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.

The research presented in this thesis was financially supported by James Cook University, School of Public Health, Tropical Medicine and Rehabilitation Sciences, the Wildlife Preservation Society of Australia and by the Australian Government, Department of the Environment, project (RFT43-04): “Experimental research to obtain a better understanding of the pathogenesis of chytridiomycosis, and the susceptibility and resistance of key amphibian species to chytridiomycosis in Australia.”

ACKNOWLEDGEMENTS

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.

Elizabeth Davidson and Brandon Sheafor offered timely advice that allowed me to start this work. Erica Rosenblum, Scott Cashins, Jeff Werner, Donna Rudd, David Cook, Anuwat Dinudom and Craig Campbell made important contributions to experimental designs and methods. Andrew Storfer, Erica Rosenblum and Mark Holdsworth offered editorial assistance. Robert Puschendorf, Diana Mendez, Noriko Iwai, HyoJin Yang translated protocols. Ari Gardner and Rebecca Webb deserve special awards for meeting countless requests with smiles. Stephen Garland, Anthony Baker, Veronica Olsen and Ruth Cambell are PCR heroes. Jeremy VanDerWal rescued scripts from multiple R (“Rrr”) dilemmas. Laurie Reilly was a valuable resource for histological headaches. Diana Mendez, Nicole Kenyon and Andrea Phillott always offered a helping hand. Belinda Tuckett, Margaret McDondald, Marcia Croucher, and Chris Brown coordinated research logistics and the finer details of university life. Keith McDonald and Michelle Neissen at QPWS made permit issues less puzzling.

Alex Hyatt, speaking of giants, welcomed me into his lab at CSIRO-AAHL and introduced me to the world of electron microscopy. I thank everybody at AAHL for their help and patience, especially Veronica Olsen, Rosey and Ellie VanDriel, Donna Boyle, Sandy Crameri, Frank Fillipi, Judy in the canteen, and (at the ARC) Gerry Marantelli and Dave Hunter. The Rose family (Andrew, Kylie, Oscar, Ruby and Angus) made life in Geelong great fun for Smoke Dog and me.

And finally…

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This accomplishment is dedicated to Wyatt Voyles, Joy Browne and Traci Voyles who have always loved me unconditionally.

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