The Ecology of Chytridiomycosis in Eastern Australia

Kerry Matthew Kriger
Bachelor of Science

Submitted in fulfillment of the requirements of the degree of
Doctor of Philosophy

December 2006

School of Environmental and Applied Sciences
Griffith University
Gold Coast, Australia
Abstract

Rapid amphibian population declines and extinctions have occurred worldwide in recent decades, often in protected areas where no obvious human disturbance can be identified. Chytridiomycosis is an emerging infectious disease caused by *Batrachochytrium dendrobatidis*, a chytrid fungus that parasitizes the keratinized epidermis of post-metamorphic amphibians and the mouthparts of tadpoles. *Batrachochytrium dendrobatidis* has been detected in over 200 amphibian species from five continents, and has been diagnosed in dead and dying frogs concurrent with population declines, extirpations and extinctions. Chytridiomycosis is thus considered a primary explanation for the global loss of amphibian biodiversity in recent decades.

To date, our understanding of chytridiomycosis host-pathogen ecology is limited. In particular, there is very little known regarding the biogeography of *B. dendrobatidis* or its prevalence in wild amphibian populations, and there is no published data whatsoever to explain the variation in disease levels between conspecific amphibian populations living at disparate locations. Without a thorough understanding of these issues, effective management of the disease will remain elusive. As such, this thesis focuses on two related goals: (1) improving the diagnostic techniques used to detect *B. dendrobatidis* in wild amphibian populations, and (2) applying these diagnostic techniques to determine both the extent to which the prevalence and severity of chytrid infections vary across ecological gradients, and the degree to which various factors limit the distribution and abundance of *B. dendrobatidis*.

Diagnosis of *B. dendrobatidis* to date has relied largely on histological examination of skin tissue stained with haemotoxylin & eosin (H&E). This method is invasive, and thus for ethical reasons it is unsuitable for sampling large numbers of amphibians, be they endangered or abundant. To assess the efficacy of a recently developed quantitative PCR assay (qPCR; Boyle et al. 2004), I sampled 101 wild juvenile *Mixophyes iteratus* by both a skin swab for use in qPCR analysis, and a toe-clip for examination by histological methods. The swab-qPCR assay detected *B. dendrobatidis* in a minimum of 14.9% of frogs, whereas histology detected infection in no more than 6.9% of frogs. I conclude that the swab-qPCR technique is the more reliable means of detecting the chytrid fungus in wild amphibians, and that it precludes
the need for toe-clipping as a means of sampling for the presence of *B. dendrobatidis* in future surveys.

While the swab-qPCR assay yields the most accurate and informative data of any available detection technique, it has yet to attain widespread use by chytridomycosis researchers, due to the relatively high costs involved. I used the results of a disease survey of 467 wild frogs from eastern Queensland, Australia to examine the necessity of triplicate assays in qPCR detection of *B. dendrobatidis*, and I describe a singlicate qPCR assay that can be used to substantially decrease costs, with no significant decrease in sensitivity. I also demonstrate that detection of *B. dendrobatidis* by use of the conventional PCR assay may lead to appreciable underestimations in disease prevalence.

Numerous laboratory experiments have demonstrated that *B. dendrobatidis* prefers cool temperatures and moist conditions, suggesting that the distribution and abundance of the fungus, and thus its effect on various amphibian populations, should be limited by high temperatures and lack of water. I thus hypothesized that chytrid infections would be most prevalent and severe in (1) cooler months, (2) frog populations at more temperate latitudes and in wetter regions, (3) high-altitude frog populations, and (4) frog species that breed in permanent streams, as opposed to ponds, ephemeral water bodies or leaf-litter.

To test the first hypothesis, I conducted disease sampling in a single population of stony creek frogs (*Litoria wilcoxii*) on 13 occasions over a 21-month period. Prevalence of *B. dendrobatidis* infection varied significantly across sampling sessions, peaking at 58.3% (in early spring) and dropping to as low as 0% on two occasions (late summer and early autumn). There was a significant negative relationship between disease prevalence and the mean air temperature in the 30 days prior to sampling. Further, by repeatedly sampling individually-marked frogs in this population, I determined that the decrease in prevalence through time is not due to high mortality of infected frogs; rather it is due to the capability of adult frogs to clear their chytrid infections.

To examine the latitudinal variation in the prevalence and severity of chytrid infections, I sampled 863 adult male stony creek frogs (*Litoria lesueuri* complex) from 31 lowland sites distributed north-south along 2315km of the Australian east coast. I found *Batrachochytrium dendrobatidis* at 77% of the sites, including sites at the northern and southern limits of the latitudinal transect. As initially hypothesized, frogs
from temperate regions had significantly more severe chytrid infections than did their tropical counterparts, often carrying an order of magnitude more *B. dendrobatidis* zoospores, suggesting that at low altitudes, temperate frogs are at higher risk of chytridiomycosis-induced mortality than are tropical frogs. The prevalence and severity of *B. dendrobatidis* infections were significantly greater at sites with high rainfall (>33mm in the 30 days prior to sampling) and cool temperatures (stream temperature one hour after sunset less than 23°C). Interestingly, while climatic variables explained a great deal of the variation in the prevalence and severity of *B. dendrobatidis* infections between infected and uninfected sites, frog snout-vent length was consistently the best predictor of infection levels across infected sites. Small frogs were both more likely to be infected, and carried more severe infections than larger frogs, suggesting either that frogs can outgrow their chytrid infections, or that the disease induces developmental stress that limits growth.

To examine the altitudinal distribution of chytrid infections, I sampled three stream-dwelling frog species (*Litoria wilcoxii, L. pearsoniana*, and *L. chloris*) along altitudinal transects in southeast Queensland, Australia. Infections were found in all the populations examined, and contrary to my initial hypothesis, I found no consistent evidence that high-altitude frogs were more likely to be infected than were lowland frogs. Further, frogs from lower altitudes carried fungal infections as severe as their high-altitude counterparts. I did find evidence, however, that chytrid infections persist longer into summer in upland as compared to lowland areas, suggesting that montane amphibian populations remain susceptible to disease outbreaks for longer periods than do lowland populations. Further, at high altitudes, temperatures optimal for chytrid growth and reproduction coincided with frog metamorphosis, the life-stage at which frogs are most susceptible to chytrid infections. I propose that while *B. dendrobatidis* can infect frogs at any altitude in the subtropics, complex relationships between the timing of metamorphosis and chytrid thermal optima render montane amphibian populations more susceptible to chytrid-induced mortality than their lowland counterparts, and may account for the differential population-level response to the presence of *B. dendrobatidis*.

To examine the influence of breeding habitat on a frog’s likelihood of acquiring chytrid infection, I sampled frogs from five different breeding habitats (permanent-ponds, permanent-streams, ephemeral-ponds, ephemeral-streams, terrestrial) in the Numinbah Valley of southeast Queensland. Infections were not evenly distributed
across the ecological guilds, being almost completely restricted to frogs breeding at permanent water bodies. Of these frogs, stream-breeders were significantly more likely to be infected than were pond breeders, though the severity of frogs’ infections did not differ significantly between the two guilds. *Batrachochytrium dendrobatidis* was detected on only one of the 117 frogs that were found at ephemeral water bodies or terrestrial sites. These findings provide strong support for the hypothesis that *B. dendrobatidis* was responsible for many of the unexplained disappearances of stream-breeding amphibian populations in recent decades.

This information is a major addition to our understanding of chytridiomycosis host-pathogen ecology, and will directly assist amphibian conservation programs by (1) providing a reliable, cost-effective method of disease diagnosis, (2) providing baseline data on infection levels, from which to judge the effectiveness of future management action; (3) allowing wildlife managers to more accurately predict those species, populations, and locations most likely to be affected by chytridiomycosis, and (4) informing the design of disease studies and survey protocols, and the timing of release/collection of amphibians involved in captive breeding programs. The information gained from this research will also contribute significantly to our general understanding of disease ecology and population declines.
Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Kerry M. Kriger

21 April 2007
Acknowledgements

Thanks to Marc Hero for conceiving a brilliant PhD project that was perfectly suited to my interests and kept me continually intrigued. Marc provided excellent advice on many aspects of my project, and always knew the right person for me to contact when I needed outside assistance. Marc's comments on my grant applications, manuscripts and presentations were invaluable, and the rapidity with which he read over and commented on my drafts no doubt contributed to my finishing my candidature in a timely manner, and with several chapters already published. Further, Marc was a good friend, and his general approach to conservation served as inspiration that will likely last far into the future. Thanks Marc.

On numerous occasions I interrupted Kevin Ashton to ask his thoughts on a PCR problem I was having; I can't remember a single time that he didn't stop what he was doing and walk to the lab to help me out. Great thanks to Kevin for helping me through some pretty frustrating times, and enabling me to learn more than just the absolute essentials of PCR. His thorough mastery over every aspect of PCR never failed to amaze me, and my PhD was significantly improved by having him as an associate supervisor.

Harry Hines and Ed Meyer answered a seemingly endless barrage of questions in the early period of my candidature, and provided invaluable advice on experimental design. My thesis has been greatly improved by their suggestion that I use temperature loggers and collect rainfall data. They also provided valuable comments on various drafts I have written, and through their wealth of frog knowledge served as inspiration in my formative days.

Thanks to Alex Hyatt, Veronica Olsen, and Donna Boyle for developing the qPCR technique on which my thesis depended, providing chytrid standards, and for providing excellent qPCR training. It would not be an overstatement to say that my research would not have been possible without their assistance.

Nor would this thesis have been possible without the substantial financial support received from a variety of organizations, and for which I am extremely grateful.
Specifically, I would like to thank the Eppley Foundation for Research, the National Geographic Society's Committee for Research & Exploration, Queensland Smart State Funding for PhD Research, Peter Daszak and the Consortium for Conservation Medicine, the Ecological Society of Australia, the Peter Rankin Trust Fund for Herpetology, the Australian Society of Herpetologists, Paul Waite and the Gold Coast Association of Postgraduates, Rick Speare and the Department of Environment and Heritage Chytridiomycosis Mapping Protocol Tender RFT63/2000, and the Natural Heritage Trust Fund. I would like to think their grants have been put to good use.

The fieldwork for this thesis was made both possible and enjoyable by innumerable volunteers who accompanied me to some pretty amazing places: to beautiful rainforest streams on warm moonlit nights, with frogs calling all around; and also on some long, hard treks on cold rainy nights way too late at night, when frogs were elusive and there were other places we'd rather have been. Felicia Percoglou, Dave Hall, and Ushio Kawai deserve extra special thanks, for going far beyond what would be expected of any volunteer. Thanks also to Kris Murray, Josh Morris, Bert Rutten, Jonathan Pickvance, Dane Hansen, Sian Darlington, Sean Picton, Sarah Butler, Rhianna Ford, Renan Pointeau, Peter Tupper, Murray Tardent, Monique van Sluys, Laura Manuel, Luke Shoo, Louise Anderson, Jon Shuker, James Webley, Gemma Schuch, Gaby Leong, Emily Fitzpatrick, Dan McDonald, Claire Arthur, Chad Kriger, Carol Kinsella, Carla Avolio, Andrew Crindland, Alli Duffy, Albertina Lima, Shane Howard and Abraham Mijares, among others.

Thanks to Peter and David Lyons, June French, Mark at Tallanbana, and the owners of the English Tea Gardens, for allowing me to conduct fieldwork on their property.

Great thanks to Clyde Wild, Joy Cumming and Lesley Johnson, for seeing to it that I was able to eat during my PhD candidature.

Thanks to Diana Mendez, an excellent teacher who went out of her way to provide training in histological techniques. Michael Arthur gave lots of statistical advice, and has an amazing ability to make people laugh during a statistics class. Thanks to Joe Lee and the Centre for Aquatic Processes and Pollution, as well as the Heart Foundation Research Centre, for allowing access to lab space and equipment. Luke Shoo answered
many random questions of mine and provided many interesting thoughts, and John Clarke answered lots of questions and assisted with scientific permits. Sonja Wapstra assisted in preparation of slides for histology, saving me lots of time and potentially lost digits (beware the microtome). Thanks to Kirsten Parris for assistance with BIOCLIM, Jan Warnken for mapping assistance, Ruth Campbell for providing positive controls, the Bureau of Meteorology for providing climatic data, and all those who provided advice on locating frog populations. And thanks to Richard Retallick for recommending I contact Marc Hero regarding PhD supervision.

Pat Hart, Lenny Freed, Scott Fretz, and Eric VanderWerf provided an excellent introduction into ecology and fieldwork, and made my decision to pursue a PhD an easy one. Jim Monohan, Robin Gorham, and Karla Henthorn provided a solid foundation for my career in ecology by giving me an excellent introduction into the basics of biology, and answering a multitude of questions.

Great thanks to my parents for impressing upon me the importance of a good education, and always making sure I was able to receive the best education possible.

Finally, the comments of many anonymous reviewers drastically improved the quality of the following chapters.
# Table of Contents

Abstract.................................................................................. 2  
Acknowledgements.................................................................. 7  
List of Tables.......................................................................... 12  
List of Figures.......................................................................... 13  

Chapter  
1  Introduction: Chytridiomycosis and global amphibian declines.............. 16  
2  Techniques for detecting chytridiomycosis in wild frogs; comparing histology with real-time Taqman PCR.  
   Abstract.............................................................................. 48  
   Introduction......................................................................... 49  
   Methods............................................................................. 52  
   Results................................................................................ 54  
   Discussion.......................................................................... 58  
3  Cost efficiency in the detection of chytridiomycosis using PCR assay.  
   Abstract.............................................................................. 63  
   Introduction......................................................................... 64  
   Methods............................................................................. 65  
   Results................................................................................ 68  
   Discussion.......................................................................... 71  
4  Large-scale seasonal variation in the prevalence and severity of chytridiomycosis.  
   Abstract.............................................................................. 75  
   Introduction......................................................................... 76  
   Methods............................................................................. 78  
   Results................................................................................ 81  
   Discussion.......................................................................... 85  
5  Survivorship in wild frogs infected with chytridiomycosis.  
   Abstract.............................................................................. 89  
   Introduction......................................................................... 90  
   Methods............................................................................. 92  
   Results................................................................................ 94  
   Discussion.......................................................................... 98
6  Latitudinal variation in the prevalence and intensity of chytrid 
(Batrachochytrium dendrobatidis) infection in eastern Australia.

Abstract ......................................................... 100
Introduction .................................................. 101
Methods ......................................................... 102
Results .......................................................... 106
Discussion ....................................................... 117

7  Altitudinal distribution of chytrid (Batrachochytrium dendrobatidis) 
infection in subtropical Australian frogs.

Abstract ......................................................... 121
Introduction .................................................. 122
Methods ......................................................... 124
Results .......................................................... 127
Discussion ....................................................... 134

8  The chytrid fungus Batrachochytrium dendrobatidis is non-randomly 
distributed across amphibian breeding habitats.

Abstract ......................................................... 139
Introduction .................................................. 140
Methods ......................................................... 142
Results .......................................................... 144
Discussion ....................................................... 147

General Conclusion ............................................ 152
References ...................................................... 157

Appendices
1  Locations sampled ........................................... 176
2  qPCR detailed methodology ................................ 180
3  Reply to Smith (2007) ...................................... 194
4  Cophixalus ornatus .......................................... 200
### List of Tables

**Table**

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Infection status of 101 juvenile frogs as determined by 2 diagnostic</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>techniques used to detect chytridiomycosis</td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>Comparison of results of all frogs that yielded a non-negative result by</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>either Taqman PCR or histology</td>
<td></td>
</tr>
<tr>
<td>3.1</td>
<td>Prevalence of chytridiomycosis in wild frogs as determined by triplicate</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>qPCR assay, and as predicted for singlicate qPCR and conventional PCR..</td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>Results of chytridiomycosis survey of 467 wild frogs from eastern</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Queensland, Australia</td>
<td></td>
</tr>
<tr>
<td>4.1</td>
<td>Prevalence of chytridiomycosis and number of <em>B. dendrobatidis</em> zoospores</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>on adult <em>L. wilcoxii</em> in Numinbah Valley, and relevant climatic variables</td>
<td></td>
</tr>
<tr>
<td>5.1</td>
<td>Prevalence of <em>B. dendrobatidis</em> infection in <em>L. wilcoxii</em>, number of frogs</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>marked, and number of recaptured frogs with various infection histories...</td>
<td></td>
</tr>
<tr>
<td>5.2</td>
<td>Temporal pattern of <em>B. dendrobatidis</em> infection for the nine recaptured</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>adult male <em>L. wilcoxii</em> whose infection status changed during the course</td>
<td></td>
</tr>
<tr>
<td></td>
<td>of the study</td>
<td></td>
</tr>
<tr>
<td>5.3</td>
<td>Survival of infected and uninfected adult <em>L. wilcoxii</em></td>
<td>96</td>
</tr>
<tr>
<td>6.1</td>
<td>Location of sampling sites</td>
<td>110</td>
</tr>
<tr>
<td>6.2</td>
<td>Summary of multiple regression analyses</td>
<td>111</td>
</tr>
<tr>
<td>7.1</td>
<td>Location of sampling sites</td>
<td>127</td>
</tr>
<tr>
<td>7.2</td>
<td>Summary information for altitudinal transects</td>
<td>130</td>
</tr>
<tr>
<td>7.3</td>
<td>Results for tests of significant variation with altitude in either the</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>prevalence or intensity of chytrid infections</td>
<td></td>
</tr>
<tr>
<td>8.1</td>
<td>Distribution of <em>Batrachocheirum dendrobatidis</em> infection across species</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>and breeding habitats</td>
<td></td>
</tr>
<tr>
<td>8.2</td>
<td>Prevalence and intensity of <em>Batrachocheirum dendrobatidis</em> infection as a</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>function of frog breeding habitat</td>
<td></td>
</tr>
<tr>
<td>A2.1</td>
<td>Chart with swab numbers</td>
<td>183</td>
</tr>
<tr>
<td>A2.2</td>
<td>Triplicate PCR plate chart</td>
<td>190</td>
</tr>
</tbody>
</table>
List of Figures

Figure

1.1 Path diagram showing factors that may influence the number of *B. dendrobatidis* detected on an individual frog. 47

2.1 Sampling for *B. dendrobatidis*: a cotton swab is firmly run over the skin of *L. wilcoxii*. 51

2.2 Temporal distribution of sampling, and number of positive, suspicious and negative PCR results obtained on each sampling date. 55

2.3 Effect of storage time on PCR result. 57

2.4 Relationship between snout-vent length (SVL) and number of *B. dendrobatidis* zoospores found on positive, suspicious and negative individuals, as determined by Taqman PCR assay. 58

2.5 Number of animals required for sampling (if all samples yield negative diagnoses) to be 95% confident that disease is absent from a population, based on minimum expected disease prevalence. 60

3.1 Quantitation of zoospores detected on samples yielding different results in triplicate qPCR assay. 70

3.2 Costs required to analyze 100 samples for chytridiomycosis using triplicate and singlicate qPCR assays, at varying levels of disease prevalence. 71

4.1 Seasonal variation in the prevalence of chytridiomycosis in adult *L. wilcoxii* in Numinbah Valley, and mean air temperature for the 30 days before sampling. 83

4.2 Prevalence of chytridiomycosis in adult *L. wilcoxii* in Numinbah Valley, and mean air temperature for the 30 days before sampling. 84

4.3 Number of *B. dendrobatidis* zoospores detected on infected male *L. wilcoxii*, and mean air temperature for the 30 days before sampling. 84

5.1 Seasonal distribution of *B. dendrobatidis* infection for the nine recaptured *L. wilcoxii* whose infection status changed during the course of the study. 97

5.2 Number of *B. dendrobatidis* zoospores detected on infected *L. wilcoxii*
that were eventually recaptured, and those that were never recaptured. ................................................................. 97

6.1 Map of eastern Australia showing locations of sites sampled. .............................................................. 112

6.2 Relationship between latitude and the prevalence and intensity of B. dendrobatidis infections. ................................. 113

6.3 Relationship between the mean temperature of the warmest quarter and the prevalence and intensity of B. dendrobatidis infections. ..................................................... 114

6.4 Relationship between the 30-day rainfall and the prevalence and intensity of B. dendrobatidis infections. ................. 115

6.5 Relationship between mean snout-vent length and the prevalence and intensity of B. dendrobatidis infections. ......................... 116

7.1 Relationship between altitude and the number of B. dendrobatidis zoospores detected on infected frogs. .................... 131

7.2 Range of air temperatures experienced in the Nerang River catchment over a 20-month period. ................................. 133

7.3 Air temperatures in the Nerang River catchment at five altitudes. ................................................................. 133

7.4 Relationship between altitude and stream water temperature in three catchments in southeast Queensland. ................. 134

8.1 Number of B. dendrobatidis zoospores detected on infected frogs from each breeding habitat. ................................ 147

A2.1 Setting the threshold. .......................................................................................................................... 187

A2.2 Setting the baseline. .......................................................................................................................... 187

A2.3 Standard curve showing high correlation between standards and threshold cycle. ............................................. 192

A2.4 Amplification plot in logarithmic scale showing a high degree of precision within quadruplicate replicates. .................. 192

A3.1 Relationship between the number of B. dendrobatidis zoosporangia and zoospores detected on toe-clips from experimentally-infected frogs and infected wild frogs. ......................... 198
Regarding the structure and content of this thesis

All thesis chapters (excepting the introduction) have been prepared as stand alone manuscripts intended for publication in international scientific journals. At the time of thesis acceptance, Chapters 2, 3, 4, 5 and 8, and Appendices 3 and 4, have been published, and Chapters 6 and 7 have been submitted for publication. All manuscripts have been prepared and written by the author of the thesis. Co-authors who have contributed to the content of a submitted/published manuscript are acknowledged at the beginning of the relevant chapter. The journal in which the manuscript has been published (or to which it has been submitted) is also indicated, as well as the publication status of the manuscript at the time of thesis submission. For consistency, a single style of text formatting has been applied throughout, and in order to avoid undue repetition of text, a single combined reference list has been provided at the end of the thesis. Based on thesis reviewer comments, minor edits have been added to Chapters 2, 3, 4 and 5 and thus these chapters differ slightly from their published versions.