My results suggest that although *Bd* growth rate decreases at low temperatures, adjustments to the *Bd* lifecycle, such as prolonged period of high zoospore densities, may mean transmission is enhanced because the timeframe of activity for infectious zoospores may be extended. This would be especially true if the reaction norm in fecundity (zoospore production) is altered by natural selection. If adaptive adjustments in fecundity-associated traits are temperature dependent, then such adaptive changes could lead to greater disease risk in specific seasons and/or ecological zones. Resolving these points of the chytridiomycosis host-pathogen dynamic may be critical to understand the spread of the disease into naïve populations and/or to develop appropriate disease management for already impacted populations.

Pathogenesis of chytridiomycosis

Results in Chapters six and seven show that severe chytridiomycosis disrupts normal cutaneous functioning and that the extent of electrolyte imbalance is the most likely cause of asystolic cardiac arrest and mortality. Ussing chamber experiments show that electrolyte transport across the skin was significantly inhibited, demonstrating that *Bd* infection compromises osmoregulatory function in the skin of diseased frogs. The importance of amphibian cutaneous functioning in maintaining homeostasis makes amphibians especially vulnerable to the effects of epidermal infections (Wright et al. 2001) and the extent of the damage caused by *Bd* is therefore life-compromising. Blood plasma electrolyte concentrations are reduced in amphibians with severe

chytridiomycosis. A disproportionate loss of electrolytes to water indicates a change in the balance of osmolality and appears to be the most likely cause of asystolic cardiac arrest. Electrolyte supplementation may prove effective supportive care for frogs in a clinical setting, when combined with a fungicical bath or heat to kill *Bd.* However, electrolyte supplementation should be used with caution due to the risk of overdosing when plasma levels cannot be continuously monitored. Because no other biochemical or physiological parameter appears to vary significantly with chytridiomycosis, the most parsimonious explanation for the pathophysiology of chytridiomycosis is that the skin is the primary organ involved in electrolyte shifts that lead to death. These results support an epidermal dysfunction hypothesis, but they do not eliminate the toxin hypothesis. Rather, I suggest that these hypotheses may not be mutually exclusive and both may be correct; the breakdown in epidermal functioning may be caused by a "toxin" (or disruptive compound) produced by *Bd*. Currently, such a compound or compounds are unknown, but advances in *Bd* molecular research and/or additional pathophysiological research may advance provide additional details of the mechanisms of *Bd* colonization and disruption of amphibian epidermis.

Part II. Management of chytridiomycosis and recommendations

The global loss of amphibian biodiversity has been called "the greatest species conservation challenge in the history of humanity" (Zipple & Mendelson 2008). The sense of urgency to protect amphibians and mitigate the effects of chytridiomycosis is well warranted. The loss of amphibian biodiversity (Lips et al. 2006, Mendelson et al. 2006), subsequent ecosystem disturbance (Whiles et al. 2006), and looming risk for naïve amphibian populations (Woodhams et al. 2008b) justify immediate conservation efforts. However, determining the best way to respond to chytridiomycosis is challenging. Disease management protocols have been developed to enable amphibian conservation programs in several countries and can serve as guidelines to coordinate long-term amphibian conservation efforts worldwide.

The *Threat Abatement Plan: Infection of amphibians with chytrid fungus resulting in chytridiomycosis* lays out objectives for control and management of chytridiomycosis by containing the spread of *Bd* (quarantine programs) and investing in recovery programs for amphibians at high risk. The research component of the plan includes studies to improve diagnostic tools, investigate transmission and dispersal and resolve the mechanisms of pathogenesis of chytridiomycosis. This plan has served as a tool for initiating disease response management programs. An action strategy currently being applied focuses on wildlife in the Tasmanian World Heritage Area (Philips & Driessen 2008), which appears to be free of *Bd* although the risk of introduction from adjacent infected regions is high. Tasmania has three endemic amphibian species and other vulnerable species, such as *Litoria raniformis*, which are currently listed as vulnerable species (IUCN 2008). The goals of the Tasmanian program include establishing protocols for early detection and rapid response to emerging disease, managing existing disease and public outreach, education and communication (Philips & Driessen 2008). Currently the distribution of *Bd* in Tasmanian frog populations is being assessed and monitored, and upcoming experiments will test the susceptibility of Tasmanian amphibians to *Bd* (personal communication- Annie Phillips). With ongoing monitoring and knowledge of species susceptibility, the Tasmanian Department of Primary Industries will be better prepared for an outbreak of chytridiomycosis and should stand a better chance of protecting the endemic species of Tasmania. The program should stand as a model for effective preparatory action plans for other wildlife diseases in other parts of the world.

The *Amphibian Conservation Action Plan* (ACAP) was published in 2007 (Gascon et al. 2007). It addresses the many factors affecting amphibian populations and it not limited to disease-related declines; infectious diseases, especially chytridiomycosis, are listed among other causes of delines including environmental contamination and climate change. Some of the objectives of ACAP centre on *ex situ* breeding programs and preserving amphibian genetic materials. The stated strategies for dealing with disease in the wild amphibian populations include research to understand “tolerant species” such as bullfrogs and potentially selecting for amphibian resistance to *Bd* (Gascon et al. 2008).

These plans are cornerstones for amphibian conservation by identifying strategies for mitigating the effects of chytridiomycosis. However, perhaps the most significant step forward came with the recognition of *Bd* by The World Organisation for Animal Health (OIE). Achieving status as a notifiable disease required several important steps. In 2006 the Aquatic Animal Commission (AAC) reviewed a report from an *ad hoc* group amphibian disease (Barry Hill, Andrew Cunningham, Peter Daszak and Rick Speare). The group identified *Bd* and Ranavirus as pathogens of "particular importance" for amphibians. An important point noted was that although the complete distribution of *Bd* was unknown, many countries are likely to be free of *Bd*, but at risk for spread due to infected animals in the international frog trade (OIE 2006). A survey questionnaire was dispatched to member countries and reconsidered by the AAC before recommendations were forwarded to the OIE. In 2008 the OIE determined that *Bd* met the relevant parameters for criteria (including consequences, spread and diagnosis- repeatable and robust means of diagnosis) for an aquatic animal disease (Article 1.2.2.1, OIE 2008) and *Bd* was listed as a "notifiable disease" (Article 1.2.3.4 OIE 2008).

The significance of the listing of *Bd* cannot be understated. The recognition of *Bd* as a wildlife disease of concern means that mechanisms for international cooperation can be utilised to mitigate the threat of chytridiomycosis to amphibians worldwide. The Aquatic Animal Health Code specifies general provisions for all listed aquatic diseases including notification systems, obligations and ethics, guidelines for risk analysis, import and export procedures and contingency plans for international trade, for all member countries (Sections 1.2 – 1.6, OIE 2008). More specifically for *Bd*, detailed requirements must be met for OIE member countries to be identified as *Bd*-free or to have free status for particular zones. There are further guidelines for maintaining *Bd*-free status and for importation from free or "not declared free" zones (Articles 2.4.1.6-2.4.12, OIE 2008). These specifications will apply to all amphibian taxa (Anura, Caudata and Gymnophiona) and all amphibian products (viable and non-viable animals and parts of animals from all life stages) used for aquaculture, human consumption, laboratory use, industrial and pharmaceutical use, and the pet trade (Articles 2.4.1.7-2.4.12, OIE 2008). In short, the recommendations for amphibian movements are fairly comprehensive. However,

compliance may prove challenging. Methods for surveillance and diagnosing *Bd* are available (OIE 2008) and may lead to the creation of standard diagnostic laboratories to facilitate OIE member countries in limiting the spread of *Bd*.

Each of the plans described above (*The Threat Abatement Plan*, *The Tasmanian Disease Management Plan* and *The Amphibian Conservation Action Plan*) place an emphasis on the importance of disease research including research on the epidemiology, transmission and pathogenesis of chytridiomycosis and the OIE Aquatic Animal Health Code lays out recommendations for international cooperation to limit the spread of *Bd*. However, I suggest that none of these plans adequately addressed the importance of research on *Bd* virulence itself.

Substantial advances in virulence research are achievable, but depend on access to pathogen isolates with a range of spatiotemporal origins. I suggest three priority targets for pathogen collection and cryopreservation: 1) isolates from geographically and taxonomically diverse amphibian hosts, 2) isolates preserved during and following *Bd* emergence and 3) isolates collected at finer scales from populations experiencing different responses to infection. Such isolates will be a key resource for laboratory experiments or genetic studies aiming to identify pathogenicity factors. They will also help us to advance chytridiomycosis research with an underlying goal of implementing viable amphibian management and conservation.

In the case of amphibian declines due to chytridiomycosis, the ability to fully confront the threat will hinge on coordinating multiple mitigation strategies including careful screening for the pathogen in naïve populations (Skerratt et al. 2008) and in international amphibian trade (Australian Department of Environment and Heritage 2006; Gascon et al. 2007), identifying and preserving vulnerable amphibians (Mendelson et al. 2006, Gascon et al. 2007) and most importantly, advancing basic disease research (Australian Government 2006, Mendelson et al. 2006, Gascon et al. 2007). I suggest that to fully confront disease threats, particularly in the case of amphibian declines, insight into host-pathogen interactions and co-evolution is critical and we must also collect and preserve

the pathogen for basic disease research. It is important to urge managers and researchers to participate in a collaborative effort to archive *Bd*. Confronting disease threats and implementing effective conservation action will require a better understanding of the disease, the host *and the pathogen.*

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APPENDIX

List and copies of published work

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

This is a published copy of Chapter six. The contributions of all co-authors are outlined in the preface.

2. Woodhams DC, Kilburn VL, Reinert LK, **Voyles J**, Medina D, Ibáñez R, Hyatt AD, Boyle DG, Pask JD, Green DM (2008) Chytridiomycosis and amphibian population declines continue to spread eastward in Panama. EcoHealth 5, 268-274

Doug Woodhams wrote this paper based on our shared observations from field work in the Republic of Panama. Additional data was contributed by other co-authors. I was primarily involved with data collection and editing of the manuscript.

3. **Voyles J**, Cashins SD, Rosenblum EB, Pushendorf R (2009). Preserving pathogens for wildlife conservation: a case for action on amphibian declines. Oryx 43: 527-529.

This paper was written as a short communication with recommednations for conservation generated for Chapter eight.

4. **Voyles J**, Young S, Berger L, Campbell C, Voyles WF, Dinudom A, Cook, Webb R, Alford RA, Skerratt LF, Speare R (2009). Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. Science 326, 582-585.

This is a published copy of Chapter seven. The contributions of all co-authors are outlined in the preface.

5. Rosenblum EB, **Voyles J**, Poorten TJ, Stajick JE (in press) The deadly chytrid fungus: A story of an emerging pathogen. PLOS Pathogens.

Erica Rosenblum wrote this paper as an invited review. I contributed background information from my literature review (Chapter two) and I was primarily involved with the writing and editing of the manuscript.

6. **Voyles J**, Richards-Hrdlicka K, Cashins SD, Rosenblum EB, Hyatt AD, Berger L, Skerratt LF (in review). *Batrachochytrium dendrobatidis*: Requirement for further collection and archiving. Disease of Aquatic Organisms.

This paper was written as a short communication with recommednations for conservation generated for Chapter eight. Katy Richards-Hrdlicka contributed additional background information and anlayses from a literature review.

COPIES OF PUBLICATIONS NOT INCLUDED