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JAMES COOK UNIVERSITY AUSTRALIA

Investigating disease ecology, pathogenesis and population persistence of frogs threatened by chytridiomycosis to improve management outcomes

Thesis submitted by

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BA, BSc, MSc Tulane University

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for the degree of

Doctor of Philosophy

in the College of Public Health, Medical, and Veterinary Sciences
James Cook University

Preface

This thesis is structured as a series of connected papers that have been published or are in preparation for publication at the time of thesis submission. Each paper is designed as an individual, stand-alone paper and for this reason there are some unavoidable repetitions, particularly within the methods and background material.

Each chapter is a collection of papers or manuscripts as listed below. If published, the whole citation is included preceding the manuscript, and the manuscript is displayed in the format of publication. If in review or in preparation, the paper has been formatted to fit the chosen journal.

CHAPTER 1

Introduction and basis for the study

CHAPTER 2

Disease ecology of a threatened and declining species and the use of reintroduction as a management technique

Paper 1: Brannelly LA, Hunter DA, Lenger D, Scheele BC, Skerratt LF, Berger L. 2015. Dynamics of chytridiomycosis during the breeding season in an Australian alpine amphibian. PloS ONE 10(12):e0143629.

Paper 2: Brannelly LA, Hunter DA, Skerratt LF, Scheele BC, Lenger D, McFadden MS, Harlow PS, Berger L. 2015. Chytrid infection and post-release fitness in the reintroduction of an endangered alpine tree frog. Animal Conservation. doi:10.1111/acv.12230.

Paper 3: Brannelly LA, Berger L, Skerratt LS. (2014) Comparison of three widely used marking techniques for adult anuran species *Litoria verreauxii alpina*. Herpetological Conservation and Biology. 9(2): 428-435.

CHAPTER 3

The effects of disease on reproduction

Paper 1: Brannelly LA, Webb R, Skerratt LF, Berger L. Amphibians with infections disease increase their reproductive effort: evidence of the terminal investment hypothesis. In Press at Open Biology.

CHAPTER 4

Pathogenesis of disease: changes in hematopoietic tissue

Paper 1: Brannelly LA, Webb R, Skerratt LF, Berger L. Effects of chytridiomycosis on hematopoietic tissue in the spleen, kidney and bone marrow in three diverse amphibian species. In review at Pathogens and Disease.

CHAPTER 5

Pathogenesis of disease: epidermal apoptosis

Paper 1: Brannelly LA, Roberts AA, Skerratt LF, Berger L. Epidermal apoptosis in frogs with chytridiomycosis. In review at Microbes and Infection.

CHAPTER 6

Differences in susceptibility between populations

Paper 1: Brannelly LA, Hunter DA, Marrantelli G, Skerratt LF, Berger L. Population differences in survivorship after disease of a functionally extinct amphibian species.

This study will be combined with a larger project investigating specific MHC alleles as a protective immune mechanism against *Bd*.

CHAPTER 7

Alternative treatment options for endangered alpine species in captivity

Paper 1: Brannelly LA, Berger L, Marrantelli G, Skerratt LF. Low humidity is a failed treatment option for chytridiomycosis in the critically endangered southern corroboree frog. Wildlife Research. 42:44-49.

Paper 2: Brannelly LA, Skerratt LF, Berger L. 2015 Treatment trial of clinically ill corroboree frogs with chytridiomycosis with two triazole antifungals and electrolyte therapy. Veterinary Research and Communication 39:179-187.

CHAPTER 8

Conclusions and management implications

Acknowledgements

First and foremost I would like to thank my supervisors, Lee Berger, Lee Skerratt and David Hunter. These three incredible scientists and researchers brought me to Australia and gave me the opportunity and the support to work on these projects, and the freedom to expand my skill sets. Lee Berger provided me the support I needed to get started, the freedom to explore new ideas when they presented themselves, and suggestions for new techniques to trial. She was always available when I needed to work through ideas, results, and importance. Lee Skerratt provided me the statistical and big picture support, and facilitated collaborations with other researchers. David Hunter provided excellent field support and his passion and commitment to frog conservation maintained my commitment to the project.

I am thankful to Taronga Zoo staff and volunteers for their dedication to merging management and scientific research in the field. Michael McFadden and Peter Harlow facilitated the reintroduction and their commitment, passion, humour and encouragement were invaluable. The Amphibian Research Centre and Gerry Marrantelli are dedicated to merging management and research as well, and provided me with animals and crickets throughout the course of my research. I would like to thank Vicki Eldridge, Trish Swain, Rhonda O'Neill, Robyn Mouat, Mararet Vella-Bonavita, SnowyHydro and National Parks and Wildlife Services who provided accommodation, access, and support during the field season. I would also like to thank Daniel Lenger for being amazing, and willing to drop his life and come into the field with me for months to catch some frogs, listen to podcasts, and bring crappy American movies to the Australian outback. His efficiency, humour, and relaxed nature made for an excellent field season.

I would like to thank the One Health Research Group, and particularly Laura Grogan, for creating weekly lab meetings to keep the group in touch and provide support. I thank Laura and Gerardo Martin for providing excellent advice on modelling, and teaching me these difficult mathematics in layman's terms, which is truly a skill. I thank Diana Mendez and Marcia Dos Santos for help with dissections and techniques. I thank Alex Roberts for her optimistic attitude and incredible helpfulness and dedication to mentorship. I thank Ben Scheele for always being available to bounce ideas and his enthusiasm for knowledge, exploration and conservation. Finally I thank Rebecca Webb for being a wealth of knowledge and always eager to help. Without her I would still be processing those slides. Also, the frogs really enjoyed the music choices.

I would like to thank the wealth of knowledge I received from meeting different academics at conferences and around the university. Gary Williams and Iyke Emato provided support through hiring me to tutor, which sharpened my skills, and also

provided statistics and SPSS help when I was struggling. I thank Frank Pasmans, An Martel, Constantin Constantinoiu, Cori Richards-Zawacki, Louise Rollins-Smith, and Rick Speare with career and techniques advice and encouragement. I thank Linda Johnson, Karen Reeks and Maria Forzan for assistance with pathology and histological techniques.

Without the help of my volunteers I would have been unable to complete the laboratory studies. Their enthusiasm and dedication to knowledge and conservation has been incredibly helpful, and the frogs very much appreciate their dedication to their health and well-being. I thank Dylan Tegtmeier, Miranda Stewart, Lorenzo Bertola, Ket Fossen, Cam de Jong, Jennifer Hawkes, Sophie Percival, Megan McWilliams, Trisha Knavel, Naomi Harney, and Lexie Edwards for their dedication and persistence.

And finally I would like to thank David Featherstone, Sarah Gierz, Wiebke Weßels, Daphné Behrens, Sybille Hess, Emily Wood, Debbie Bower, and Izzy Riofrio for giving me motivation to continue and most importantly, reminding me to take breaks, explore this beautiful country and refresh. Your support enabled me to maintain productivity: especially in the difficult and frusterating parts that every researcher must experience. I would like to thank my family, especially my sisters Heather Brannelly and Haley Brannelly, and my parents Donna Saber and Kevin Brannelly for supporting me from across the world. I would like to give special thanks to the Cohort Doctoral Studies Program, Melissa Crow, Jenni Judd, and David MacLaren for support and providing access to resources and providing a safe space for us PhD candidates to have a yarn and re-motivating us to be productive. Finally, thank you Rob Bergstresser for helping me edit this thesis for grammar and clarity.

Statement on the contribution of others

My research was funded by the Australian Research Council (grants FT100100375, LP110200240, DP120100811 to LFS and LB), the Taronga Conservation Science Initiative and the Wildlife Preservation Society of Queensland. My stipend and fees as a PhD student at James Cook University were funded through the James Cook University International Research Scholarship. Travel and conference attendance were funded in part through the Graduate Research Scheme at James Cook University awarded to me in 2015 and 2016.

All experimentation was conducted at James Cook University. Animals were housed in DB070, all dissections and histological examination were conducted in the Bruce Copeman Parasitology laboratory, histological preparation was conducted in the veterinary pathology laboratory and all genetic and protein work was conducted in Pharmacology and Medicine Faculty Laboratory. Taronga Zoo and the Amphibian Research Centre captive raised the animals utilised in my research.

My advisors Drs Lee Berger, Lee Skerratt and David Hunter gave advice throughout the project on study design, research methodology, data collection techniques and analysis as well as manuscript preparation. Each project involved collaborators, and they have been included as co-authors on the papers. Other collaborators not listed as a co-author of specific publications have been included in the acknowledgements following each paper in the text. I performed the majority of the research and wrote all papers, with editing help and approval by all listed collaborators. A detailed account of each co-author's contribution to the paper is listed below, under the paper citation.

CHAPTER 2

Paper 1: Brannelly LA, Hunter DA, Lenger D, Scheele BC, Skerratt LF, Berger L. 2015. Dynamics of chytridiomycosis during the breeding season in an Australian alpine amphibian. PloS ONE 10(12):e0143629.

LAB, DAH, LFS, LB conceived and designed the research. LAB, DAH, DL and BCS collected the data. LAB and LFS analysed the data. LAB wrote the manuscript and LAB, DAH, DL, BCS, LFS and LB edited the manuscript. DAH, LFS and LB provided funding, reagents and materials for the research.

Paper 2: Brannelly LA, Hunter DA, Skerratt LF, Scheele BC, Lenger D, McFadden MS, Harlow PS, Berger L. 2015. Chytrid infection and post-release fitness in the reintroduction of an endangered alpine tree frog. Animal Conservation. doi:10.1111/acv.12230.

DAH, LFS, MSM, PSH, LB conceived and designed the research. LAB, DAH, DL and BCS collected the data. LAB analysed the data. LAB wrote the manuscript and LAB, DAH, LFS, BCS, DL, MSM, PSH and LB edited the manuscript. DAH, LFS and LB provided funding, reagents and materials for the research, and DAH, MSM and PSH provided the animals for the release.

Paper 3: Brannelly LA, Berger L, Skerratt LS. (2014) Comparison of three widely used marking techniques for adult anuran species *Litoria verreauxii alpina*. Herpetological Conservation and Biology. 9(2): 428-435.

LAB, LB and LFS designed the experiment; LAB collected the data, analysed the data and wrote the manuscript. LAB, LFS and LB edited the manuscript. No funding was needed to perform this research.

CHAPTER 3

Paper: Brannelly LA, Webb RJ, Skerratt LF, Berger L. Amphibians with infections disease increase their reproductive effort: evidence of the terminal investment hypothesis. Open Biology. In Press.

LAB conceptualised the experiment, LAB, LSF and LB designed the experiment, LAB and RJW performed the experiment and collected the data, LAB analysed the data and wrote the manuscript. LAB, RJW, LFS and LB edited the manuscript. LFS and LB provided funding, reagents and materials for the research.

CHAPTER 4

Paper: Brannelly LA, Webb R, Skerratt LF, Berger L. Effects of chytridiomycosis on hematopoietic tissue in the spleen, kidney and bone marrow in three diverse amphibian species. In review.

LAB, LSF and LB conceptualised and designed the experiment, LAB and RJW performed the experiment and collected the data, LAB analysed the data and wrote the manuscript. LAB, RJW, LFS and LB edited the manuscript. LFS and LB provided funding, reagents and materials for the research.

CHAPTER 5

Paper: Brannelly LA, Roberts AA, Skerratt LF, Berger L. Epidermal apoptosis in frogs with chytridiomycosis. In review.

LAB, LFS and LB designed the project, LAB and AAR collected the data, LAB analysed the data, LAB and AAR wrote the manuscript, LAB, AAR, LFS and LB edited the manuscript. LFS and LB provided funding, reagents and materials for the research.

CHAPTER 6

Paper: Brannelly LA, Hunter DA, Marrantelli G, Skerratt LF, Berger L. Population differences in survivorship after disease of a functionally extinct amphibian species.

DAH, GM, LFS and LB designed the experiment, LAB collected and analysed the data and wrote the manuscript. LAB, LFS and LB edited the manuscript. DAH, LFS and LB provided funding, reagents and materials for the research, and DAH and GM provided animals for the research.

CHAPTER 7

Paper 1: Brannelly LA, Berger L, Marrantelli G, Skerratt LF. Low humidity is a failed treatment option for chytridiomycosis in the critically endangered southern corroboree frog. Wildlife Research. 42:44-49.

LAB conceptualised the research, LAB, LB, GM and LFS designed the experiment. LAB collected and analysed the data and wrote the manuscript. LAB, LFS and LB edited the manuscript. LFS and LB provided funding, reagents and materials for the research, and GM provided animals for the research.

Paper 2: Brannelly LA, Skerratt LF, Berger L. 2015 Treatment trial of clinically ill corroboree frogs with chytridiomycosis with two triazole antifungals and electrolyte therapy. Veterinary Research and Communication 39:179-187.

LAB, LFS and LB conceptualised and designed the experiment. LAB collected the data. LAB and LB analysed the data. LAB wrote the manuscript. LAB, LFS and LB edited the manuscript. LFS and LB provided funding, reagents and materials for the research.

Abstract

Amphibians are currently experiencing the greatest decline in biodiversity of all vertebrate taxa globally. While there are many reasons for declines, recently disease has been identified as the primary cause of catastrophic population crashes. One disease in particular, chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis*, *Bd*, is considered the worst pathogen to cause biodiversity loss. Declines of amphibians due to *Bd* infection began in the 1970s, and since then, numerous species have gone from being widespread and at high density to contracted populations near extinction. Much effort and research has been aimed at reversing these population declines but the disease is widespread with high prevalence and impacts. The purpose of this thesis is to explore how *Bd* infection affects declining amphibian species in Australia, with a focus on pathogenesis of the disease, mechanisms of population persistence and how understanding disease ecology can inform management techniques.

There were five major components of this project. **Aim 1**: to explore the disease ecology of a threatened and declining species and trial reintroduction as a management technique. **Aim 2:** to investigate the effect of disease on reproduction. **Aim 3**: to investigate underexplored mechanisms of pathogenesis of disease in susceptible species, specifically lymphocyte depletion and apoptosis. **Aim 4:** to investigate population differences on susceptibility of infection in a susceptible and endangered species. **Aim 5**: to explore new potential treatment options for captive colonies. I focused my investigations on declining or endangered alpine species of Australia, using two species: *Pseudophryne corroboree* and *Litoria verreauxii alpina*. In each chapter I used one or both of these species to explore the aims.

For the first aim, I trialled an amphibian reintroduction and explored disease ecology in wild populations of *L. v. alpina*. Captive raised animals were released into sites that held current populations and sites from which the species had been extirpated. Reintroduced animals initially fared well after reintroduction suggesting that reintroduction could be used as a management technique. However, infection increased throughout the breeding season and recovery from infection was low, suggesting that all animals succumbed to disease at the end of the breeding season, whether reintroduced or extant. These results suggest that while the populations are persisting at certain sites, the populations are experiencing near population turnover each year and disease resistance is not evolving.

For the second aim, I investigated the effect of disease on reproduction, specifically gametogenesis. Surprisingly, both spermatogenesis and oogenesis increased in experimentally infected animals. Therefore, increased reproduction might be an important mechanism of population persistence, and might be more important than immunity in these species. However, although increased reproduction and high recruitment may be effective in enabling population persistence in the short term, it

appears a precarious strategy as it relies on annual success. These results also suggest that it is important to explore novel and understudied mechanisms of population persistence, which may impact management priorities.

Pathogenesis of *Bd* infection on understudied susceptible species was explored in Aim 4 by examining two potential aspects of the immune response in experimentally infected animals: hematopoietic tissue depletion and epidermal apoptosis. Quantifying hematopoietic tissue using histology showed leucocytes were depleted in infected animals, but this effect varied among species and tissue types examined (ie spleen, bone marrow and kidney). My results appear to confirm previous *in vitro* studies showing *Bd* releases immunosuppressive compounds. Assessing epidermal apoptosis using TUNEL assays and caspase activity revealed that apoptosis was suppressed early in infection, but then apoptosis increased reaching high levels at terminal stages of disease. These aspects of pathogenesis varied between individuals and species, suggesting that animals that can resist hematopoietic depletion and modulate epidermal apoptosis will be more likely to minimise infection risk and outcomes.

The fourth aim was to investigate population differences in susceptibility to *Bd* infection in remnant populations of a critically endangered species. I exposed *P. corroboree* sourced from four populations, and found that one population was more resistant, exhibiting decreased infection load and increased survivorship. This population can be utilised to identify mechanisms of resistance for incorporating into captive breeding programs.

The final aim was to explore new treatment options, because maintaining disease free colonies of amphibians is imperative to the conservation effort. I explored decreased humidity as a treatment and found it to be unsuccessful. I also trialled an intensive combination of electrolyte replacement and antifungal chemotherapy in an attempt to cure frogs with late stage disease, but only one frog recovered. But, there was an increased survival time, suggesting that there is potential to treat frogs with severe chytridiomycosis.

Research over the past decade on the impacts, outcomes and immune mechanisms of chytridiomycosis on amphibians has shown species vary widely. Although it is easier to use model species, it is important to study susceptible and declining amphibians to develop targeted and context specific conservation solutions. My research on pathogenesis has produced evidence for improved captive breeding and treatment. Field and laboratory studies on disease ecology, potential reservoir hosts and mechanisms of population persistence will inform management opportunities targeted at protecting these declining species. For example I have shown that supporting annual reproduction may be more feasible and effective than reducing mortality rates.

Table of contents

CHAPTER 1

Introduction and basis for the study

Amphibians are currently experiencing the greatest biodiversity decline of all vertebrate taxa (Stuart et al. 2004). Chytridiomycosis, a skin disease caused by the chytrid fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), is one of the major causes of this dramatic decline (Skerratt et al. 2007). *Bd* is the worst pathogen on record for causing biodiversity loss: it infects over 600 species of amphibian and has caused declines in over 300 species of which about 200 species have become critically endangered or extinct (Skerratt et al. 2007; Wake & Vredenburg 2008). Chytridiomycosis was first identified in 1998 (Berger et al. 1998; Longcore et al. 1999), but the disease had been causing amphibian declines upon arrival in Australia as early as the 1970s (Berger et al. 1999). It has been detected in museum samples from Asia as early as 1902 (Fisher 2009), and endemic lineages have been found in Asia, Africa, Europe and Brazil where it may not have caused declines. One lineage, the Global Pandemic Lineage (GPL) has spread recently and is responsible for epdemics and population declines. The origin of *Bd* is unknown at this point and through recent genetic analyses it appears that the fungal species *Bd* has been in existence for thousands of years but the GPL emerged as a severe amphibian pathogen, likely by sexual reproduction between strains, and assisted in spread by globalisation (Farrer et al. 2011; Schloegel et al. 2012).

In Australia, six Queensland frog species became extinct in the decades since Bd arrived, but there are currently six other species in eastern Australia that have undergone slower declines and are now on the brink of extinction (Skerratt et al. 2016). Four of these are alpine species that have persisted despite high mortality rates, and intervention is now urgent. As management methods are lacking, research aimed at reducing the impacts of *Bd* and producing sustainable populations is needed (Woodhams et al. 2011; Scheele et al. 2014b).

This chapter only contains information that explains the overall approach of my project, while detailed literature reviews are included within the other chapters.

Pathogenesis of *Bd*

The amphibian chytrid fungus is not host specific and it is unclear what qualities of the host allow for *Bd* infection to cause the disease chytridiomycosis. The pathogen

infects the upper layers of the epidermis, where it reproduces and accumulates. There is little observed inflammation at site of infection (Berger et al. 1998), which is unlike other fungal pathogens of amphibians (Speare et al. 1997), suggesting that the pathogen is able to suppress the immune response. *In vitro* studies showed cultured *Bd* causes apoptosis of lymphocytes from the spleen and reduced proliferation (Fites et al. 2013). Further, experimentally infected frogs *in vivo* had a reduced immunoglobulin and splenic lymphocyte response to antigenic stimulation with sheep red blood cells, and lower blood lymphocyte counts (Young et al. 2014), and a reduced response to PHA injection (Fites et al. 2014).

The dermis and epidermis are immensely important for water, ion and oxygen regulation in amphibians (Heatwole 1994), and *Bd* infection disrupts those important pathways by damaging the skin: it causes hyperkeratosis (or thickening of the stratum corneum) erosions, and ulceration in extreme cases (Berger et al. 1998). Chytridiomycosis ultimately causes death by heart failure (Voyles et al. 2007). Progress of infection varies between species and individuals, but in many species infection load increases exponentially at about weeks 2-5. Frogs appear normal until a threshold is reached where they succumb to severe chytridiomycosis 3-12 weeks after infection (Voyles 2009; Brannelly et al. 2015d). Terminal chytridiomycosis presents as an apparently acute condition- frogs may be in good body condition and show non-specific signs such as loss of appetite, red legs, splayed legs, irregular skin slough and slow or abolished righting reflex (Pessier & Mendelson 2010).

Since the discovery of *Bd* in 1998 (Berger et al. 1998; Longcore et al. 1999), researchers have investigated immune mechanisms against *Bd* infection in a wide range of amphibian species. Some mechanisms explored are antifungal bacterial flora on the skin surface (Conlon 2011a, 2011b), antimicrobial skin peptides (AMPs) (Rollins-Smith & Conlon 2005), major histocompatibility complex (MHC) (Savage & Zamudio 2011), and splenic lymphocyte activity (Ramsey et al. 2010). Despite the decades of investigation, neither a single immune mechanism nor a suite of mechanisms have yet been identified to explain the variation in susceptibility of amphibian hosts. While there is much research devoted to finding immune mechanisms against the pathogen in a variety of convenient or model species, more direct research is needed on susceptible and declining species. Pathogenesis of the disease has been investigated in terms of pathology of the skin and explored in species with relatively stable populations (Voyles et al. 2009;

Peterson et al. 2013; Baitchman & Pessier 2013). However, host species differences, pathogenesis in declining species, and unique mechanisms of pathogenesis need further investigation.

Disease ecology and management efforts

Epidemics occur when *Bd* enters a system, but in many cases populations can persist with the continued presence of the disease and over time it behaves more like an endemic disease. Many populations appear stable with reduced distributions and abundances, and sometimes population rebounds occur (Voordouw et al. 2010; Lam et al. 2010). Persisting populations may be assisted by refugia, such as warmer, dryer habitat or lower elevation where the environmental factors assist in reducing disease intensity and enable the individuals to overcome infection (Puschendorf et al. 2011). However, even when the pathogen becomes endemic in a region, mortality rates from disease remain high.

Understanding host-pathogen dynamics and interactions within the environment is immensely important to understanding impacts of disease on both individuals and populations (Murray et al. 2009). One of the most effective methods to understanding disease ecology is by conducting intensive capture mark recapture (CMR) studies. CMR studies enable a greater understanding of survival, recapture probability, and disease exposure and spread within a population. Other disease ecological factors that can be explored through CMR are habitat factors such as water body dynamics, temperature, humidity, population density and disease dynamics and reservoir hosts (Briggs et al. 2005; Murray et al. 2009; Phillott et al. 2013). CMR studies are a useful tool for management purposes because impacts of disease on population persistence are revealed and the results can inform management practices for threatened and declining species.

Management efforts are underway in many countries because amphibian species are declining at an alarming rate, often due to *Bd* infection (Skerratt et al. 2007; Griffiths & Pavajeau 2008). While understanding the disease ecology of at risk species is immensely important in devising optimal management, these studies are intensive, expensive, and usually require a long time frame. Therefore, pro-active interventions to reduce extinction risk usually need to begin before research to mitigate the impacts of *Bd* are complete. However, it is important to trial management methods following the

scientific method, to collect evidence for future management efforts (Dodd Jr. & Seigel 1991; Sarrazin & Barbault 1996).

A major focus of my PhD research was devoted to investigate understudied endangered species, in order to inform management decisions. I am interested in bridging the gap between applied research and management for declining species. Amphibian species are declining globally partly because of *Bd,* and species in decline need practical management solutions to meet their conservation objectives.

Protecting captive colonies

Management efforts involving captive colonies and reintroduction efforts are currently underway in zoos and other captive facilities around the world for endangered and declining amphibian species. Maintaining the health of these colonies represents a challenge, in particular reducing effects of chytridiomycosis in terms of mortality and biosecurity. While there are successful therapies for treating *Bd* infection in captive colonies, no single treatment has been successful across species and age classes (Berger et al. 2009, 2010; Woodhams et al. 2012; Brannelly et al. 2012). Because treatments are not universally successful, it is important to trial specific treatments for endangered and declining species before an outbreak within a colony occurs.

Thesis aims

There were five major components of this thesis which are outlined in the schematic Figure 1:

Aim 1: To explore the disease ecology of a threatened and declining species and trial reintroduction as a management technique.

I endeavoured to understand factors that enable some populations of declining and susceptible amphibian species to persist with *Bd* while other populations are extirpated. I conducted an intensive CMR study of two populations during the breeding season. Through this study I assessed disease impact, survival and recapture rates. I also assessed two potential reservoir hosts (an amphibian and a crayfish species) within the habitats. I

determined factors that influenced survivorship and gained knowledge on disease ecology that can be used to improve management outcomes.

In addition to investigating disease ecology, I trialled the use of reintroduction as a management technique. I directly assessed reintroduction as a management technique for captive raised amphibians susceptible to *Bd* infection by assessing survivorship and disease presence. Variables assessed in this study included the effects of population of origin on recapture rate and survivorship, as well as the environmental factors that influence reintroduction success.

Aim 2: To investigate the effect of disease on reproduction.

Results from Aim 1 showed *Litoria verreauxii alpina* have been able to persist despite remaining highly susceptible to *Bd* infection, even after over 20 years of coexisting with the pathogen. High recruitment appears to be an alternative strategy to mitigate high mortality rates. Mounting an immune response to fight disease is costly for an organism, and can cause trade-off of investment from another life history trait, like reproduction. In some non-amphibian cases, an organism will actually increase reproductive effort when their survival is threatened by disease (Bonneaud et al. 2004; Velando et al. 2006; McCallum et al. 2013). I explored gamete production in males and females through an infection experiment and histological examination of the gonads in order to assess reproductive investment in response to disease. Investing resources in reproduction over immunity can have major implications for the evolution of disease resistance in declining species. If infected animals are increasing reproductive efforts and producing more offspring before succumbing to disease, it is possible that population level selection for disease resistance or tolerance is minimised. Therefore increased reproductive effort might be an important mechanism for population persistence and explain high susceptibility and mortality of populations with endemic *Bd* infection.

Aim 3: To investigate underexplored mechanisms of pathogenesis of disease in susceptible species.

Despite over a decade of research into pathogenesis of *Bd* infection, effective mechanisms of immunity and explanations for differences between species and individuals remain unclear. I explored two aspects of pathogenesis: lymphocyte depletion and epidermal apoptosis. To investigate lymphocyte depletion I quantified the density of

hematopoietic tissue in different organ types and different species. I explored apoptosis as a pathology of *Bd* through an infection experiment and sampling epidermal tissue as disease progressed. Through these studies I aimed to assess species differences in susceptibility and pathology of disease. Such information using underexplored mechanisms of pathogenesis expands the knowledge of disease progression in susceptible and declining species and may be useful in determining individual variation in disease susceptibility.

Aim 4: To investigate population differences in susceptibility to* Bd *infection in a susceptible and endangered species.

Many endangered species now occur in very small populations and it is unknown if resistant phenotypes are present, which could be used to understand and bolster immunity. Extensive reintroduction efforts are currently under way using *Pseudophryne corroboree*, but their susceptibility has not been quantified. To investigate population differences in susceptibility to *Bd* infection in remnant populations of this critically endangered species, I exposed *P. corroboree* sourced from four populations in captivity.

I monitored susceptibility by infection level, time until death, and survival. Understanding the differences in susceptibility between individuals and populations is important for improving survival of reintroduced animals in the presence of *Bd* in the environment.

Aim 5: To explore new potential treatment options for captive colonies.

I explored novel treatment regimens to treat chytridiomycosis and *Bd* infection: non-chemotherapeutic through drying treatment regimes, and intensive therapy to treat late stage chytridiomycosis. Establishing and maintaining disease free colonies represents a significant challenge to amphibian management facilities. The current chemotherapeutic methods for treating *Bd* infections, although effective, can be harmful to the animals being treated and no single treatment method has been successful across species and age classes of amphibians. Exploring novel treatments for target species is important for maintaining healthy captive colonies.

Study system

In Australia, the alpine amphibians have been heavily impacted and are still undergoing population declines (i.e. *Litoria booroolongensis*, *Litoria spenceri* and *Pseudophryne corroboree, Philoria frosti, Litoria spenceri*) (Osborne et al. 1999, Skerratt et al. in press). Montane regions of temperate and tropical environments are often undisturbed or pristine habitats within protected areas. However, despite minimal human impact, populations of montane amphibians have declined the most severely (Osborne et al. 1999; Carey et al. 1999). Chytridiomycosis is a primary cause of amphibian declines in alpine regions of Australia (Hunter et al. 2009a). *Bd* thrives in cooler alpine climates (Berger et al. 2004; Murray et al. 2011). Additionally, alpine amphibians often aggregate to breed and have a shorter breeding season than their lowland counterparts, due to the shorter period of ideal temperatures for activity. Because the pathogen favours cooler temperatures and can spread quickly throughout the populations during the aggregate breeding season, alpine species have been greatly affected by *Bd.* However, the alpine amphibians have declined more slowly than tropical species, so that although there have been no extinctions in temperate areas to date, there are now more extant alpine species at high risk (Skerratt et al. 2016). One possible explanation for the slow decline is that alpine species often have slower life histories due to energy availability of cooler environments for ectotherms.

Study species

Litoria verreauxii alpina

The critically endangered alpine tree frog, *L. v. alpina,* is native to the upland regions of New South Wales and Victoria, Australia above 1200 m. *Litoria v. alpina* occurs in sub-alpine woodland, grasslands, and bog environments. Breeding populations occur in streamside pools and calling occurs from the late winter to early summer (Gillespie et al. 1995). Eggs are laid in pools around submerged vegetation in large jelly-like clumps; free-swimming larvae hatch within a few days (Gillespie et al. 1995). Tadpoles have been recorded from November to January and metamorphose from December to January (Hunter et al. 1998). During the non-breeding season, individuals

disperse from the breeding habitat and hide amongst leaf litter, under logs or stones (Gillespie et al. 1995).

Once widespread, *L. v. alpina*'s distribution has contracted 80% of its former range since the 1980s (Osborne et al. 1999). The decline is due primarily to *Bd* infection (Hunter et al. 2009a), and despite significant declines, some populations are persisting in the presence of the pathogen (Osborne et al. 1999). Historically, the species was common in ephemeral wetland habitats, but appears to have been extirpated from these areas (Osborne et al. 1999), and all current populations occur near permanent water bodies, many of which are artificial, such as small damns and reservoirs (Osborne et al. 1999). *Litoria v. alpina* is unique in having an uninfected and non-declined population nearby infected areas, presenting a unique opportunity for evolutionary biology.

A previous laboratory experiment showed that while the mortality rate is high in this species, susceptibility to chytridiomycosis varied among clutches and populations and appeared to be evolving as frogs from a naïve population had slightly lower survival than a long exposed (>20yrs) population (Bataille et al. 2015). In the closely related *L. v. verreauxii*, populations that declined are re-expanding, which suggests genetic resistance or other mechanisms of population persistence may be developing (Scheele et al. 2014a). Understanding the mechanisms by which some populations are persisting with *Bd* could be important for enabling the recovery of the species, and could serve as a model for other species in similar peril.

Pseudophryne corroboree

The southern corroboree frog, *P. corroboree*, is functionally extinct in the wild, with only a handful of individuals remaining. The species is endemic to the Kosciuszko National Park in New South Wales, Australia and occupies small pools, sphagnum bogs, wet heath and wet tussock grasslands at an altitude of 1300-1760m (Osborne 1990). *Pseudophryne corroboree* reach sexual maturity at 4-6 years, and the oldest animal found in the wild was nine years old but this species is known to survive much longer in captivity (Hunter 2000). Annual survivorship is between 50 and 60% for adults (Hunter 2000).

The breeding season begins with males calling from December – March. Males call from small nests in the moss or other soft vegetation on the edge of breeding pools (Osborne 1990). The species has relatively low fecundity, with females laying around 30

eggs per clutch per year, and males guard the nests (Hunter 2012). Males can care for multiple clutches at one time and are territorial (Hunter 2000). Egg laying usually occurs in mid summer, and embryos develop through to Gosner stages 27 at which point eggs enter a diapause and await a flooding before hatching, between March and September. Survivorship from egg laying to metamorphosis is estimated to be 20%, but decreases to 0% survivorship during drought years when pools desiccate (Hunter 2000).

The primary cause of decline is chytridiomycosis (Hunter et al. 2009b). *Pseudophryne corroboree* have been the focus of a population monitoring program since 1986 and the decline was not halted (Hunter 2012). Currently, *P. corroboree* is being sustained by captive assurance colonies in New South Wales and Victoria Australia with the hope of reintroduction, and some active reintroductions and other recovery methods underway (Hunter 2012). In adult frogs, *Bd* prevalence in the field was typically about 30%. However, infection and susceptibility trials had not been conducted prior to the commencement of my PhD research. Understanding pathogenesis, immunity and variation between individuals and populations is immensely important to the conservation effort and reintroduction of this functionally extinct species.

Throughout this thesis I explored disease impact on these two species that are endangered due to chytridiomycosis, and endemic to the Australian Alps: *Litoria verreauxii alpina* and *Pseudophryne corroboree.* Throughout this thesis I used either or both species to gain insights that can be used to improve their conservation. They inhabit the same region but exhibit different disease ecology; while *L. v. alpina* has almost complete annual mortality and a high turnover, *P. corroboree* has a lower mortality rates but also lower recruitment rates.

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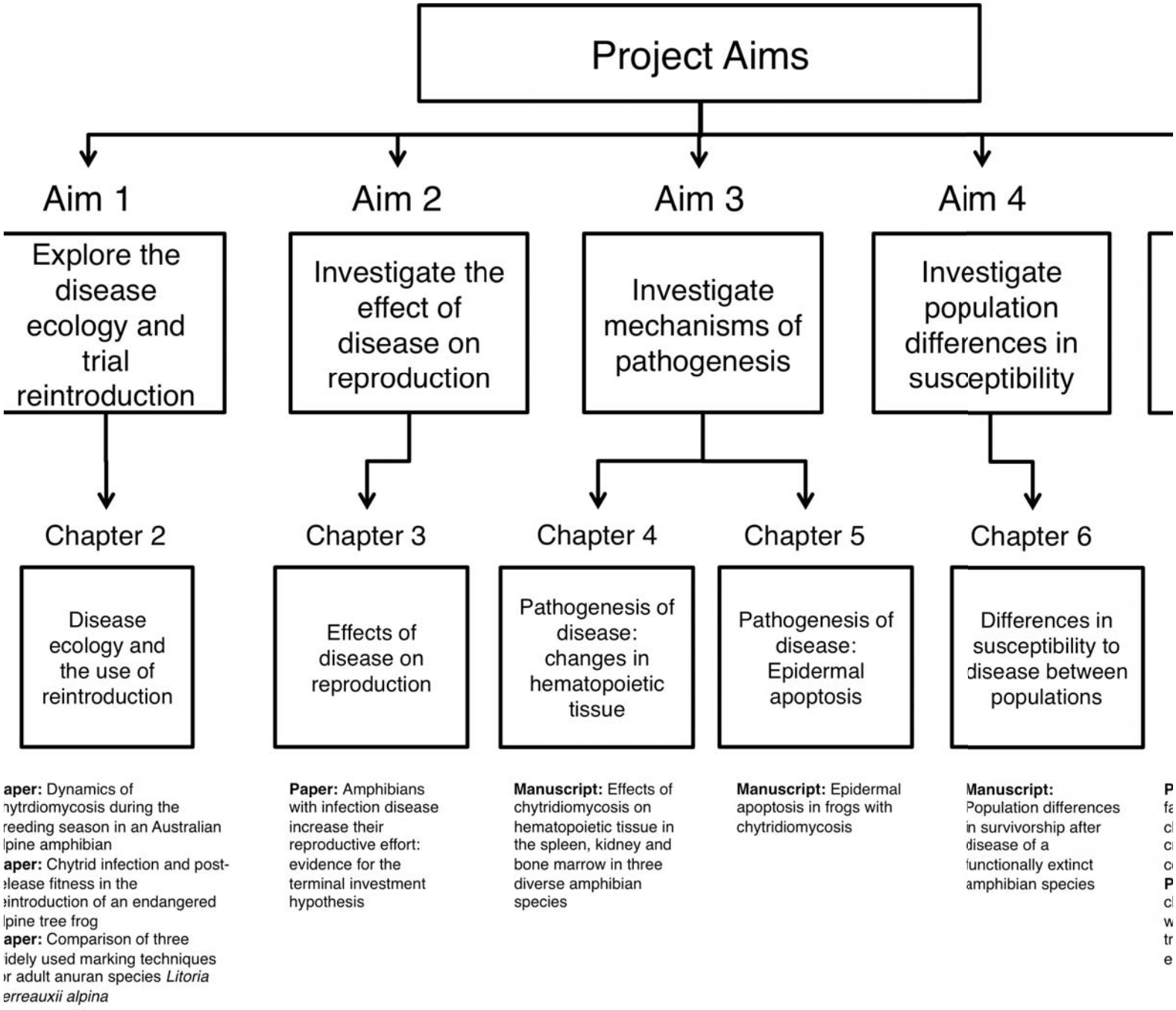
Figure Legend

Fig. 1 Schematic of the project aims and chapters.

The project aims and how they are sepatated between chapters and publications.

Figure

Figure 1



CHAPTER 2

Disease ecology of a threatened and declining species and the use of reintroduction as a management technique

Introduction

In Chapter 2 I investigated the first aim of this thesis: to explore the disease ecology of a threatened and declining species and trial reintroduction as a management technique.

For this study I explored the disease dynamics of the alpine tree frog, *Litoria verreauxii alpina* during the breeding season at two sites. I explored survivorship, recapture rates and disease transmission and recovery through an intensive capture mark recapture study over the 12-week breeding period of this species. I further investigated factors that may impact disease dynamics, such as population density and reservoir hosts. This study is **Paper 1** of this chapter: "Dynamics of chytridiomycosis during the breeding season in an Australian alpine amphibian," which has been published in PLoS ONE.

Concurrently with the exploration of disease dynamics during the breeding season in *L. v. alpina*, I trialled reintroduction as a management technique. While it is imperative that disease ecology of a species is studied to determine disease impacts and identify management techniques for a specific system, such research is time intensive and costly, and species at risk of decline and extinction cannot wait for all data to be gathered before action is taken. Sometimes, it is better, provided that resources including surplus individuals of the target species are available, to trial management options while the information is gathered. For this reason we trialled a reintroduction of the endangered *L. v. alpina* where animals were captive raised from three different populations of origin and released into two paired sites. We assessed the efficacy of using reintroduction as a management technique for this species and determined factors that influence the success of reintroduction. This study is **Paper 2** of this chapter: "Chytrid infection and post-release fitness in the reintroduction of an endangered alpine tree frog," which has been published in Animal Conservation.

Because both **Paper 1** and **Paper 2** of Chapter 2 were capture mark recapture studies, identifying a method for identifying individuals is imperative. I ran a marking method trial where I explored three different marking options: passive integrative transponders, visual implant elastomers, and toe clipping. This marking study is **Paper 3** of this chapter: "Comparison of three widely used marking techniques for adult anuran species *Litoria verreauxii alpina*," which has been published in Herpetological Conservation and Biology.

Paper 1

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Dynamics of chytridiomycosis during the breeding season in an Australian alpine amphibian

Short Title: Disease dynamics during the amphibian breeding season

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Abstract

Understanding disease dynamics during the breeding season of declining amphibian species will improve our understanding of how remnant populations persist with endemic infection, and will assist the development of management techniques to protect disease-threatened species from extinction. We monitored the endangered *Litoria verreauxii alpina* (alpine treefrog) during the breeding season through capture-mark-recapture (CMR) studies in which we investigated the dynamics of chytridiomycosis in relation to population size in two populations. We found that infection prevalence and intensity increased throughout the breeding season in both populations, but infection prevalence and intensity was higher (3.49 and 2.02 times higher prevalence and intensity, respectively) at the site that had a 90-fold higher population density. This suggests that *Bd* transmission is density-dependent. Weekly survival probability was related to disease state, with heavily infected animals having the lowest survival. There was low recovery from infection, especially when animals were heavily infected with *Bd.* Sympatric amphibian species are likely to be reservoir hosts for the disease and can play an important role in the disease ecology of *Bd*. Although we found 0% prevalence in crayfish (*Cherax destructor*), we found that a sympatric amphibian (*Crinia signifera*) maintained 100% infection prevalence at a high intensity throughout the season. Our results demonstrate the importance of including infection intensity into CMR disease analysis in order to fully understand the implications of disease on the amphibian community. We recommend a combined management approach to promote lower population densities and ensure consistent progeny survival. The most effective management strategy to safeguard the persistence of this susceptible species might be to increase habitat area while maintaining a similar sized suitable breeding zone and to increase water flow and area to reduce drought.

Key terms

Capture-mark-recapture, density-dependence, endangered species, population monitoring, reservoir host, wildlife disease

Introduction

The amphibian disease chytridiomycosis (caused by the fungal pathogen *Batrachochytrium dendrobatidis, Bd*) is a major cause of amphibian declines globally and has been called the most devastating threat from disease to biodiversity [1]. In-depth ecological studies are important for determining disease impact in the wild because many factors can affect disease dynamics. Breeding habitat plays an important role in the prevalence of *Bd* infection, with higher prevalence more often associated with permanent water bodies [2,3]. *Bd* is known to exhibit density-dependent disease transmission [4–6]. *Bd* infection tends to peak seasonally [7–11], and the peak is often attributed to optimal temperature conditions for *Bd*. However, other factors might play a role [12]. Aggregate breeders often experience dramatic increases in infection prevalence during their short breeding season [13], likely due to both increased density of animals and increased frequency of contact due to breeding behaviour. Intensive population monitoring throughout the breeding season can shed light on the ecological impact of *Bd* on declining species and can inform management decisions.

Capture-mark-recapture (CMR) studies are an effective ecological tool to determine effects of disease on populations and individuals in the wild and guide management decisions [14]. The in-depth analysis of CMR data allows for greater understanding of how survival and recapture probability are directly affected by disease and the probability of an animal gaining an infection or recovering from infection [15]. CMR studies in amphibian populations with prevalent *Bd* infection have uncovered factors that influence disease dynamics including a dependence on disease prevalence, infection intensity, temperature, population density, and reservoir hosts [5,14,15]. Infection intensity is known to play an important role in disease dynamics, yet CMR studies often do not separate animals with heavy versus light infection [5,16]. It is important, particularly for pathogens with a strong relationship between infection burden and disease impact, to conduct CMR analyses that include infection load in order to obtain a true understanding of the disease dynamics [5].

Here, we conducted a CMR study to monitor the effects of prevalence and intensity of *Bd* infection in the endangered alpine treefrog, *Litoria verreauxii alpina*, with the purpose of better understanding the ecology of chytridiomycosis during the breeding season. Our aim was to identify opportunities for intervention that would promote the

recovery of this and similar species. *Litoria v. alpina* is an aggregate breeder and native to the upland regions of the Australian Alps above 1200 m. Once widespread, *L. v. alpina*'s distribution has declined by over 80% of its former range since the 1980s [17] due primarily to chytridiomycosis [18]. Despite major declines, some populations persist in the presence of the pathogen [17,18]. While adults are known to have high mortality due to the disease, tadpoles and juveniles do not; therefore, high recruitment plays an important role in population persistence [19]. Although this species is endangered, populations have not been intensively monitored for chytridiomycosis during the breeding season when animals are thought to become infected.

A second aim to this study was to screen for potential *Bd* reservoir species. Disease reservoirs can play an important role in the disease dynamics of co-occurring susceptible species [5]. A possible amphibian reservoir species present in the Australian Alps is the widespread and abundant common eastern froglet, *Crinia signifera* [18,19]. Recent research of non-amphibian hosts of *Bd* identified the North American crayfish, *Procambarus clarkii* [20,21]. While *P. clarkii* is not present in Australia, the commercially farmed and invasive crayfish species *Cherax destructor* is widespread throughout Australia [22] and might be involved in the ecology of chytridiomycosis.

Materials and methods

Study site

We conducted an intensive 10-week CMR study of two populations of *L. v. alpina* during their 12-week breeding season (see S1 File) [23]. Study Site 1: Oglivies Dam is a 0.17 hectares low elevation (1382m) site (S1 File) [23]. Sampling occurred between 1-Sept-2013 through 6-Nov-2013 (weeks 2-11 of breeding). Study Site 2: Sponar's Creek (S1 File) [23] is a 1.8 hectares high elevation (1515m) site. Sampling occurred between 25-Sept-2013 and 25-Nov-2013 (weeks 1-10 of breeding) (S1 Fig). Air and water temperatures were recorded every two hours at each site using iButtons (S2 Fig).

Field survey

Animals were captured during one to three nights each week over 10 weeks, resulting in the capture of up to 50 new animals on each survey night (S1). Animals were

captured with a new, clean, gloved hand and kept individually in a new plastic zip bag. Animals were set aside while we completed our collection and then processed and returned to the site of capture each night. Animals found in amplexus were held together in one bag. Animals were photographed (for individual identification, see S1 File), swabbed for *Bd* (see below), weighed to the nearest 0.01g and snout to venter length (SVL) was measured to the nearest 0.02mm. Waders and boots were dried between sites to prevent the spread of *Bd*.

Testing for Bd

We tested for *Bd* infection by using skin swabs and a qPCR assay [24]. The swabbing protocol was standardized by performing 45 strokes on the venter and limbs with a sterile rayon-tipped swab (MW-113, Medical Wire & Equipment, Wiltshire, United Kingdom). Genomic DNA was extracted from the swabs using the Prepman Ultra (Applied Biosystems®, Life Technologies Pty Ltd, Carlsbad, California, USA) and a bead beater to break the fungal cell walls for two minutes, and then the extract was diluted 3:47 in PCR water. Extracted DNA was then analysed using quantitative real time PCR following Boyle *et al.* [24]. We conducted the analysis in singlicate to maximise both cost efficiency and test accuracy [25,26] including a positive and negative control and a series of dilution standards (to estimate infection load in zoospore equivalents, ZE).

To test for inhibition of the swab DNA, a subset of 20 samples was haphazardly selected and an internal positive control (VIC^{TM} IPC, Applied Biosystems®, Life Technologies Pty Ltd, Carlsbad, California, USA) was added to the qPCR reaction. No inhibition was detected in those samples. Because many samples returned high (>1000ZE) infection loads and prevalence was high, we concluded that inhibition due to high zoospore loads was unimportant. We prioritized resources to increase sample size rather than including IPC's in every reaction.

Reservoir hosts

Common eastern froglets (*Crinia signifera)* were collected at both sites (n=93) between 21-Sept-2013 and 21-Nov-2013 (S2). Animals were collected and individually stored in a clean plastic bag, and swabbed for *Bd* using the same protocol as for *L. v. alpina* and then released.

The western blue claw yabbie, *Cherax destructor*, was present at both sites where *L. v. alpina* were sampled, but it proved difficult to capture. Instead, crayfish were collected from a nearby site that supported populations of *L. v. alpina* and *C. signifera*: Kiandra (S1 Fig; S2 Table) (Lat. -35.867, Long. 148.498: Elevation 1358m). Frogs at this site have similarly high *Bd* prevalence [19]. Animals were collected with baited (Fancy Feast™, Nestlé Purina PetCare, St, Louis, Missouri, USA) minnow traps between 1-Oct-2013 and 25-Nov-2013. Traps were left open for 2 – 5 days before collection. Animals were collected individually from the traps with inverted plastic zip bags, and euthanized by freezing for at least two hours (n=94).

The gastrointestinal (GI) tract was tested for *Bd* presence following Brannelly *et al.* [20]. Because faecal matter inside of the GI tract causes PCR inhibition, it was removed with a sterile swab. The inside of the GI tract was then swabbed using 30 strokes with a new sterile MW113 swab, and the GI tract was swabbed again with a second swab as a backup. We followed the same extraction and qPCR protocol as for the frogs except that we diluted DNA extraction samples 1:10. We included an IPC in each sample to test for inhibition. Samples were analysed in singlicate, but inhibited samples were reanalyzed in triplicate. When all samples returned negative results for *Bd*, we selected a subset of 24 backup swabs and re-extracted the DNA using Qiagen DNeasy Blood and Tissue Kit using a final elution volume of 200µl. The samples were then analyzed following the above stated qPCR protocol, but the DNA sample was undiluted. No evidence of inhibition was observed.

Statistical analysis

Survival, recapture probability, and disease state were examined using the statistical software M-SURGE [27]. The Conditional Arnason–Schwarz model was used. M-SURGE is a program designed specifically for multi-state CMR studies and can be used to analyse low recapture rates. We determined survival (S), recapture (r) and state change (Ψ) probabilities. The independent variables tested were site (s: Oglivies Dam and Sponar's Creek), time in weeks (t), current *Bd* state (f), and *Bd* state of previous capture (to). The two *Bd* states were *Bd* positive and *Bd* negative. Only data from week 3 through week 10 of the breeding season were included, because both sites were sampled for those eight weeks. We measured goodness of fit as "ĉ" with the program U-CARE, and ĉ=0.707. Below a value of "1", ĉ represents under distribution of the data; therefore, ĉ not

adjusted, and ĉ=1 was used in analysis. Because females were never recaptured, they were excluded from the analyses in M-SURGE. While typically only two disease states are investigated [15,28–30] (*Bd* negative and *Bd* positive) as above, we decided to further explore the effect of infection intensity. To explore infection intensity, we analysed the data using a three-disease-state model in which the three states were *Bd* negative, low infection (<350 zoospore equivalents, ZE) and high infection (>350ZE). The goodness of fit was ĉ=0.661, and it was not adjusted.

Population size for each population was analysed using a Program MARK POPAN model. Variables explored were (t) time in weeks, (Φ) survival probability, (p) recapture probability, and (Pent) probability of entry into the population. Because males were more conspicuous than females and tend to be present at the breeding site for an extended period, the "super-population size" calculated through POPAN refers to all the males that came to breed within the 2013 breeding season. Population density was estimated as the super-population size of males divided by site area.

Model selection was based on Akaike's information criterion (AIC), with the best fitting model indicated by the lowest AIC value. The seven best fitting models were chosen for analysis. We did not perform model averaging.

Infection intensity over the breeding season was assessed using ANCOVA in SPSS (v21), and individuals are covariates. The data were transformed $Log_{10}(N+1)$ for a normal distribution, and only positive animals were included in the analysis. Infection prevalence was assessed using a logistic regression in which change in infection over time was compared between populations. Prevalence between the two sites within specific weeks was compared using Pearson's Chi-Squared test in SPSS (v21), and odds ratios were calculated in Microsoft Excel. Because female capture rates were low, infection intensity and prevalence analyses only included males. To compare overall infection status of males and females between the two sites, ANCOVAs in SPSS (v21) were used, in which sex, site, week, site*sex and week*sex were the factors analysed, and individual was a covariate.

Body condition defined as mass/SVL was analysed using ANCOVAs in SPSS (v21). Mass/SVL is an appropriate measure for body condition in this species because it is highly correlative (Female: Pearson's correlation=0.817, $p < 0.01$; Male: Pearson's correlation=0.669, $p < 0.01$) and linear (Linear Regression ANOVA: Female: Mass=-5.819 + 0.272(SVL) $p < 0.01$; Male: Mass=-1.598 + 0.121(SVL), $p < 0.01$) [31]. We

compared site and time, and individuals were covariates. Effect size was determined using the averaged Cohen's *d* statistic across time and was calculated in Microsoft Excel. The effect of infection intensity on body condition was correlated using linear regression analysis in SPSS (v21), and time was not included.

Animal ethics and permits

This study was carried out in strict accordance with the recommendations of Animal Ethics. The protocol was approved by the Animal Ethics Committee at James Cook University (Application A1880). All necessary permits obtained for the described study complied with all relevant regulations. Amphibian and crayfish collection permits were issued by NPWS Wildlife Licencing and Management.

Results

Population density

The best supported model to estimate population abundance of males over the course of the breeding season was Φ(.)p(t)pent(t) for both sites (Oglivies Dam; AIC=439.318, parameters=16: Sponar's Creek; AIC=816.459, parameters=17) (Table 1). The outcomes explored were Φ=survival probability, p=recapture probability and pent=probability of entry into the population. The variable that could affect each outcome was t=time in weeks, or (.) indicating that time was not an important variable in predicting the outcome.

The population estimate for Oglivies Dam (0.17 hectares) was 2725 males (95% CI, 1712-4505); and the estimated population density was 16,031 males per hectare (95% CI, 10,070-25,498) (Table 2). Sponar's Creek (1.8 hectares) population size was smaller with 319 males (95% CI, 277-381); and the estimated population density was 177 males per hectare (95% CI, 154-212) (Table 2). The population density of *L. v. alpina* at Oglivies Dam was on average 90.53 times higher than at Sponar's Creek.

Recaptures

A total of 459 animals were captured at both sites over the course of the breeding season. At Oglivies Dam, 241 animals were captured, 18 were female, and 16.60% of

males were recaptured (Table 2). At Sponar's Creek, 218 animals were captured, 21 were female, and 36.24% of males were recaptured (Table 2).

Disease dynamics

Between recaptures, the disease state detected for some animals often changed (Table 3). The most common disease state change was from *Bd* negative to low infection intensity at Sponar's Creek, and from low infection intensity to high infection intensity at Oglivies Dam. It was less common for an animal at either site to reduce or clear infection (Table 3). Therefore, infection prevalence increased each week throughout the course of the breeding season at both sites (Logistic Regression: Exp(B)=1.313, $p<0.001$) and also differed significantly between sites (Logistic Regression: Exp(B)=0.319, $p<0.001$). Oglivies Dam had higher infection prevalence than Sponar's Creek throughout the breeding season (Odds ratio=3.50). Oglivies Dam infection prevalence was greatest at weeks 3 (Chi-Squared: $\chi^2=4.800$, p=0.028; Odds ratio=3.25) and 4 (Chi-Squared: $\chi^2=8.422$, p=0.004; Odds ratio=4.98) (Fig 1a).

Similarly, infection intensity increased throughout the course of the breeding season (ANCOVA: $F_{11, 346}=6.216$, $p < 0.001$) and differed between the two sites (ANCOVA: $F_{1,346}=6.002$, p=0.015), with Oglivies Dam having 2.02 times greater average infection intensity (d=0.48) (6,698±16,522ZE) than Sponar's Creek (3,315±10,034ZE) (Fig 2b).

Males had higher overall infection intensities than females (averaged across sites: males 5,497±736ZE; females, 2,730±2,513ZE) (ANCOVA: by sex, $F_{1,597}=612.369$, $p<0.01$; by sex*week $F_{10,597}=2.335$, p=0.01), but infection intensity did not vary by site for each sex (ANCOVA: by sex*site, $F_{1,597}=1.289$, p=0.257).

Body condition was not affected by infection intensity (Linear regression ANOVA: $F_{1, 580}=0.62$, p=0.432). Male body condition did not vary between sites (ANCOVA: $F_{1,580}=0.812$, p=0.368), but did decrease by an average of 10.51% from week to week throughout the course of the breeding season (ANCOVA: $F_{11, 580}=4.984$, $p < 0.001$; d=0.1051). Body condition of the females did not vary between sites (ANCOVA: $F_{1, 36}=0.245$, p=0.626) or through time (ANCOVA: $F_{11, 36}=1.37$, p=0.26).

CMR analysis

Two-disease-state model

Using a two state analysis (*Bd* positive and *Bd* negative) the two best fit models were Model 1) S(g)p(g*t)Ψ(to*f*t) (AIC=927.706, deviance=867.706, parameters=30), and Model 2) S(.)p(g*t)Ψ(to*f*t) (AIC=927.927, deviance=869.972, parameters=29) (Table 4). The outcomes explored were (S) survival probability, (p) recapture probability, and (Ψ) state change probability. The variables affecting each probability were (g) site, (t) time in weeks, (to) state of previous capture, (f) state of capture, and (.) indicates that no variable affected the probability. Neither model suggested that disease state influenced survival. In Model 1, survival probability per week differed between sites: animals at Oglivies Dam had a lower estimated survival probability per week (0.721, 95% CI, 0.555-0.843) than Sponar's Creek (0.861, 95% CI, 0.726-0.935). In Model 2 survival probability was the same for all animals (0.821, 95% CI 0.722-0.890). Recapture and disease state change probability were equivalent in models 1 and 2. Recapture probability differed between sites and weeks but not for different disease states (Fig 2a). Recapture rate was lower earlier in the season for Oglivies Dam, while it was higher early in the season for Sponar's Creek. Towards the end of the sampling period, both sites had similar weekly recapture rates. The probability of an animal changing infection state was dependent on time and disease status but not site (Fig 2b). Early in the season, there was a higher probability of staying *Bd* negative or clearing infection, but after week 7 the chance of staying *Bd* negative or clearing infection dropped to a very low probability. Early in the season animals were not likely to gain *Bd* infection or remain *Bd* positive. But after week 7 the likelihood of maintaining infection or becoming infected was high.

Three-disease-state model

When including three-disease-states in the CMR analysis (*Bd* negative, "low infection" with intensity of infection under 350ZE, and "high infection" with intensity of infection above 350ZE), the best fit model was S(g*f)p(g*to+t)Ψ(to*f) (AIC=1027.424, deviance=979.424, parameters=26) (Table 4). Disease status and site were important factors influencing weekly survival. Uninfected animals from both sites had similar survival probabilities per week (Oglivies Dam: 0.766, 95% CI, 0.273-0.966; Sponar's Creek: 0.760, 95% CI 0.552-0.891), and when infected with low *Bd* loads (Oglivies Dam: 0.994, 95% CI, 0.003-1.0; Sponar's Creek: 0.997, 95% CI, 0.001-1.0). In contrast, when carrying a high *Bd* infection, weekly survival at Oglivies Dam was lower (0.460, 95% CI, 0.232-0.706) than survival at Sponar's Creek (0.771, 95% CI, 0.423-0.939). Recapture probability was dependent on week, site, and state at previous capture (Fig 2c). Recapture

probability was higher overall at Sponar's Creek than at Oglivies Dam, but infection intensity impacted recapture rates differently at the two different sites. At Oglivies Dam, animals were more likely to be captured if they had high *Bd* infection intensity; but at Sponar's Creek, *Bd* negative animals had the highest recapture probability. Infection state change probability was dependent on state of infection at current capture, and state of infection at previous capture, but not time or site (Fig 2d). While some animals remained *Bd* negative over the breeding season, most became infected. Once lightly infected, some animals appeared to clear the infection; however, if the animals showed a high intensity of infection, they were less likely to become *Bd* negative at the next capture.

Reservoir hosts

A total of 94 *C. destructor* crayfish were sampled and all tested negative for *Bd* (Fig 1a). A total of 93 *C. signifera* individuals were tested for *Bd*. Infection prevalence was 100% (Fig 1a), and infection intensity was consistently high (3875±6691ZE) throughout the breeding season (Fig 1b).

Discussion

Density-dependence

Our study supports the concept of density-dependence of *Bd* infection because the 90–fold higher density site, Oglivies Dam, had 3.49 and 2.02 times higher prevalence and intensity, respectively, throughout the breeding season than the Sponar's Creek site. This pattern has also been shown in studies of *Rana muscosa*, in which *Bd* infection also increased with increased population density [5,6,32]. The transmission and spread of *Bd* is known to have density-dependent disease transmission characteristics in other studies [4–6]. At higher population densities, uninfected animals are more likely to come in contact with infected animals and the pathogen in the environment, leading to an increase in direct and indirect transmission [33].

Disease dynamics

Infection intensity and prevalence at the beginning of the season were lower at both sites, and then it increased until the end of the breeding season (Fig 1). The increase in infection intensity and prevalence is consistent with what has been observed for other

aggregate breeders [13,34]. However, some *L. v. alpina* animals entered the breeding season with an infection suggesting that they are becoming infected outside the breeding season. These infected animals might have overwintered with infection after exposure to infection in the previous season; or, more likely, they gained infection before the breeding season while they were dispersed in terrestrial habitats. The abundance of terrestrial reservoirs could be a key factor determining exposure in the non-breeding season (see Reservoir Hosts section below). However, overwintering ecology and infection susceptibility are unknown in this species [23].

CMR analysis

Recapture rates differed between the two sites. This was expected because they differed significantly in population density [30,35,36]. Animals in the smaller population, Sponar's Creek, were more likely to be recaptured as recapture effort was relatively greater per individual (Table 2). Uninfected animals were more likely to be recaptured than infected animals at Sponar's Creek (Fig 2c), which is a pattern observed in other frog species with *Bd* [14,15]. However, recapture probabilities were different at Oglivies Dam, where heavily infected animals were more likely to be recaptured. This is consistent with CMR analysis of other wildlife diseases [28]. While the heavily infected category for this study was >350ZE, animals likely do not become ill until a much higher infection load develops [37]. Oglivies Dam animals had higher infection intensities, and more animals were likely experiencing clinical signs of chytridiomycosis in the heavily infected category compared with animals at Sponar's Creek. Heavily infected animals might be more easily recaptured because they are more lethargic. Therefore, if there is large heterogeneity in infection burden, it is important to include infection intensity in analyses to help tease apart its effects on recapture rates.

The results from the infection state change analysis suggest that the *Bd* transmission and recovery dynamics were similar in both populations. The two-disease-state analysis suggested that early in the season, some animals were able to recover from infection, but as the breeding season progressed, animals were less likely to recover and unlikely to remain *Bd* negative. The higher potential for recovery early on might occur because there were fewer heavily infected animals early in the season, which would lower transmission rates. When the recapture analysis was expanded to include three disease states, it showed that animals were very unlikely to recover from high infection

intensity as seen by the high proportion of animals remaining at high infection intensity (Fig 2d). In contrast, recovery from low infection is more likely.

Results of analysis of factors influencing survival depended on whether infection intensity was included. In the two-disease-state model without considering infection intensity, the best-fit model suggested that disease state does not influence survival; however, the lower weekly survival probability for Oglivies Dam is confounded by the higher proportion of infected individuals with higher infection intensities as demonstrated by the three-disease-state model. In the three-disease-state model, survival of the highly infected animals differed between the sites, with Oglivies Dam animals having lower survival (0.46 weekly survival) than the highly infected animals at Sponar's Creek, and the low infected and uninfected animals at both sites had similar weekly survival rates (0.76–0.99). It is possible that the site differences in survival suggested by the model were not due to site characteristics, but rather they represented differences in infection intensity between the sites that our model was unable to discern using the high intensity threshold of 350ZE. The lower survival of heavily infected animals at Oglivies Dam might be because Oglivies Dam animals were more heavily infected than Sponar's Creek animals. These results support previous reports of the progression of *Bd* infection in susceptible species. Infection intensity builds over several weeks, and animals succumb to chytridiomycosis and die when heavy infection burdens are reached [6,13,38]. Analysing field data using multistate models is important to characterize disease dynamics in affected populations [39,40].

Differences in the sexes

A limitation of this study is that females were never recaptured and so we were unable to determine the effect of disease on their behaviour and survival. However, for *L. v. alpina,* males are likely to be the drivers of *Bd* within the system due to their breeding behaviour. Males tend to be present in the breeding ponds for weeks at a time, whereas females arrive to mate and deposit their eggs and then return to their non-breeding habitat. Males may have higher infection intensity throughout the breeding season because they spend more time in the breeding habitat increasing their chance of pathogen exposure, either through direct contact with other animals, or through indirect transmission in the aquatic environment. While these results suggest that females have lower levels of infection in the breeding pond during capture than males, female *L. v.*

alpina have similar low year-to-year survivorship similar to males [19]. This suggests that total infection rates and disease outcomes are similar in both sexes.

Reservoir hosts

Reservoir species likely play an important role in infection dynamics of *L. v. alpina*. Infected *L. v. alpina* die after the breeding season resulting in almost complete population turnover every year [19]. Tadpoles and juveniles leave the pond uninfected [19] and most first time breeders enter a pond uninfected. Under such circumstances, reservoir hosts are the likely cause of pathogen persistence. Two potential reservoir species were analysed in this study: an invasive crayfish species, *C. destructor*, and a sympatric frog species, *C. signifera*. We did not find crayfish carrying *Bd* infection, suggesting that *C. destructor* is not a reservoir species in the Australian Alps. However, we found 100% prevalence and high intensity of *Bd* infection in *C. signifera*. Intensity of infection in *C. signifera* is higher than *L. v. alpina* throughout the breeding season (Fig 1b). Additionally *C. signifera* do not vary in infection prevalence or intensity throughout the season suggesting that this species is tolerant of infection and is likely to be contributing to persistence and spread of this deadly pathogen similar to other reservoir hosts of this pathogen [41]. *Litoria v. alpina* can be found in close contact with *C. signifera* during the breeding season and in shared hibernation places during the non-breeding season. It is likely that infection in *L. v. alpina* is perpetuated by direct and indirect transmission of *Bd* from *C. signifera*.

Management implications

Recent laboratory studies suggest that *L. v. alpina* might be evolving an immune response to fight infection [37], and selection for resistance is a possibility. However, artificial selection for disease resistance to chytridiomycosis has not been successfully attempted, and it is costly in terms of the research required [42,43]. In addition, the higher density site had the lower survivorship when heavily infected might reduce the opportunity for the evolution of resistance mechanisms that could permeate the population. Therefore, alternative management strategies might be more efficient in the short term to help secure the conservation of species [42]. Our study supports the density-dependence of *Bd*. Our site with higher population density of *L. v. alpina* had higher infection prevalence and intensity and lower survival for heavily infected frogs. In order

to promote survivorship and decrease effects of disease, management strategies might aim to decrease population densities. Such management techniques might include increasing the area of the water body while maintaining the same size of suitable breeding habitat within the pond or decreasing environmental disease transmission by increasing water replacement using increased flow into and out of the ponds that *L. v. alpina* inhabit.

Litoria. v. alpina are highly susceptible to *Bd* infection, rarely recover from infection, and have near complete population turnover each year in populations with endemic *Bd* infections [19]. Therefore, high recruitment enables some populations to persist despite the impact of chytridiomycosis, and is crucial to the persistence of *L. v. alpina* [15,19,44]. While reproductive output in persisting populations is sufficient to maintain populations, it is a risky mechanism for population persistence. Drought is a high risk factor for decreased progeny survival as evidenced by *L. v. alpina* having been extirpated from all ephemeral water bodies [23]. Management strategies to ensure consistent annual survival of progeny by reducing the effects of drought by increasing water body size and water input would promote population persistence [23]. Increasing water body size and water input (while maintaining breeding habitat size) might serve to both promote lower population densities and enable consistent recruitment. This strategy might be effective in sustaining some reintroduced populations. Because *C. signifera* appears to be maintaining infection within the system, reducing their abundance or reintroducing *L. v. alpina* into sites where they are absent could promote *L. v. alpina* conservation.

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Figure legend

Fig 1. Infection prevalence and intensity across sites for *Litoria verreauxii alpina, Crinia signifera* and *Cherax destructor*.

(a) Prevalence of infection. Error bars indicate 95% confidence intervals. (*) indicates time-points where prevalence is significantly different at each site for *Litoria verreauxii alpina*, using Pearson's Chi-Squared test. (b) Infection intensity transformed to $\log_{10}$ scale. Error bars indicate standard error. Sites are Oglives Dam (OD) and Sponar's Creek (SC).

Fig 2. Recapture probability and State change probability.

Conditional Arnason-Schwarz model in which outcome probabilities are (S) survival, (p) recapture, (Ψ) state change, and the variables that can influence the outcomes are (g) site, (t) time in weeks, (to) state at previous capture, (f) state at capture. Panels (a) and (b) represent the two-disease-state model, *Bd* positive (Bd+) and *Bd* negative (Bd-), in which the best model was S(g)p(g*t)Ψ(to*f*t). Panels (c) and (d) represent the three-disease-state model, *Bd* negative (Bd-), low infection intensity of >350ZE (Low) and high infection intensity of >350ZE (High), in which the best model was S(g*f)p(g*to+t)Ψ(to*f). (a) Recapture probability per week in a two-disease-state model. Factors included in the best model for recapture probability were site and week, Error bars indicate 95% confidence interval. (b) Probability of changing state per week in a two-disease-state model. Factors included in the best model for state change probability were week, infection state at current capture, and infection state at previous capture, and error bars indicate 95% confidence interval. (c) Recapture probability per week in a three-disease-state model. Factors included in the best model for recapture probability were site, state of infection and week. Error bars indicate standard error, and only one error bar included for figure clarity. (d) Probability of changing state in a three-disease-state model, error bars indicate 95% confidence interval. Sites are Oglives Dam (OD) and Sponar's Creek (SC),

Figures

Figure 1

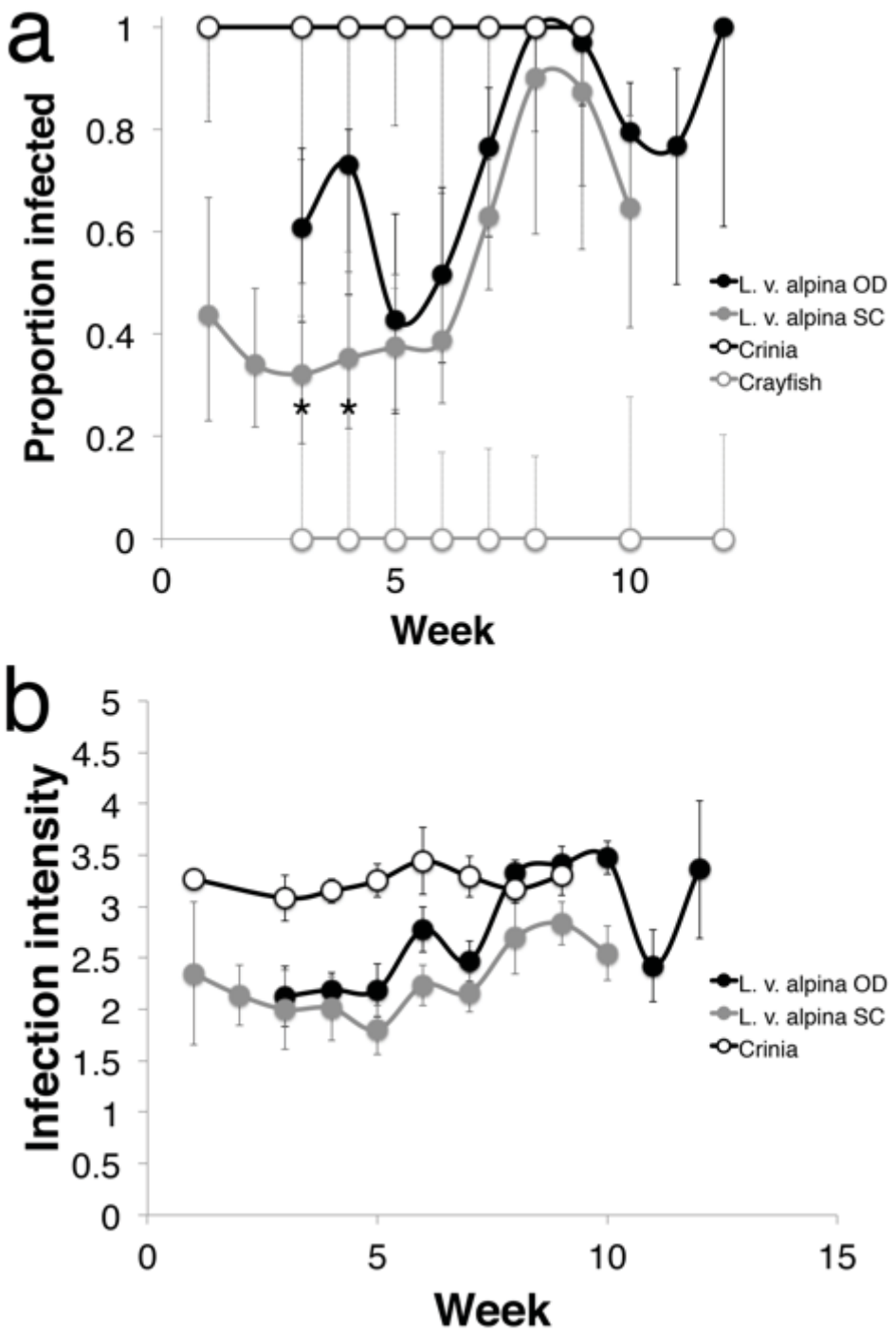
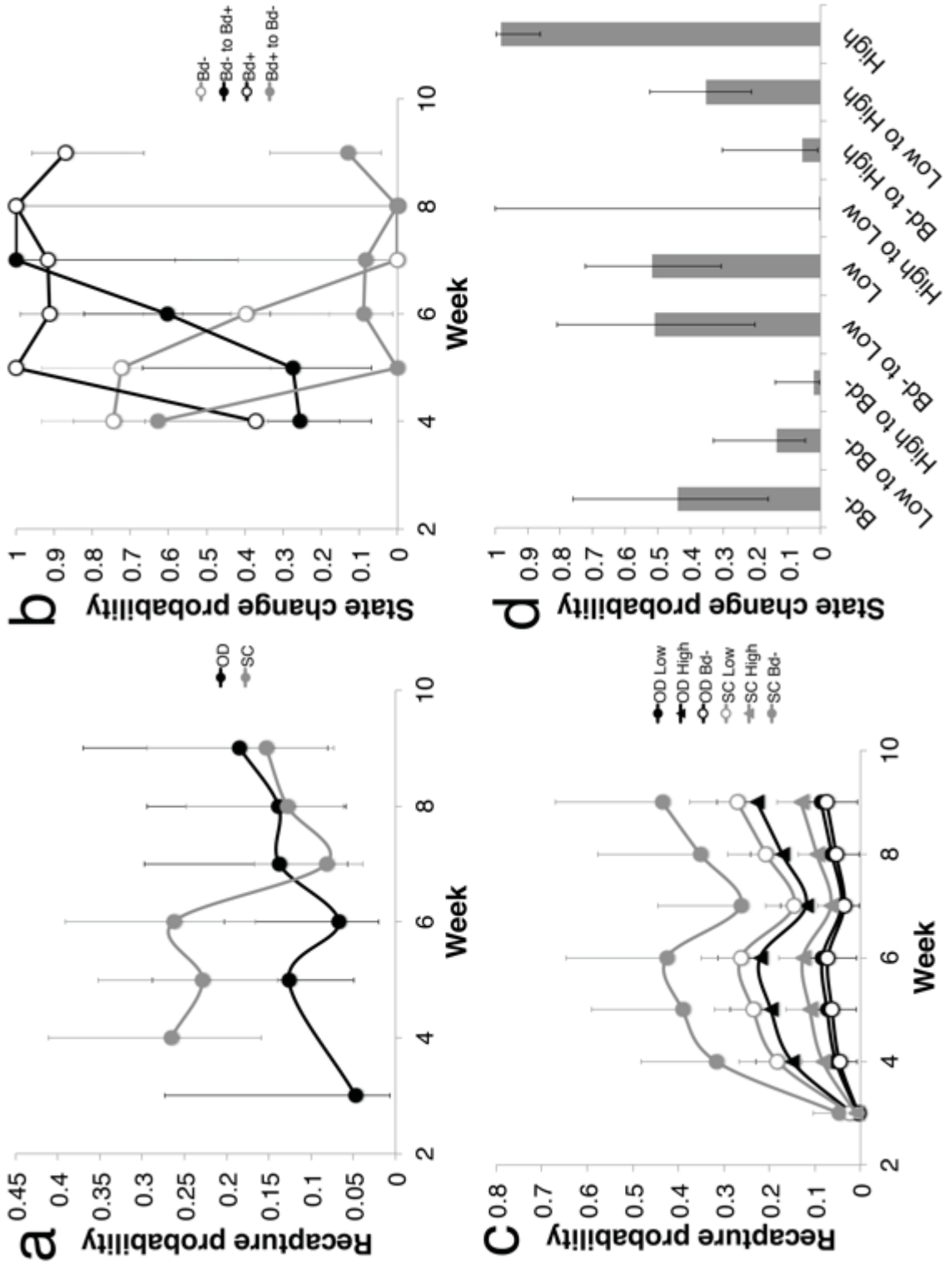


Figure 2


a
Recapture probability
0.45
0.4
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OD
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b
State change probability
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0.7
0.6
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6
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10
Week
Bd-
Bd- to Bd+
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c
Recapture probability
0.8
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SC High
SC Bd-
d
State change probability
1
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Bd-
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High to Bd-
Bd- to Low
Low
High to Low
Bd- to High
Low to High
High

Tables

Table 1. AIC ranking of best-fit POPAN models.

Model results are for the two sites: Sponar's Creek and Oglivies Dam.

Model Variables*	Parameters	Deviance	AIC	Δ AIC
Sponar's Creek				
Φ(.)p(t)Pent(t)	17	0	816.459	0
Φ(t)p(.)Pent(t)	16	0	826.993	10.534
Φ(t)p(t)Pent(t)	24	0	829.007	12.548
Φ(.)p(.)Pent(t)	8	0	830.658	14.199
Φ(.)p(.)Pent(.)	3	21818.006	23201.514	22385.055
Oglivies Dam				
Φ(.)p(t)Pent(t)	16	0	439.318	0
Φ(t)p(t)Pent(t)	23	0	449.208	9.89
Φ(t)p(.)Pent(t)	18	0	449.832	10.514
Φ(.)p(.)Pent(t)	9	0	456.482	17.164
			70049.592	69610.274
Φ(.)p(.)Pent(.)	3	67125.025	1	1

* Outcome probabilities determined are (Φ) survival probability, (p) recapture probability, and (Pent) probability of entry into the population. These outcome probabilities are determined by the variable (t) time in weeks, or no variable (.).

Table 2. Recaptured animals are each site.

Number of *Litoria verreauxii alpina* that were recaptured at the different sites (Sponar's Creek, 1.8 hectares in area: Oglivies Dam, 0.17 hectares in area) and how many times the individual was recaptured over the course of the breeding season. Population size is the super-population estimate for males.

Number of recaptures	Oglivies Dam	Sponar's Creek
0	201	139
1	40	50
2		17
3		11
5		1
Total captured	241	218
Population size	2725	319

Table 3. Disease state change over the course of the study.

The proportion of recaptured individuals that changed disease state over the course of multiple recaptures between the two sites: Oglivies Dam and Sponar's Creek. This table represents the data collected from the CMR study

.

Recaptures	Oglivies Dam	Sponar's Creek
Stay Zero	0.025	0.266
Stay Low	0.075	0.063
Stay High	0.250	0.025
Zero to Low	0.125	0.266
Zero to High	0.150	0.089
Low to High	0.275	0.127
Low to Zero	0.050	0.063
High to Low	0.025	0.013
High to Zero	0.025	0.013
Low to Zero to High		0.013
Zero to Low to High		0.051
Zero to Low to Zero to Low		0.013

Table 4. AIC ranking for Conditional Arnason–Schwarz models.

Model results for both the two-disease-state (*Bd* negative and *Bd* positive) and three-disease-state analysis (*Bd* negative, low *Bd* infection under 350ZE, and high *Bd* infection over 350ZE).

Model Variables#	Parameter	Deviance	AIC	Δ AIC
2-State				
S(g)p(g*t)Ψ(to*t)	30	867.706	927.706	0
S(.)p(g*t)Ψ(to*f*t)	29	869.972	927.972	0.266
S(g+f)p(g*t)Ψ(to*f*t)	31	867.697	929.697	1.991
S(f)p(g*t)Ψ(to*f*t)	30	869.971	929.971	2.265
S(g*f)p(g*t)Ψ(to*f*t)	32	867.377	931.377	3.671
S(f*g)p(g*t)Ψ(to*f*t)	32	867.377	931.377	3.671
S(g)p(g*t)Ψ(to*f)	18	900.469	936.469	8.763
S(g*f*to)p(g*to*t)Ψ(g*to*f*t)	67	835.799	969.799	42.093
S(.)p(.)Ψ(.)	3	987.247	993.247	65.541
3-State				
S(g*f)p(g*to+t)Ψ(to*f)	24	979.337	1027.337	0
S(g*f)p(g*to+t)Ψ(f)	21	987.779	1029.776	2.439
S(g*f)p(g*to+t)Ψ(to)	21	996.219	1038.219	10.882
S(g*f)p(g*to+t)Ψ(to*f*t)	60	921.865	1041.865	14.528
S(g)p(g*to+t)Ψ(to*f*t)	56	935.674	1047.674	20.337
S(g*f)p(g*t)Ψ(to*f*t)	62	925.296	1049.296	21.959
S(f)p(g*to+t)Ψ(to*f*t)	57	935.729	1049.729	22.392
S(g*f*t)p(g*to*t)Ψ(g*to*f*t)	113	859.464	1085.464	58.127
S(.)p(.)Ψ(.)	3	1079.595	1085.595	58.258

#Probabilities determined are (S) survival probability, (p) recapture probability, (Ψ) state change probability; and the variables that influence S, p and Ψ are (g) site, (t) time in weeks, (to) state of previous capture, (f) state of capture and no variable (.). (+) means the effect of the variables were additive, (*) means the variables are multiplied.

Supporting information

The following supporting information is available for this article online.

Supporting information legend

S1 File. Supplemental Methods. Further information on the study species, study sites, weather data collection, and individual marking methods.

S1 Fig. Map of Kosciuszko National Park, New South Wales, Australia study sites.

Oglivies Dam 35° 57' 29" S, 148° 24' 4" E: Elevation 1382m. Sponar's Creek, 36° 21' 32.4" S, 148° 30' 0" E: Elevation 1515m. Kiandra, 35° 52' 1" S, 148° 29' 53" E: Elevation 1358m, where the crayfish were collected. The white lines indicate state lines, Victoria to the West and Australian Capital Territory to the Northeast. The grey lines indicate major roadways. Scale bar=10km. Map data reprinted from Google Imagery under CC BY license, with permission from TerraMetrics, original copyright 2015.

S2 Fig. Air and water temperatures in degrees Celsius at the sites.

Sites are Oglivies Dam and Sponar's Creek. Error bars are standard error. Temperatures were collected with iButtons places at the sites.

S1 File. Supplemental methods

Study species

Litoria verreauxii alpina occurs in sub-alpine woodland, grasslands, and bog environments. Breeding populations occur in streamside pools and all current populations occur in or near permanent water bodies; many of which are artificial, such as small dams and reservoirs [1,2]. Historically the species was also common in ephemeral wetland habitats but appears to have been extirpated from these areas [1,2], and thus have resulted in the remaining populations to be isolated and distinct from each other. Calling occurs from late winter to early summer [3]. Eggs are laid in pools around submerged vegetation in large jelly-like clumps; larvae hatch within a few days [3]. Tadpoles have been recorded from November to January and metamorphose from December to January [4]. During the non-breeding season, individuals disperse from the breeding habitat and hide amongst leaf litter, logs, or stones [3]. *Litoria. v. alpina* is highly susceptible to *Bd* in the lab and succumb to infection in just three weeks [5].

Study sites

Oglivies Dam occurs at 1383 elevation and consists of two small ponds with an ephemeral creek feeding and draining. The combined water bodies occupy 0.17 hectares, and are up to 1.5m deep. The site is 20m from a seasonal road open to the public from December to May. Animals are found throughout the entire water bodies. Sponar's Creek occurs at 1515m elevation and is a large pond 5m from a main road and 50m from a hotel. The pond occupies 1.8 hectares and is fed and drained by a permanent creek. Suitable breeding habitat for *L. v. alpina* is confined to the edges of the pond, which is where animals were captured. Both sites are permanent water bodies. Study site area was measured as the water body plus a 1 m perimeter where the animals come to breed. Sampling dates were staggered between the two sites because the breeding seasons are staggered due to the differences in elevation between the two sites, with the higher elevation site beginning breeding approximately 4 weeks later than the lower elevation site. The distance between these two sites was 45.15km.

Weather data

Mean weekly air temperatures were similar for both sites but mean weekly water temperature were higher and with a steady increase over time at Oglivies Dam due to the smaller size of the pond (S. Fig. 2). Both sites temperatures were below the range for optimum *Bd* growth, which is between 13°C and 25°C [6].

Individual identification

Upon first capture a toe tip (right fourth digit) was collected and stored in 98% ethanol for a genetic sample and a marker of recapture. Inflammation at site of tipping was never observed upon recapture. Animals were individually identified by photographs of their dorsum and left side. Left side photographs were used most often for identification, and dorsum photographs were used as verification when needed. *Litoria v. alpina* are variable in colouration and pattern, but as the study was short (10 weeks at each site), pattern change was not a concern. Photographs were chosen as an identification method because a laboratory study conducted on this species demonstrated that neither passive integrative transponder (PIT) tags nor visual implant elastomer (VIE) tags were reliable tagging methods, and toe clipping, while reliable, was slow to heal and caused infection in some individuals in the laboratory [7].

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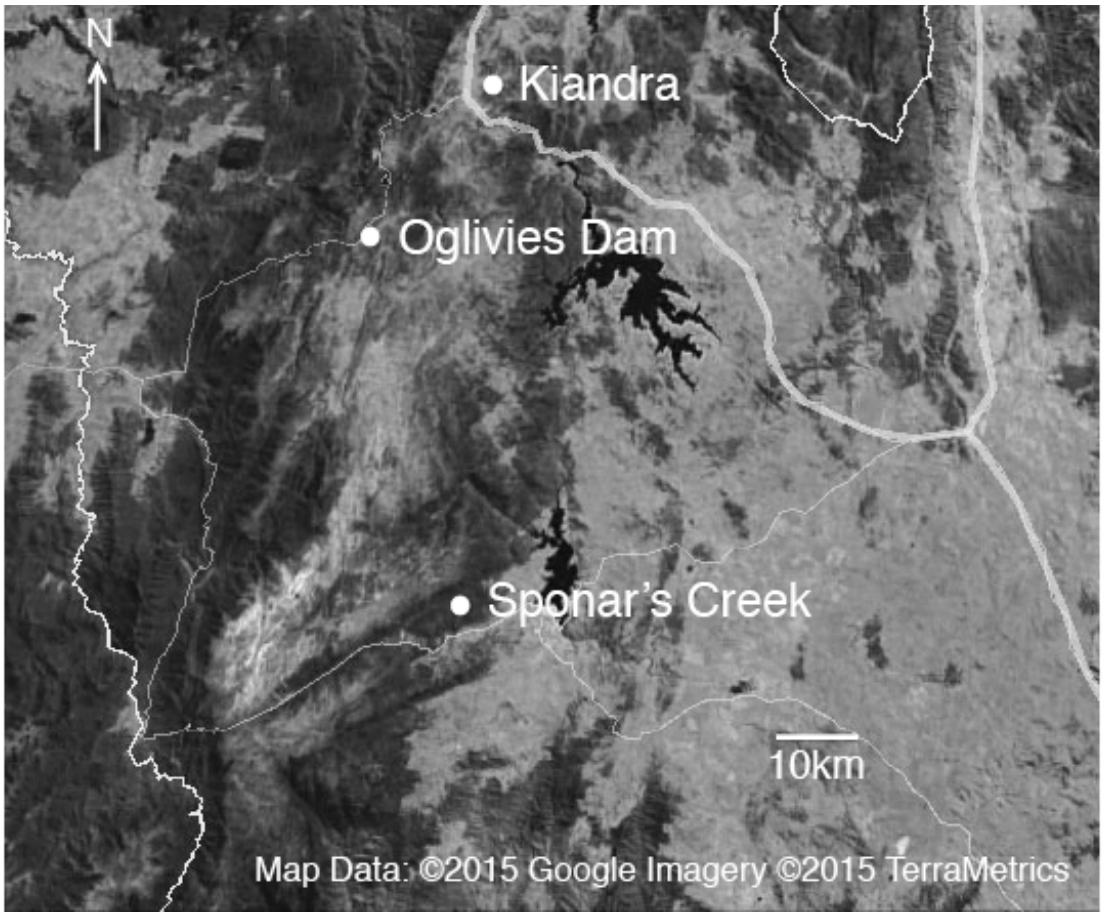
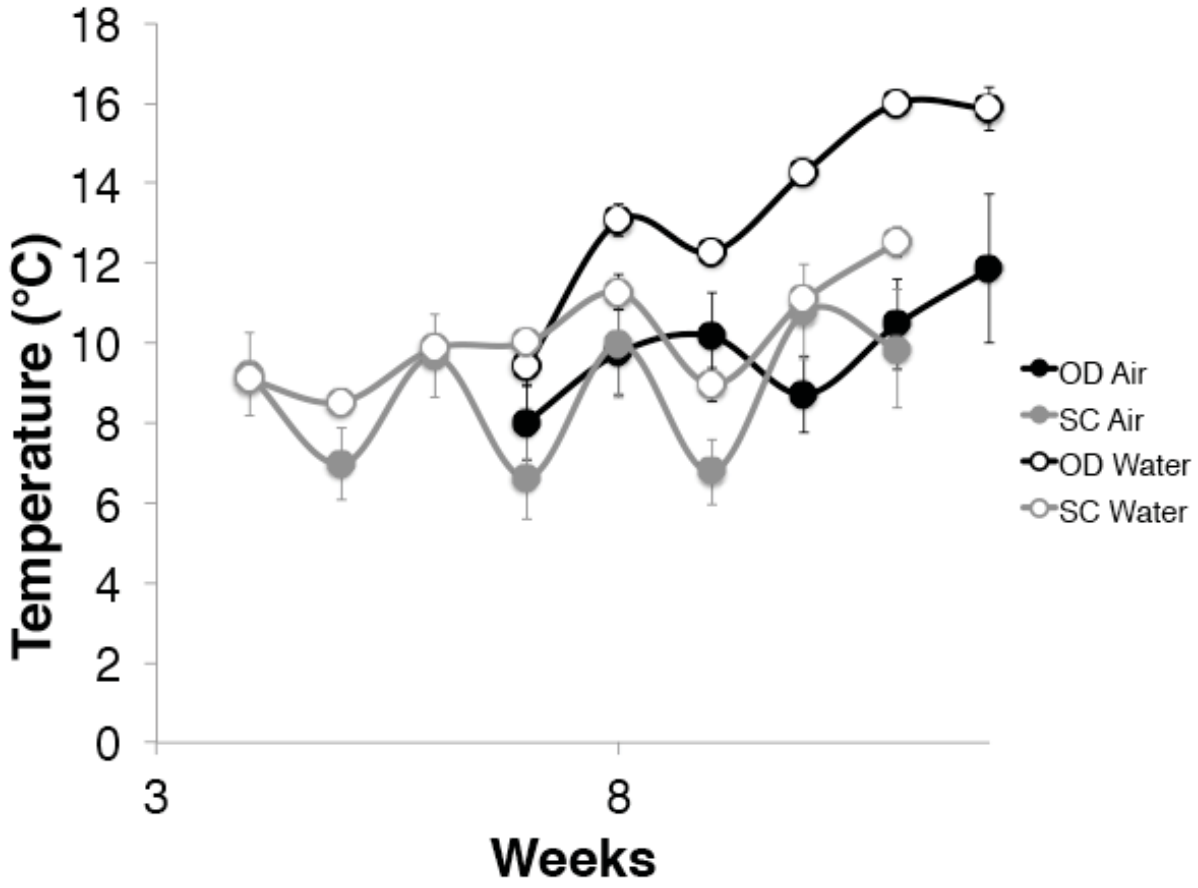
S Figures

S1 Figure



S2 Figure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Temperature (°C)
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Weeks
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SC Air
OD Water
SC Water

Paper 2.

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Factors influencing post-release fitness in the reintroduction of an endangered alpine tree frog

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Abstract

Global amphibian decline and extinction has been associated with the spread of the pathogenic chytrid fungus (*Batrachochytrium dendrobatidis, Bd*). Despite extensive research, there have been no examples of effective management abating the on-going impact of this pathogen in the wild. The endangered alpine tree frog (*Litoria verreauxii alpina*) has been extirpated from 80% of its former range due to *Bd*. We directly tested whether source population or host site influenced the efficacy of a reintroduction of *L. v. alpina*. We captive reared and released 1241 individuals from three different populations, two with a history of *Bd* exposure and one that was *Bd* naïve, into two sites where they had historically occurred, and two sites where the species currently persists. Between 6 – 9 months post release, we recaptured 4.83% of the released animals, and observed breeding at all sites. Both released and extant animals had similar susceptibility to infection; both groups increased in *Bd* infection prevalence and infection intensity throughout the breeding season. We did not detect any effect on survival by site of release; however, population of origin had a relatively large impact ($\omega = 0.454$), and animals from one *Bd* exposed population were recaptured significantly more than the animals from the other *Bd* exposed population and the *Bd* naive population. Population exposure history to the disease of reintroduced amphibians may be used to increase post-release fitness and conservation success. Selection for mechanisms of resistance should be further explored to help mitigate the impact of chytridiomycosis during reintroduction programs.

Key Words

amphibian decline; capture-mark-recapture; chytridiomycosis; Conditional Arnason-Schwartz model; *Litoria verreauxii alpina*; reintroduction

Introduction

Reintroductions and translocations are an increasingly popular conservation technique, yet despite the global effort, their success is largely unknown or unpublished. Over 700 translocations were carried out annually in the 1980s in the USA alone (Griffith et al. 1989), and the number is growing globally (Seddon et al. 2012). But, less than 50 cases per year are published (Armstrong & Seddon 2008), only half incorporate scientific and ecological theory into planning (Sarrazin & Barbault 1996; Joseph et al. 2013), and few reintroductions are adequately monitored (Dodd Jr. & Seigel 1991; Beck et al. 1994; Seigel & Dodd Jr. 2002; Germano & Bishop 2008).

Most publications on reintroduction and translocation report failure, although success rates differ among taxa (Griffith et al. 1989; Dodd Jr. & Seigel 1991; Germano & Bishop 2008; Griffiths & Pavajeau 2008; Miller et al. 2014). Reasons for failure are many and include poor release site choice (Seddon 2010), high predation (Fischer & Lindenmayer 2000; Banks et al. 2002), and unknown or failure to mitigate cause of the species decline (Kleiman et al. 1994; Fischer & Lindenmayer 2000). However, there have been a number of successes such as the American alligator, the black-footed ferret, and the natterjack toad (Elsey et al 1992; Dobson & Lyles 2000; Beebee et al. 2012). To combat reintroduction failures, the factors associated with poor establishment and persistence need to be identified and addressed (Armstrong & Seddon 2008).

Amphibians are currently experiencing the greatest biodiversity loss of all vertebrate taxa (Stuart et al. 2004). The amphibian disease chytridiomycosis (caused by the fungal pathogen *Batrachochytrium dendrobatidis*, *Bd*) is a major cause of amphibian declines globally (Skerratt et al. 2007). *Bd* is known to infect over 600 species of amphibian, and is the primary cause of over 200 amphibian species to become critically endangered or extinct (Wake & Vredenburg 2008).

While active reintroductions and captive breeding programs are established for declining amphibian species (see Stockwell et al. 2008; Hunter et al. 2010; McFadden et al. 2010), simple release methods have been unsustainable because the disease is still present (McFadden et al. 2010). Current attempts to control the disease and improve reintroduction success include restricting exposure to reservoir host species, and reintroducing into areas that are less suitable for *Bd* (Scheele et al. 2014). Alternatively,

selection for resistance prior to release has been suggested as a potential strategy that would be sustainable in the long term, and enable recovery of species throughout their former range (Woodhams et al. 2011; Scheele et al. 2014).

This study aimed to directly test the use of reintroduction as a management technique for the endangered alpine tree frog, *Litoria verreauxii alpina*, which is declining due to *Bd* infection (Hunter et al. 2009). In this pilot study we monitored the establishment phase of released animals for short-term breeding and survival, one breeding season post release. We hypothesize that if the animals return to breed after their first overwinter period, there is potential for reintroduction success.

Further we assessed two factors that may influence reintroduction post-release fitness: population of origin and release site characteristics. To examine the effects of population of origin, we released captive-reared animals from populations with different disease exposure histories and assessed the effect on their recapture rates after overwintering. Two populations had been exposed to *Bd* for a long time (>20 years), and one population was *Bd* naïve. Prior evidence from a laboratory experiment showed that while the mortality rate is high in *L. v. alpina*, susceptibility to chytridiomycosis varies among clutches and populations and appears to be associated with population exposure history (Bataille et al. 2015). Additionally, we considered release site environmental factors that might affect reintroduction success: 1) water body permanence, because while *L. v. alpina* historically occurred across diverse sites, especially ephemeral water bodies, they are now only present at permanent, man-made water bodies (Osborne et al. 1999), and 2) site elevation, because this species inhabits a range of elevations (1200m and above), and elevation has been associated with differences in population level impacts of *Bd* in other species (Sapsford et al. 2013). Lastly, we considered the effect of tagging method on recapture rate.

For reintroduction to be a successful management technique, fitness of the re-established animals should equal or exceed that of the extant animals. Over the breeding season we measured survivorship, *Bd* infection prevalence and intensity, and breeding success in released frogs that returned to breeding sites after winter, and compared with extant animals. Understanding the factors that affect reintroduction success, both in the immediate and longer term, will help optimise larger reintroduction efforts in *L. v. alpina* and other species threatened by chytridiomycosis.

Methods

Study Species

Egg masses were collected from three different populations (Fig. 1): Population A and B are long-time *Bd* exposed populations, and Population C is a *Bd* naïve population (S1). Animals were raised in captivity for 18 mo. in quarantine at Taronga Zoo, Mosman, New South Wales, Australia. Tadpoles were raised in white plastic 600 x 400 x 200 mm tubs, and post-metamorphosis, the animals were housed communally (less than 24 animals per enclosure) at 20 – 23 °C in 300 x 205 x 205 mm terraria with a gravel substrate and a fabric fern. Tadpoles were fed a variety of lettuce species and adults were fed crickets four times weekly. Enclosures were flushed once daily with carbon filtered tap water for 30 sec, and changed every 2 mo. As the animals were brought in as egg masses and kept in quarantine, risk of *Bd* exposure was low, but as a precaution one animal from each enclosure was swabbed for *Bd* to confirm negative status (see *Bd* testing below).

Preparation for release

Eight weeks prior to release all animals were tagged using a nano-passive integrated transponder (PIT) tag and a toe pad was removed for a tissue sample and stored in 95% ethanol at -20 °C. Tag retention was assessed three weeks prior to release, and when tags were expelled, a toe-clipping scheme was used following Hero (1989) (See S1; Brannelly et al. 2014). For this reason we tested the effect of tagging method on recapture rates of released frogs after overwintering. Animals were able to heal for at least one week. Animals were weighed to the nearest 0.01 g and snout to venter length (SVL) was measured to the nearest 0.02 mm.

Release

Animals were released into paired sites, which varied by water body permanence (permanent and ephemeral) at two elevations (high and lower within the species habitat range). The site in each pair with a permanent water body had a current thriving population of *L. v. alpina* (Sites 1 and 3). At the other site in each pair with an ephemeral pond *L. v. alpina* had been extirpated in 2008 (Sites 2 and 4). It must be noted that at Site 2, animals had begun to recolonise; however, population density was very low: seven

extant males and no females were found during the monitoring. Sites 1 and 2 were lower elevation, and sites 3 and 4 were high elevation. We did not expect the presence or absence of *L. v. alpina* to strongly affect the recapture rates of released frogs given the suitability of the sites for *L. v. alpina* in the past. An effect of species presence is inseparable from an effect of water body permanence.

On 13 March 2013, 1241 animals were released into the four sites in Kosciusko National Park, New South Wales, Australia (Fig 1). Animals were transported in small plastic containers (200 x 150 x 100 mm) on a damp moss substrate, at 16 – 20 °C. At each site we released an even distribution of animals from each clutch and source populations (see Table 2).

Testing for Bd

We tested for *Bd* infection by using skin swabs and a qPCR assay (Boyle et al. 2004). Swabbing was standardized for each animal (45 strokes per swab; MW-113, Medical Wire and Equipment). Genomic DNA was extracted from the swabs using a bead beater for two minutes per sample to break the fungal cell walls, and Prepman Ultra DNA extraction method (Applied Biosystems®, Life Technologies Pty Ltd) with the final extract diluted 6:100. Extracted DNA was then analysed using quantitative real time PCR following Boyle et al. (2004). We ran samples in singlicate with a positive and negative control, and a series of dilution standards (to estimate infection load of the swab in zoospore equivalents, ZE).

Field monitoring

Between September and November 2013 we monitored both the released animals, and wild animals from the extant populations. Animals were captured 1 – 3 nights a week per site throughout the breeding season (10 weeks). Extant animals were identified by photographs (see S1; Brannelly et al. 2015) and a toe tip was collected after first capture and stored in 95% ethanol.

Animals were captured with a new, clean gloved hand and kept individually in a new plastic zip bag, processed and returned to the site of capture. Animals were identified, swabbed for *Bd*, measured, weighed and breeding status was noted.

We recorded instances of mating at moment of capture, and presence of egg masses and tadpoles opportunistically. At ephemeral sites, we believe the majority of *L.*

v. alpina egg masses and tadpoles were from released animals because while seven extant males were found at Site 2, only released females were captured at both these sites.

Sites 1 and 2 were resurveyed in October 2014 for released animals and marked extant animals to determine if animals survived for one year post release.

Project costs

Reintroduction costs are rarely reported and often helpful for future research and assessing benefits of interventions (Fischer & Lindenmayer 2001). The maintenance costs for captive raising the animals was $30,000 AUD to cover food, housing units and water. Captive raising required a total of 1152 person hours, and the breeding season monitoring required 1025 person hours. Person hours do not include travel to and from remote sites, nor diagnostic *Bd* testing.

Statistical analysis

To determine which factors influenced whether released animals were captured after overwintering, null hypothesis testing methods using Chi-Squared Tests in SPSS (v21) were used. Recaptured animals and never-recaptured animals were analysed as the dependent variable, and the data was aggregated to analyse one independent variable at a time. The independent variables were population of origin, tagging method, elevation at site of release, water body permanence at site of release, and site of release. Effect size was determined using Cohen's ω statistic, and odds ratios were calculated following Altman (Altman 1991), both using Microsoft Excel.

To determine if the fitness and behaviour of the released animals compared to the extant animals over the course of the breeding season, survival, recapture and state change probability were assessed using the Conditional Arnason-Schwartz model and statistical software M-SURGE (Choquet et al. 2004). We determined survival (S), recapture (r) and state change (Psi) probabilities. The independent variables tested were group (g, either extant or released), time in weeks (t), current *Bd* state (f), and *Bd* state of previous capture (to). The two *Bd* states were *Bd* positive and *Bd* negative. Week 1 was the start of the breeding season for each particular site, and Week 10 was the end: $\hat{c}$ = 0.833 and was not adjusted. Because females only return to the pond briefly to lay, they were never captured more than once during the breeding season, and were excluded from the analyses in M-SURGE. Site characteristics were not included as covariates in the

analysis because site is unlikely to confound infection dynamics (see Infection Dynamics in Results), and because sample size of the released animals was too small to investigate the effect of site through CMR analysis.

Model selection was based on Akaike's information criterion (AIC), with the best fitting model indicated by the lowest AIC value. The seven best fitting models were chosen for analysis. We did not perform model averaging.

Infection intensity between released and extant animals over the breeding season was assessed using ANCOVA in SPSS (v21), where individuals are covariates. Infection prevalence was assessed using a logistic regression where change in infection over time was compared between populations.

Body condition was analysed using the independent and paired t-tests in SPSS (v21) where individual was a covariate and body condition was defined using the standard allometric estimate (See S1). Effect size was determined using the Cohen's *d* statistic, which was calculated in Microsoft Excel.

Results

Factors influencing immediate reintroduction success

Of the 1241 individuals released, 4.83% of animals were recaptured at least once during the first breeding season (61 animals: 14 female and 47 male) (Table 2). There was a significant effect of population of origin (Chi-Squared: $\chi^2_2 = 12.961$**, p = 0.002**, $\omega = 0.454$) (Table 2). Animals originating from one of the *Bd* exposed populations (Population B) were recaptured more than those from the other *Bd* exposed population (Population A), and the *Bd* naïve population (Population C) (Odds Ratio: Population B to Population C = 4.069, 95% CI 1.803 – 9.1844; Population B to Population A = 1.802, 95% CI 0.94 – 3.423). There was no observable difference between Population A and C (Chi-Squared: $\chi^2_1 = 0.951$, p = 0.229). We were unable to detect an effect of release site (sites combined by elevation: $\chi^2_1 = 0.373$, p = 0.428; sites combined by water body permanence: $\chi^2_1 = 0.757$, p = 0.792; sites analysed individually: Chi-Squared: $\chi^2_3 = 5.048$, p = 0.168) or tagging method (Chi-Squared: $\chi^2_1 = 0.003$, p = 0.954) on recapture after release.

Factors effecting longer term reintroduction success

Capture-mark-recapture model

Two models were the best fit for the data (Table 1). The first model was S(f*g)p(t)Psi(to*f*t) (AIC = 1542.148, deviance = 1480.148), and the second best model was S(f)p(t)Psi(to*f*t) (AIC = 1542.340, deviance = 1484.340). These two models only differ in the weekly survival estimation, where Model 1 suggests that released animals have a different survival probability compared to extant animals, and Model 2 suggests that both released and extant animals have the same weekly survival probability (Fig 2A). In Model 1, both extant and released animals have similar survival probability when *Bd+*, but when *Bd-*, released animals had a much lower survival than extant animals. Recapture probability was determined by week (Fig. 2B). State change probability was determined by the disease state at previous capture and state at current capture, and week (Fig 2C). Throughout the breeding season the probability of remaining infected was high, and the probability of clearing infection was low. As the season progressed, the probability of remaining uninfected decreased, while the probability of gaining an infection increased.

Infection dynamics

Overall infection intensity was high in the first week of the breeding season across sites (10,003.56 ZE; SD = 29,347.95), then dramatically decreased by week three (722.595 ZE; SD = 1,616.13), and then proceeded to gain intensity throughout the rest of the season (6,070.54 ZE; SD = 14,640.2 in week 10) (Fig. 3A). There was no difference between extant and released animals (ANCOVA: $F_{1, 749} = 0.92$, $p = 0.761$). Infection intensity was not different between elevation (ANCOVA: $F_{453} = 1.27$, $p = 0.26$) or water body permanence (ANCOVA: $F_{453} = 0.053$m $p = 0.818$).

Infection prevalence increased throughout the breeding season (Logistic Regression: Exp(B) = 1.245, **$p < 0.001$**) and differed between extant and released animals (Logistic Regression: Exp(B) = 0.546, **$p = 0.015$**). Infection prevalence of extant individuals followed a similar pattern to infection intensity where prevalence began high (57.7%), declined (32.8%), and then increased again (77.8%), while infection prevalence of released animals only increased throughout the season (56.3 – 100%) (Fig. 3B).

Body condition

Recaptured released animals did not differ from non-recaptured released animals in body condition prior to release (males only, t-test: $t_{424} = -0.024$, $p = 0.981$) (Fig. 4A), but body condition decreased by 17.94% from release to recapture, (Paired t-test: $t_{56} = 6.719$, **$p < .001$**, $d = .876$) (Fig. 4B). There was no observed difference between released and recaptured males body condition and extant males (t-test: $t_{478} = -0.635$, $p = 0.526$) but released females had 43.78% lower body condition than extant females (t-test, variances not equal: $t_{16.542} = 8.597$, **$p < 0.001$**, $d = 2.98$) (Fig. 2). Released females were significantly smaller than extant animals (SVL released: 33.6 mm SD = 2.87; SVL extant: 40.4 mm SD = 2.87; t-test: $t_{48} = 7.704$, **$p < 0.001$**), while there was no difference in the size of the males (SVL: 32.8 mm SD = 2.2; t-test: $t_{478} = 0.098$ $p = 0.098$).

Breeding

We found evidence of released animals breeding at all sites. All released females that were captured were gravid, and one was even found in amplexus with an extant male. Released males were often found actively calling, and all had darkened nuptial pads and vocal sacs. Egg masses were observed from Week 2 – 10 at sites 1, 2 and 3. Tadpoles were observed at site 1, 2 and 4 during weeks 6 – 10.

Frog colour observation

During the breeding season, we noticed that the released animals were consistently different in colour to the extant animals. While colouration and dorsal pattern of *L. v. alpina* is highly variable, ranging from brown to solid green, the released animals were brighter on the dorsum, and in extreme cases they displayed a teal tint. The venter was also lighter than the extant animals (Fig. 5).

Resurvey of sites 1 and 2

During resurvey period in 2014, unmarked extant males were found at both sites, but no marked released or extant animals from the survey in 2013 were found.

Discussion

This study directly tested factors that may influence reintroduction success for a frog species endangered because of *Bd* infection. We recaptured 4.83% of released

animals that were participating in breeding activities 6 – 9 months after release, which included male calling, successful laying and fertilising of egg masses, and tadpole production. This breeding success, albeit in only a small proportion of the total released frogs, suggests it is possible to use captive rearing and release of *Bd* threatened animals to establish breeding populations in the wild. This is a key step in developing the capacity to conserve populations of threatened frogs, although in many cases further intervention to mitigate *Bd* will be required to enable populations to persist (Kleiman et al. 1994; Fischer & Lindenmayer 2000; Armstrong & Seddon 2008; Scheele et al. 2014).

Our study also suggests that the captive reared and released individuals that survived through to breeding had comparable fitness to extant animals. Recapture rates were similar among released and extant animals. Released males had similar body condition compared with extant males, suggesting that they had no trouble feeding after release. While the released females were smaller than extant females, and had lower body condition, this is likely due to the released females returning to breed at a younger age. In the wild, females generally reach sexual maturity at three years of age (Scheele et al. 2015). Captive rearing may have allowed females to mature faster (O'Regan & Kitchener 2005; Ritz et al. 2010), but at a smaller body size.

Population of origin

Population of origin appeared to impact post-release recapture of individuals. We recaptured more animals (7.22%) that originated from Population B, a population previously exposed to *Bd* (Table 2). Post-release survival is likely to have been influenced by mortality associated with *Bd* infection. Population B may have been recaptured more because they are more resistant to *Bd* infection; evidence from a laboratory experiment suggests that susceptibility to chytridiomycosis varies among populations and is associated with population exposure history (Bataille et al. 2015). These results should be investigated further, as they may inform the capacity to selectively breed for resistance, which may be critical for recovering wild populations of *Bd* threatened frog species (Woodhams et al. 2011; Scheele et al. 2014). However, frogs from various populations may differ in fitness due to selection from other factors, not just disease.

Site of release

We did not detect an effect of environmental factor on post-release survival. This was unexpected because *L. v. alpina* had previously been extirpated from the ephemeral sites during their initial decline, and perhaps the small number of recaptures explains why an effect was not detected. However, the original extirpation may have been due to lower recruitment at ephemeral sites, rather than higher adult mortality, which fits in with our consistent recapture rates among sites. Successful recruitment is important to the survivorship of *L. v. alpina* populations in the longer term (Scheele et al. 2015), and drought is common in the Australian Alps, which can prevent tadpoles from successfully metamorphosing from ephemeral water bodies. We found seven extant animals at Site 2 for the first time since the species had been extirpated from the site, suggesting some population re-expansion of *L. v. alpina* may be occurring. We expect that this small number of animals had little to no effect on factors assessed among release sites.

Infection dynamics

Infection intensity for both released and extant animals was high at the start of the breeding season, decreased and then rose again (Fig 3A). Some animals were *Bd* negative when they arrived at the water body, and gained infection during breeding, after exposure to *Bd* through contact with an infected animal or pond water. Some animals appeared to enter the breeding habitat already heavily infected (Figure 3A, 3B). The *L. v. alpina* breeding season starts around snowmelt following the end of winter torpor. Infection intensity was high when breeding commenced; therefore, it is likely that a proportion of the breeding adults maintained *Bd* infection since the previous autumn. We suspect that the animals coming into the breeding population with high infection burdens died within the first few weeks of the breeding season, explaining the drop in intensity around week three. Few wild *L. v. alpina* recover from infection (Fig. 4A), which is supported by laboratory infection experiments showing high susceptibility (Bataille et al. 2015).

Post-release survivorship

In amphibians, survivorship of animals released into the wild from captivity is often very low or unknown and variable among species and between release events (Denton et al. 1997; McFadden et al. 2010; Muths et al. 2014). Only a small proportion (4.83%) of the released *L. v. alpina* was recaptured during the breeding season, which may suggest migration and/or high mortality occurred in the six months after release.

Currently, there is little information on survivorship of *L. v. alpina* over winter because they are too small to be fitted with radio trackers for an extended period. Overwintering is known to cause high mortality (Bradford 1983; Anholt et al. 2003; Swanson & Burdick 2010), which suggests low overwinter survival for *L. v. alpina* likely explains the low return rate.

During the breeding season released animals had a lower survival probability than extant animals when *Bd*-negative according to one of the best fit models (Fig. 3A). Possible explanations for lower survival are starvation/dehydration and predation. Released and recaptured males were in similar body condition to the extant males, suggesting that food/water was not a limiting resource at any site of release. But, predation of released animals is a major concern for reintroductions; it is one of the most reported causes of project failures (Banks et al. 2002). Predation of amphibians is also affected by colouration and camouflage, which can be affected by the captive environment (Norris & Lowe 1964; Van Buskirk 2011). Colouration can be influenced by captive diet (Ogilvy et al. 2012) and some species can undergo an ontogenetic change to match their environment (Fernandez & Bagnara 1991; Garcia et al. 2003). In this recapture study, we could reliably distinguish released animals from extant animals based on their lighter colouration. It appears that the animals developed their colouration to match their captive environment, which may have reduced their camouflage and increased their predation risk (see Fig. 5). We urge captive managers involved with reintroductions to design enclosures to match natural habitats.

Conclusions

This study demonstrates the potential for reintroduction success in *L. v. alpina*. Released animals returned and participated in breeding activities in their first breeding season. The released animals had similar recapture, and infection state change probability to extant animals, and similar survivorship, especially when *Bd*+. Population of origin of released animals seemed to have an effect on overwinter survivorship: one *Bd* exposed population had higher recapture rates and possibly survivorship than animals originating from a *Bd* naïve population.

Extant populations of *L. v. alpina* appear to be stable, but adults are still susceptible and succumb to disease at high rates (Scheele et al. 2015). In order for management techniques to be successful, populations would need to rebound and re-

establish into extirpated sites, and could occur if released animals were more disease resistant than the extant animals. Therefore, released animals would need to have improved fitness compared with extant animals in survival post infection for population rebound to be assisted by the reintroduction. Laboratory infection experiments suggest that susceptibility to chytridiomycosis may be associated with population exposure history (Bataille et al. 2015). The laboratory and field data suggest that future research should explore selection for mechanisms of resistance to help mitigate the impact of chytridiomycosis during reintroduction programs.

However, it appears that not all persisting populations with endemic *Bd* infections have effective mechanisms against disease. Future research also needs to address other mechanisms, such as maximized reproductive output prior to clinical infection, which may be evolving to promote species persistence. Differences in recruitment across sites may be more important than adult mortality in explaining the distribution of declines (Scheele et al. 2015).

We noticed a difference in colouration between the released animals and the extant animals, which may have increased predation for the released animals. Predator avoidance is important for captive raised and released animals; therefore, artificial habitat colouration and diet should mimic the natural environment (Van Buskirk 2011; Ogilvy et al. 2012). There is hope for recovery in this species through reintroductions, but the study also highlights the difficulty of using reintroductions as a management technique. More research into mechanisms of persistence needs to be conducted to maximize conservation success.

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Tables

Table 1*:* The top four best fit models based on AIC, and a selected assembly of other models trialled to demonstrate the relative importance of each variable analysed.

Models Tested#	Parameters	Deviance	AIC	ΔAIC
S(f*g)p(t)Psi(f*to*t)	31	1480.148	1542.148	0
S(f)p(t)Psi(f*to*t)	29	1484.34	1542.34	0.192
S(f*g)p(.)Psi(f*to*t)	23	1498.5756	1544.576	2.428
S(g)p(.)Psi(f*to*t)	21	1503.17	1545.17	3.022
S(f)p(to)Psi(f*to*t)	22	1502.799	1546.799	4.651
S(.)p(t)Psi(f*to*t)	28	1492.03	1548.03	5.882
S(f)p(to*t)Psi(.)	21	1506.283	1548.283	6.135
S(f*g*t)p(to*g*t)Psi(f*to*g*t)	83	1410.279	1576.279	34.131
S(.)p(.)Psi(.)	3	1597.134	1603.134	60.986

#Probabilities determined are (S) survival probability, (p) recapture probability, (Ψ) state change probability; and the variables that influence S, p and Ψ are (g) site, (t) time in weeks, (to) state of previous capture, (f) state of capture and no variable (.). (*) means that the effect of the variables were multiplicative.

Table 2. Released animals that were recaptured at least once during the breeding season.

Number of animals recaptured / number of animals released. Columns are sites of release (Sites 1 – 4) and rows are population of origin (Populations A, B and C).

	Site 1	Site 2	Site 3	Site 4	Total
Population A	5 / 89	4 / 94	3 / 92	1 / 98	3.49%
Population B	8 / 139	13 / 136	13 / 140	6 / 139	7.22%
Population C	0 / 74	3 / 81	2 / 83	2 / 76	2.23%
				Total	4.83%

Figure legend

Figure 1. Map of Kosciuszko National Park, New South Wales, Australia.
Populations A, B and C are sites of population origin. Sites 1 – 4 are sites of release. Population A: Kiandra, 35.867°S, 148.498°E: Elevation 1358m. Population B: Eucumbene, 36.041°S, 148.729°E: Elevation 1154m. Population C: Grey Mare, 36.317°S, 148.260°E: Elevation 1521m. Site 1: Oglivies Dam, 35.958°S, 148.401°E: Elevation 1382m. Site 2: Oglivies Ephemeral, 35.960°S, 148.399°E, Elevation 1331m. Site 3 Sponar's Creek, 36.359°S, 148.486°E, Elevation 1515m. Site 4: Piper's Creek, -36.372°S, 148.452°E, Elevation 1543m. The white lines indicate the borders of adjacent states and territories, Victoria to the West and Australian Capital Territory to the Northeast. The grey lines indicate major roadways. Map was adapted from Google Earth.

Figure 2. Probabilities of survival, recapture and state change for the two best fit Conditional Arnason-Schwartz models.
1) S(f.g)p(t)Psi(f.to.t) and 2) S(f.g)p(t)Psi(f.to.t) where S = survival probability, p = recapture probability, Psi = probability of states change; g = group (released v extant), f = state (either *Bd*+ or *Bd*-), to = state of previous capture (either *Bd*+ or *Bd*-), and t = time. **A.** Survival probability per week. Estimates from Model 1: released animals in black, extant animals in grey, *Bd*- animals with circles and *Bd*+ animals with squares. Model 2: no difference between extant and released animals, with *Bd*- is open black circles and *Bd*+ as open black squares. **B.** Recapture probability each week. Models 1 and 2 have the same estimates. **C.** Probability of changing disease state per week. Models 1 and 2 have the same estimates. Probability of remaining *Bd*+ is represented as a black circle, remaining *Bd*- is the grey circle, probability of clearing infection is the black square and the probability of gaining infection is the grey square. Error bars are 95% confidence intervals. Error bars indicate 95% confidence intervals

Figure 3. *Bd* infection dynamics.

A. Intensity of *Bd* infection of animals throughout the course of the breeding season. Extant and released animal infection loads are combined. The average zoospore loads are only for *Bd* infected animals. **B.** The proportion of *Bd* infected animals over the 10-week breeding season. Extant animals (Grey); Released animals (Black). The time point at which the two groups diverge (*). Error bars indicate 95% confidence intervals.

Figure 4. Body condition of animals.

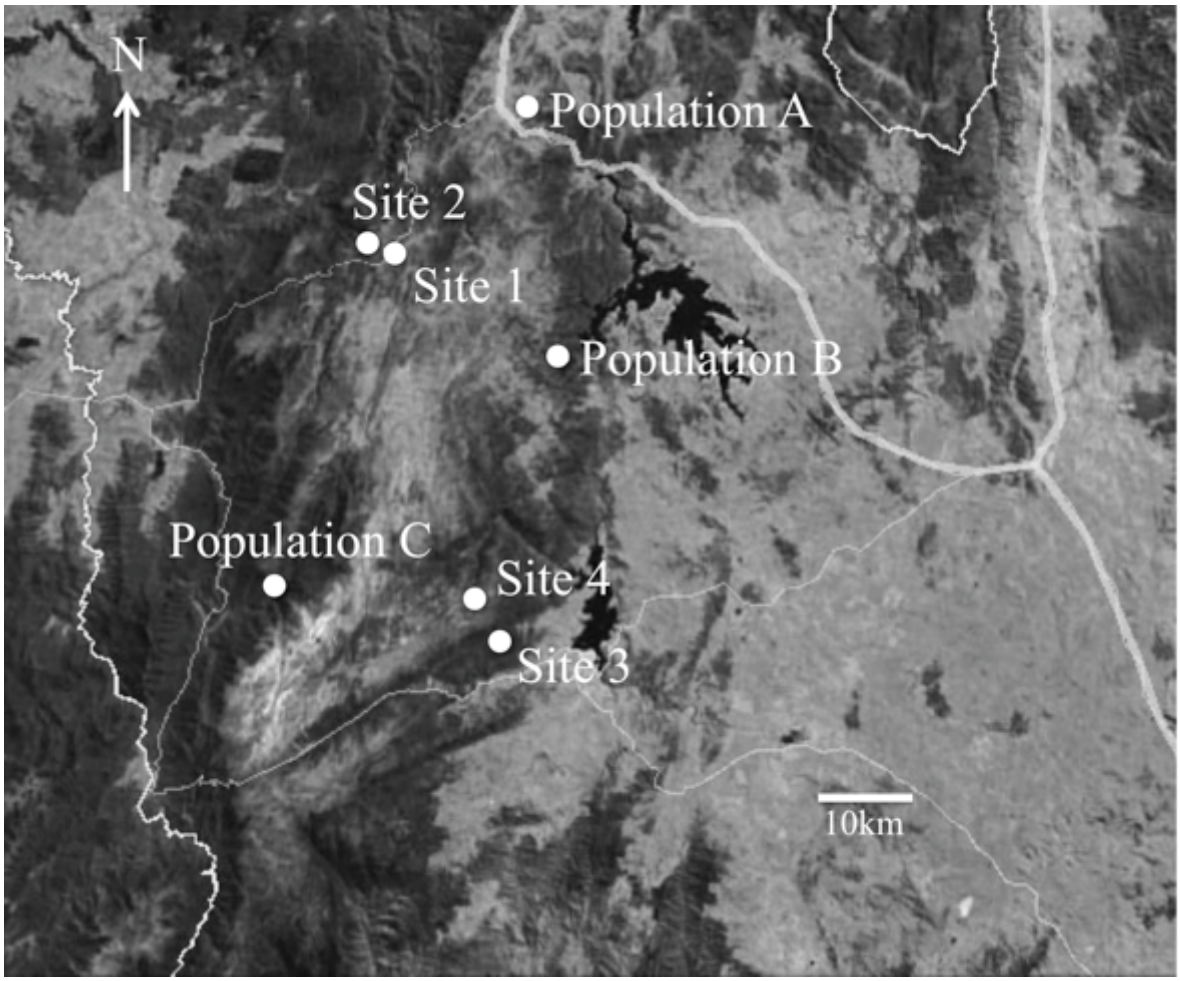
Body condition is the ratio Log_{10} (mass (g)) / Log_{10} (SVL (mm)). **A.** Change in body condition from release to recapture, males and females combined. The body condition of recaptured animals at release (Black Circle), and the body condition of the animals that were recaptured at first recapture (Black Square). Error bars indicate standard error. **B.** The body condition of released animals and extant animals separated by sex during the breeding season. Males, all combined (Open Circle); Extant Females (Grey Triangles); Released Females (Black Triangles). Error bars indicate standard error.

Figure 5. Examples of released and extant animal colouration.

A. A released animal, teal in colour. **B.** An extant animal, green, similar in shade to the moss in the environment. Photographs by D. L.

Figures

Figure 1



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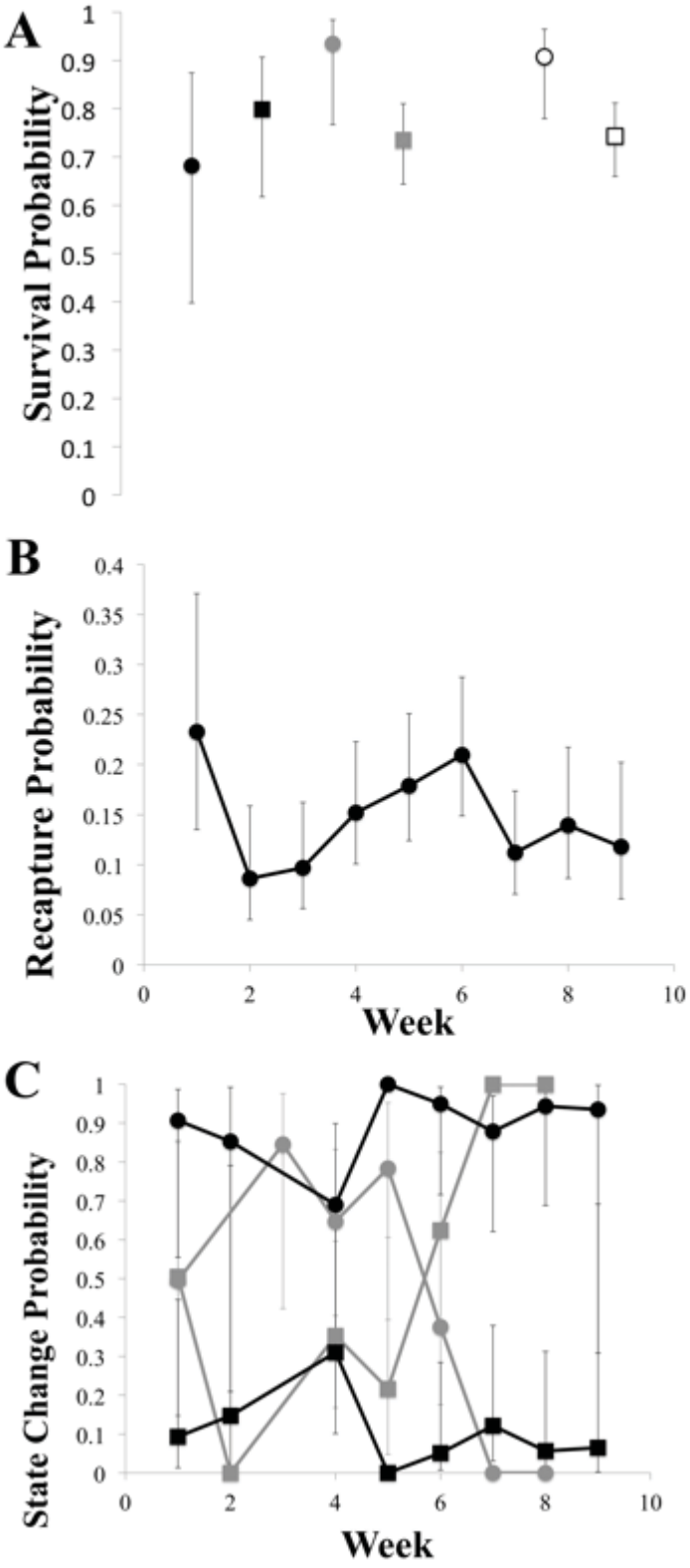
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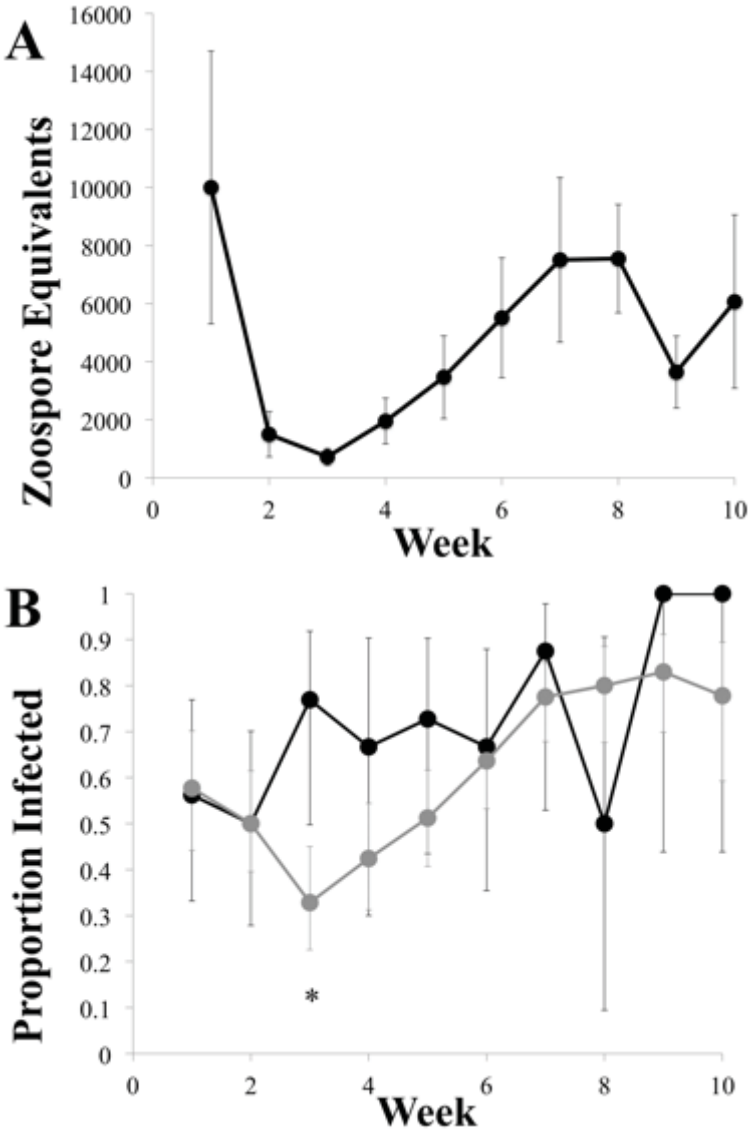
Survival Probability
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
B
Recapture Probability
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
2
4
6
8
10
Week
C
State Change Probability
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
Week

Figure 3.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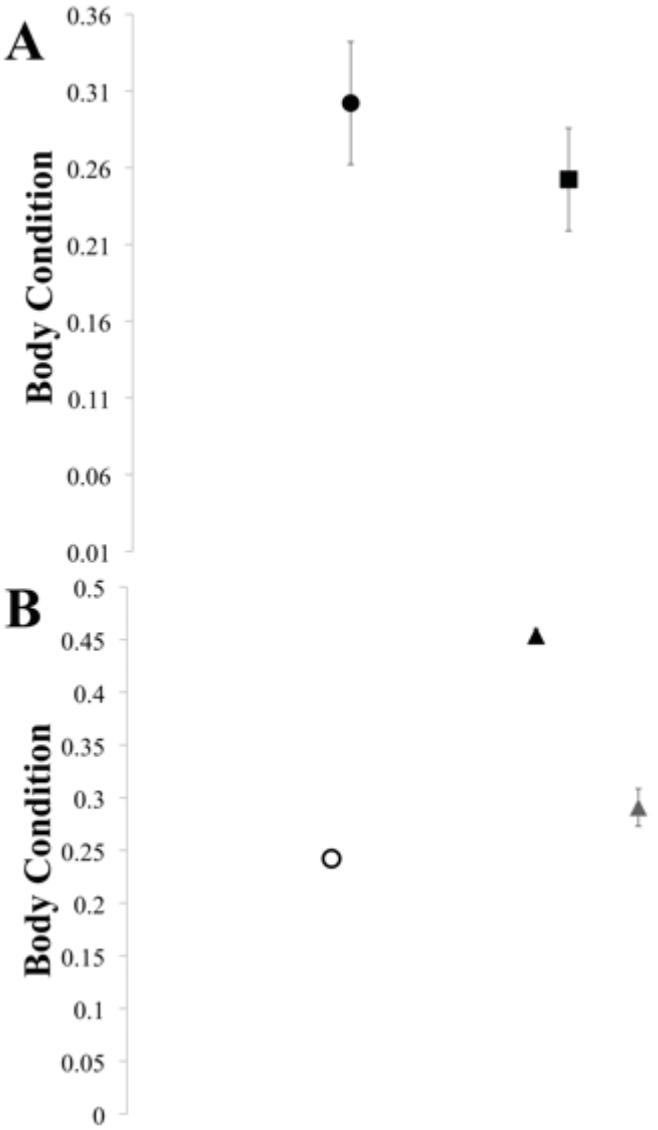
Zoospore Equivalents
16000
14000
12000
10000
8000
6000
4000
2000
0
0
2
4
6
8
10
Week
B
Proportion Infected
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
0
2
4
6
8
10
Week

Figure 4.


A
Body Condition
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0.31
0.26
0.21
0.16
0.11
0.06
0.01
B
Body Condition
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0

Figure 5.

Supporting information

Appendix S1

Study species ecology

Litoria verreauxii alpina occurs in sub-alpine woodland, grasslands, and bog environments. Breeding populations occur in streamside pools, and all current populations occur in or near permanent water bodies, many of which are artificial, such as small dams and reservoirs (Osborne & Hollis 1999; Scheele et al. 2015). *Litoria. v. alpina* is highly susceptible to *Bd* in the lab and succumb to infection in just three weeks (Bataille et al. 2015). Populations are persisting and appear to remain stable in spite of high *Bd* infection prevalence and intensity, but life history strategies have shifted in exposed and infected populations. *Litoria v. alpina* in *Bd* naïve populations have a long life span, with some individuals surviving five-eight years, yet in *Bd* exposed populations, there is nearly complete population turnover at the end of their first breeding season (Scheele et al. 2015). Population persistence appears to be assisted through high recruitment (Scheele et al. 2015).

Marking methods

While photo identification is appropriate for *L. v. alpina* in short duration experiments (Brannelly, unpublished data), pattern permanence is unknown in the long term (>8mo.); therefore, a permanent marking method was required. Animals were tagged using a nano-passive integrated transponder (PIT) tag (Nonatec^{TM}) injected into the coelomic cavity into the left venter angled toward the anterior, and sealed with tissue adhesive (Vetbond^{TM}, 3M). Tag retention was checked three weeks prior to release. Tags were expelled in 60.6% of animals, an alarmingly high rate (see Brannelly et al. 2014). Many tags were expelled through the point of entry still attached to the tissue adhesive, but many tags were expelled after the point of entry healed. Toe clipping is the most reliable marking method for this species (Brannelly et al. 2014), and was used in cases where tags were expelled, following Hero (1989). A maximum of four toes were removed from each animal, which is known to have few consequence on survival (Grafe et al. 2011), and only two per left or right side. The first digit on the forelimbs and the second digit on the hind-limbs were not removed.

We did not notice any PIT tags expelled between release and recapture. Released animals were easy to discern from the extant animals due to their colouration both dorsal

and ventral. The missing toe pad on the front left forth digit served not only as a genetic sample (see above) but also as an indicator of release. Using the combination of both colouration and the missing toe pad, we are confident that no recaptured animal expelled a PIT tag between release and the breeding season. However, it mused be noted that only 21 of the 60 animals recaptured after release we marked using PIT tags.

Photo identification was used for identifying extant animals because the breeding season only lasted ten weeks and dorsal and side patterns do not change within that short time, making it a less invasive and reliable method. Photos of the dorsum and the left side were taken. Side photos were primarily used to identify individuals and the dorsal photos were used to double check identities. Toe tips were removed from extant animals upon first capture, and there were no instances of wound infection upon recapture.

Body condition calculation

The best body condition estimate was determined between three different methods of estimation: standard isometric (BC = M/L), standard allometric (BC = Log(M)/Log(L) and scaled allometric (BC = Log(M)/Log(L_0/L) (Peig & Green 2009) where BC is body condition, M is mass in g, L is snout to vent length (SVL) in mm and L_0 is the population average SVL. Correlation and linear regression analysis was carried out for each of the three BC estimations in SPSS v21 for the extant *L. v. alpina* populations alone, and males and females were assessed separately to determine the best method to calculate body condition for *L. v. alpina*. High linear correlation was demonstrated for all methods in both males (n = 580) and females (n = 37), with no one method outperforming the others. The standard allometric analysis was performed for all individuals: BC = log(M)/log(L).

Standard isometric regression and correlation for males: Linear regression ANOVA: $F = 438.882$, $p < 0.01$ (Fig S2A). Standard isometric regression and correlation for females: Linear regression ANOVA: $F = 9.443$, $p < 0.01$. Standard allometric regression and correlation for males: Linear regression ANOVA: $F = 438.472$, $p < 0.01$ (Fig S2B). Standard allometric regression and correlation for females: Linear regression ANOVA: $F = 8.249$, $p < 0.01$. Scaled allometric regression and correlation for males: Linear regression ANOVA: $F = 438.472$, $p < 0.01$. Scaled allometric regression and correlation for females: Linear regression ANOVA: $F = 8.249$, $p < 0.01$.

Additional literature cited

Grafe, T. U., Stewart, M. M., Lampert, K. P., Rödel, M.-O. (2011). Putting toe clipping into perspective: A viable method for marking anurans. *J. Herpetol.*, 45, 28–35.

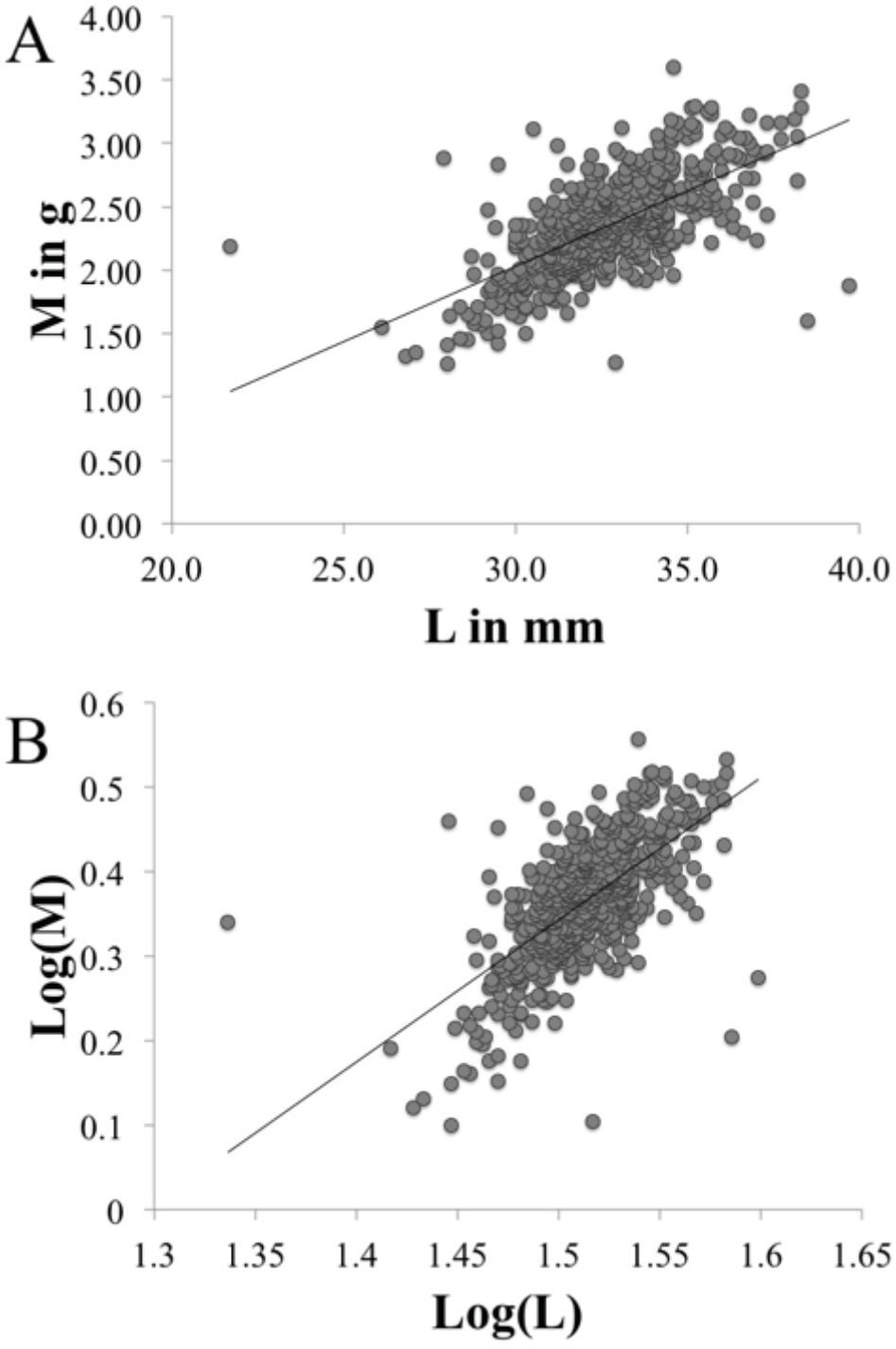
Osborne, W., Hunter, D., Hollis, G., 1999. Population declines and range contraction in Australian alpine frogs, in: Campbell, A. (Ed.), Declines and Disappearances of Australian Frogs. Biodiversity Group Environmental Australia, Canberra, ACT, pp. 145–159.

Supplementary figure legend

Supplementary Figure 1. Linear regression of mass and length in male *L. v. alpina*. A) Mass (M) compared to length (L) with the trendline representing the linear correlation, M = -1.54 + 0.12 L. B) Logarithmic conversion of the same data as A. Log (M) compared to Log(L) with the trendline representing the linear correlation M = -2.18 + 1.69 L.

Supplementary Figure

Supplementary Figure 1



Paper 3

Citation: Brannelly LA, Berger L, Skerratt LS. (2014) Comparison of three widely used marking techniques for adult anuran species *Litoria verreauxii alpina*. Herpetological Conservation and Biology. 9(2): 428-435.

Comparison of three widely used marking techniques for adult anuran species *Litoria verreauxii alpina*

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Abstract

Effective marking techniques are required for both laboratory and field studies of adult amphibians, especially when individuals cannot be identified based on color or pattern. We compared the efficacy of four marking techniques (toe clipping, visual implant elastomer (VIE) tags, and passive integrative transponder (PIT) tags injected into two locations (the body cavity and subcutaneously) in the endangered alpine tree frog, *Litoria verreauxii alpina*. The most effective marking method was toe clipping, with 96.1% correct identifications. The second best marking method was PIT tags injected subcutaneously, where tags were retained in 73.3% of animals after six weeks, but tag retention might decrease over time due to tag expulsion. PIT tags injected into the body cavity were poorly retained (33.3%). The least successful marking method was VIE tags as individuals were correctly identified only 18.4% of the time. We conclude that toe clipping may remain the most effective marking method for some amphibian species, where modern tagging techniques are unreliable due to low tag retention and high tag movement. Researchers should conduct marking trials before implementing large scale marking schemes in unstudied species, and they should publish negative results as well as desired outcomes.

Keywords

marking methods; visual implant elastomer tags; passive integrative transponder tags; toe clip; *Litoria verreauxii alpina*

Introduction

Identifying individual amphibians is essential for management (e.g. captive breeding plans) and for field and laboratory based research. Research includes capture-mark-recapture studies that track individual frog behavior and survival, and are important for conservation, population dynamics and ecological studies (McCarthy & Parris 2004). Marking is necessary when individuals cannot be distinguished based on physical features such as color or pattern (Donnelly et al. 1994). Currently, three marking techniques are commonly used for amphibians: toe clips, passive integrated transponder (PIT) tags, and visible implant elastomer (VIE) tags. All three of these tagging methods have been successful in a variety of taxa (see Donnelly et al. 1994; Davis & Ovaska 2001; Penney et al. 2001; Woods & James 2003; Curtis 2006; Waudby & Petit 2011; Hamel et al. 2012), but their effectiveness on anuran species needs to be further assessed in a greater variety of species (Funk et al. 2005, Phillott et al. 2007).

Recently, many animal ethics committees have become skeptical of toe clipping as a valid and ethical method for marking amphibians, and some have refused to approve toe clipping for marking (B. Scheele pers. comm.; L. Brannelly pers. obs.; see Perry et al. 2011, Correa 2013). Although the primary purpose of ethics boards is to restrict the pain and discomfort of research animals, the perception of pain and suffering of amphibians during toe clipping is predominantly an anthropomorphized intuition (Langkilde & Shine 2006; Fisher et al. 2013), particularly after May (2004) called the practice “barbaric.” Even so, toe clipping remains the most widely used marking method for anurans, and has been defended by many as simple, cost effective, having relatively minor health impacts, and the operationally best method for particular species (e.g., Phillott et al. 2007, 2008, 2010, 2011, Perry et al. 2011, Correa 2013). Moreover, it does not increase measurable distress above that experienced from handling alone (Kinkhead et al. 2006; Fisher et al. 2013).

Alternative marking methods for amphibians, such as VIE tags and PIT tags, are relatively new, and some of the few studies assessing their use have demonstrated marking failure (i.e. Brannelly et al. 2013; Tracy et al. 2011). To date, no study has assessed the efficacy of these three marking methods on a single species to determine the most reliable marking method to be used in capture-mark-recapture studies. However,

animal ethics boards are enforcing the use of these methods over toe clipping (Funk et al. 2005; Phillott et al. 2008; Perry et al. 2011; Correa 2013).

In this study we tested four marking techniques on the alpine tree frog, *Litoria verreauxii alpina*. *Litoria v. alpina* is an endangered subspecies endemic to the alpine regions of Mt. Kosciuszko National Park in New South Wales and Victoria, Australia, where population monitoring is an important part of their conservation. Two study trials were conducted to assess the most effective marking technique for this species before a regime was broadly implemented. The first was a preliminary trial to determine the efficacy of PIT tags injected into the coelomic body cavity of the animals, and tag retention was monitored for four months post injection. After these coelomic PIT tags failed and our animal ethics committee rejected our toe clipping application, we conducted a second study in which we compared the efficacy of toe clips, VIE tags, and subcutaneously injected PIT tags during a six weeks trial period.

Material and Methods

Study species

Litoria verreauxii alpina were raised in captivity for 24 mo from wild collected eggs. Animals from two different populations and a total of four different clutches were haphazardly selected for each marking regimen. Fifteen animals were used for each marking trial. They were housed communally (3 – 9 individuals per terrarium) in 36 x 21 x 21 cm terraria on a gravel substrate with a layer of moss covering half the terraria. Temperatures ranged from 18-22°C. Animals were watered with aged tap water daily and fed crickets three times weekly. Adult *Litoria v. alpina* are small, with snout to vent length (SVL) ranging from 26.5 – 38.8 mm and mass ranging from 1.7 – 5.5 g. Body condition (SVL / mass) was recorded before each animal was marked, and each time the animal was examined for marking method success.

Infection control

During tagging or marking, instruments (needles or scissors) were sterilized with 95% ethanol. Animals were handled with nitrile gloves that were changed frequently and always between terraria. Animal enclosures were disinfected every eight weeks with 10%

bleach solution, rinsed twice, and allowed to dry for at least 24 h before reuse. Gravel substrate was autoclaved before use and after enclosure changes.

Study One

Animals were tagged and were checked at two days, one week, eight weeks and 16 weeks after injection. Animals were checked for tag loss and state of healing. All animals were healthy at the start of the experiment.

Coelomic cavity injected PIT tag

Nano-PIT tags (1 x 8 mm) (Nonatec^{TM}) were injected with a 20-gauge needle into the coelomic cavity. Tags were injected into the left ventral surface with the PIT tag injected toward the anterior. A veterinary adhesive (Vetbond^{TM}) was applied to the site of injection. The researchers were well trained in this tagging method, having successfully performed this procedure on >1000 animals previously.

Study Two: Mark method comparison

The efficacy of three marking methods was assessed: PIT tag (subcutaneous injections), toe clip, and VIE tag. Animals were checked two days after marking and then weekly for six weeks to assess ease of identification and state of healing.

Toe clipping

Animals were toe-clipped according to the Hero (1989) scheme, in which three toes were removed at the 2nd phalange, a maximum of two from each side, and not the second digit on the foot or the thumb.

VIE tagging

Two colors of visual implant elastomer, blue and red, were injected into the ventral thigh, subcutaneously, using a 29 gauge needle (Northwest Marine Technology^{TM}). Between one and five tags were implanted per individual, with a maximum of three into each thigh. The procedure was supervised by S. Sapsford, who successfully used it to mark *L. rheocola* (Sapsford et al. 2013; Fig. 1). Each tag was 1 – 2 mm in diameter. Red and blue were chosen because they could be visualized without a UV light. Tag movement, obscurity, and expulsion were measured at each examination. Tags were obscured when the tag migrated to a highly pigmented portion of the body where that tag could not be visualized, or when two tags combined. Tag expulsion occurred when the tag was expelled from the body through the skin, which was detected

through an open wound, as a result of this expulsion, and the expelled tag found in the terrarium. Identity of individuals was based on position and order of VIE tags in the thigh. Identification was not possible when tags migrated so that the order of the tags varied from implantation. Photographs were taken of each animal at each time point to document tag movement and to verify each animal's identity in combination with body size and enclosure number.

PIT tagging

Nano-PIT tags (Nonatec^{TM}) were injected with a 18-gauge needle. The PIT tag was injected into the left axillary region pointing posteriorly and gently massaged down away from the injection site (as per S. Clulow pers. comm.).

Nonatech^{TM} secures their tags in the needles using a bio-safe silicone plug, which enters the animal at point of tag injection. Under ideal circumstances, the silicone is a round and smooth bead that is intended to enter the animal and to remain implanted with the PIT tag. However, when the silicone was placed in the needle, it did not always conform to a smooth bead shape. In Study One, in instances when the PIT tags were expelled from the animal through the injection site, the PIT tag came out attached to the silicone plug, which had been inadvertently glued to the veterinary adhesive. Therefore, in Study Two, the silicone plug was removed prior to implantation and the veterinary adhesive was not used with the aim of reducing the chance of expulsion.

Ethics

The study was approved by James Cook University Ethics application A1880.

Results

Study One: Coelomic cavity PIT tag injections

PIT tagged animals were monitored for four months, and during that time, 66.7% (10 of 15) of animals lost their tags. All tags were recovered from the animal enclosures. 53.3% of the tags were expelled in the first two months after injection (8 of 15); 25% those tags (2 of 8) were expelled within the first two days through the site of implantation (Figure 2). All injection site wounds had healed completely within the first week after tagging.

Study Two: Marking methods compared

Each animal was checked a total of seven times after the initial implantation; two days after marked, and then weekly over six weeks.

Subcutaneous PIT Tag injections

During the six weeks the animals were monitored, 26.7% of the tags (4 of 15) were expelled. The tags were expelled across the skin, not through the site of implantation. All injection site wounds had healed completely within the first week after tagging. 25% of the expelled tags were expelled at week three after implantation, 25% were expelled at week four, and 50% were expelled at week six. All retained tags were successfully read.

VIE tags

A total of 41 VIE tags were injected into the 15 animals. Animals were correctly identified in 18.4% of the 103 total identity checks, and only 6.7% (1 of 15) of animals were identified based on VIE tags at all identity checks.

29.2% of tags moved at some point during the first six weeks after implantation (Fig. 3A), and 63.1% of all identity checks had movement of at least 1 tag compared to the check before (Fig. 3B), resulting in potential inability to identify the animal based on tags alone. The most common movement was between the dorsal and ventral surfaces, followed by movement to the other leg, tag obscurement, and finally tag expulsion (Figure 3A). 26.7% of animals expelled tags (8 tags from 4 animals). Of the tags that were obscured, 17.2% became visible at a later check. Of the tags that migrated away from the site of implantation, 5.8% of tags migrated back so that the animal could be correctly identified again.

Toe Clipping

96.1% of identity checks resulted in animals being correctly identified based on toe clips. Although clips remained consistent, in four instances a frog was incorrectly identified due to human error (i.e. removal of the second toe on the right hand was recorded as removal of the second toe from the left hand). For most animals it took under two weeks for wounds to heal (66.7% of animals), but full healing for 20% of the animals took up to five weeks. In 13.3% of animals (2 of 15) healing did not occur after six weeks and resulted in whole foot inflammation.

Discussion

The purpose of this study was to assess the mark retention and reliability of three of the most widely used and discussed tagging methods for amphibians to determine the best marking method for *L. v. alpina*. Based on our data, by far the most reliable marking method for identifying *L. v. alpina* was toe clipping, which resulted in 96.1% correct identifications during the six week second study. However, healing time was lengthy and 13.3% of the animals had infections six weeks post marking. The high infection rate observed in this species in captivity appears to be rare for anurans, and many species heal from toe clipping without complications. Although there are a few reports of high infection rates associated with toe clipping, both in captivity and in wild animals (see Golay & Durrer 1994; Lemckert 1996; Reaser & Dexter 1996; Williamson & Bull 1996), it appears to be species dependent. Toe clipping should be trialed before broadly implementing in capture-mark-recapture studies of a new species.

This study demonstrates that VIE tagging is not a viable marking method for this species as animals were correctly identified based on tags only 18.4% of the time. Because of their extensive subcutaneous lymphatic system, amphibians have little connective tissue between the dermis and the underlying muscle compared to other vertebrates (Farquhar & Palade 1965), which makes subcutaneous tag movement more likely. To combat tag movement, VIE tags should be placed in a region where movement is unlikely. Although other studies suggest low tag movement in the thigh (Moosman & Moosman 2006; Sapsford et al. 2013), this region was not suitable for *L. v. alpina*. To help reduce the risk of tag movement, tagging frogs in the webbing or toes of the hind feet has been suggested (Hoffman et al. 2008; Nauwelaerts et al. 2000), although using this technique in small animals or those with minimal webbing may not be feasible. Success of VIE tags in adult anurans appears to be species dependent and highly variable.

PIT tags do not seem to be a viable option for this frog species, as there was a high expulsion rate from both implantation sites. When injected into the coelomic cavity, 66.7% of tags were expelled within four months, (with 53.3% during the first 8 weeks), while 26.7% of animals expelled tags within just six weeks of subcutaneous implantation. Both methods have unacceptably low success rates for use in large-scale capture-mark-recapture studies.

PIT tag retention and ideal implant location have been extensively studied in other vertebrate groups (Kaemingk et al. 2011; Hamel et al. 2012, 2013), but not in amphibians. Injection sites that have been successful in fish (Hamel et al. 2012, 2013) and salamanders (Unger et al. 2012), such as sections of muscle that are not directly involved in locomotion, are not large enough to incorporate a PIT tag in most species of frog. Most researchers inject PIT tags subcutaneously in anurans, even though it has been suggested as a poor tagging location in fish species (Clugston 1996). Blomquist et al. (2008) tested three different dorsal subcutaneous injection sites in a large frog species and found that above the scapula had the highest retention. Multiple studies have found that tags implanted subcutaneously into the dorsum show no evidence of rejection (Brown 1997; Newell et al. 2013). In *L. v. alpina,* injections into the dorsum are impractical because the small size of adults increases risk of accidental damage to the spinal cord during injection. Although subcutaneous ventral sites of injection have been successfully used in other small anurans (S. Clulow pers. comm.), *L. v. alpina* seems to expel tags more readily.

PIT tags have been expelled through the digestive tract in species of snake (Roark & Dorcas 2000, Pearson & Shine 2002) and frog (Tracy et al. 2011). Possibly, tags injected into the coelomic cavity in *L. v. alpina* were engulfed and expelled by a similar mechanism resulting in the high failure rates found in this study. Interestingly in this study, the same proportion of animals (26.7%) marked with VIE and subcutaneously injected PIT tags expelled tags during the six weeks. None of the tags were expelled through the entry site, and all resulted in an open wound through the dermis. Because of the way the tags were expelled, we believe that tags would continue to be expelled over time. Tag retention seems to be much lower in *L. v. alpina* than species used in other published studies (i.e. Brown 1997; Newell et al. 2013), but may not be the exception as data from few marking studies are accessible.

Our incorrect identifications of the toe clipped individuals were based on human recording error, not a failed marking technique. Human recording error is a potential concern for all marking techniques. The error experienced in this study is less than what others have reported for toe clipping (e.g. Kenyon et al. 2009). In this study, full healing occurred within two weeks of the marking procedure, although in toe clipped individuals the healing time was longer and risk of infection was higher. Although we sterilized instruments prior to toe clipping according to standard protocol, treating animals with

topical antibiotics after the procedure could be considered in future experiments to prevent infections.

Laboratory studies of marking efficacy can be adequately translated into the field as long as the mark is retained, and behavior and survival is minimally affected. Mark retention is unlikely to alter between laboratory and field settings but impact on behavior and survival is a concern (Donnelly et al 1994) as marking can predispose to infection or increase predation (Schmidt & Schwarzkopf 2010). With invasive marking techniques, as long as the animal heals properly, behavior is likely to return to normal (Lemckert 1996; Schmidt & Schwarzkopf 2010; Sapsford et al. 2014).

Conclusions

This study was the first to assess the efficacy of three popular marking techniques, toe clipping, VIE tags, and PIT tags, in a single anuran species. Based on our results, the most reliable marking method for *Litoria verreauxii alpina* is a toe clip scheme. The least successful marking technique is VIE tags, followed by PIT tags implanted into the body cavity. The second best marking method is PIT tags injected subcutaneously, but retention during even the short term was too low to be useful for mark-recapture studies in which individuals need to be identified with high certainty. Although toe clipping remains the most effective marking method for this species, *L. v. alpina* was susceptible to infection after toe clipping. Therefore, another identification method, such as pattern recognition, is worth assessing as a safer option, although it is time intensive and changing patterns and colors would reduce accuracy (Donnelly et al. 1994; Kenyon et al. 2009).

To be appropriate, a marking methods need to be both effective (in the sense tested in our study) and morally acceptable. Based on non-scientific perceptions, some animal ethics boards are increasingly rejecting toe clipping as a legitimate marking technique (Funk et al. 2005, Phillott et al. 2008; Correa 2013), although other marking techniques have not been thoroughly tested in a wide range of species (Funk et al. 2005, Phillott et al. 2007). Choice of method should be based on evidence; therefore, we urge field researchers and captive managers to publish their successes and failures with marking techniques.

Acknowledgements

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Figure legend

Figure 1. Photographs of frog thighs with VIE tags.

A) and B) are individual 1 and C) and D) are individual 2. A) and C) are VIE tags on the day of implantation (Day – 0). B) shows tag recombination at Day 14. D) shows tag migration from the venter to the dorsum on Day 14.

Figure 2. The proportion of PIT tags expelled from the animals each time the animals were checked.

A total of 10 tags were expelled in the first 4 months, n = 15.

Figure 3. VIE tag movement, obscurity, or expulsion that occurred during the course of the experiment (n = 15).

A) The proportion of VIE tags that moved during the six week study, and to where tags moved. B) The proportion of identification checks that resulted in misidentification based on VIE tags alone and quantification of tag movement.

Figures

Figure 1

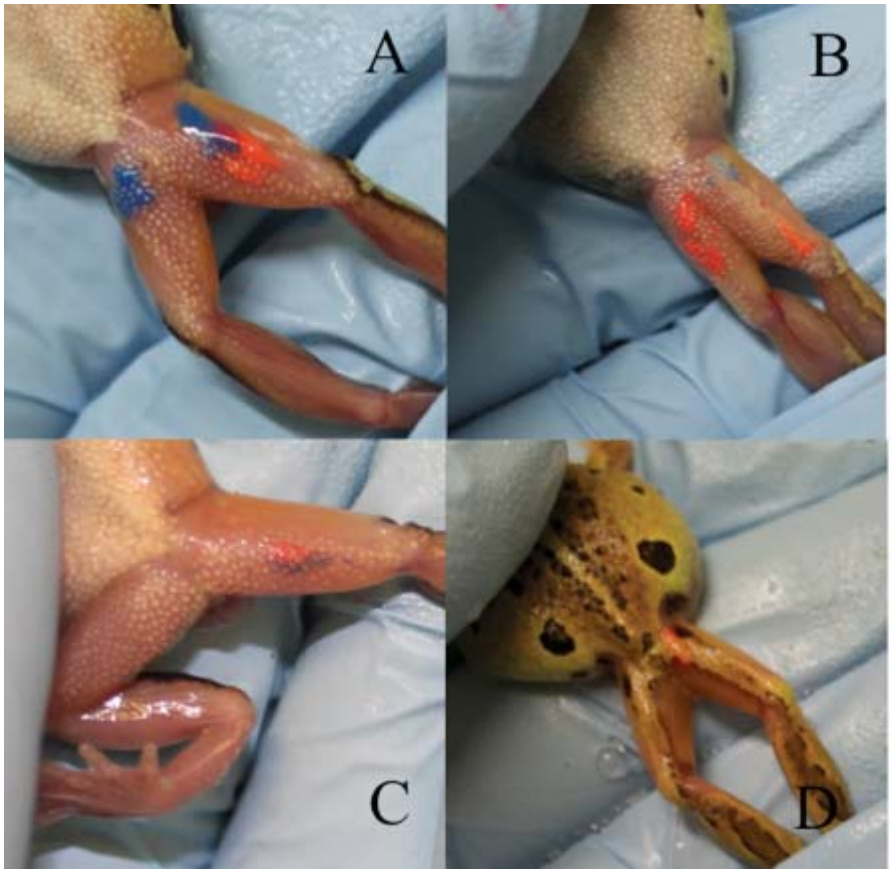


Figure 2

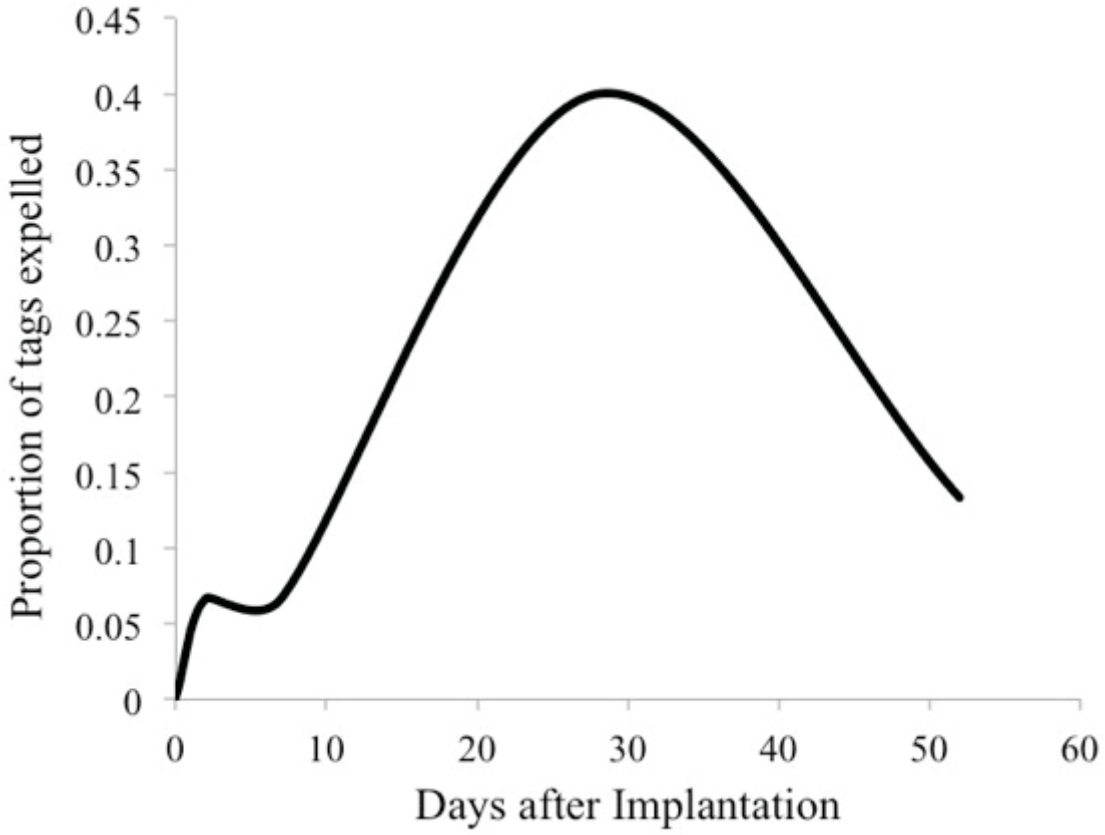
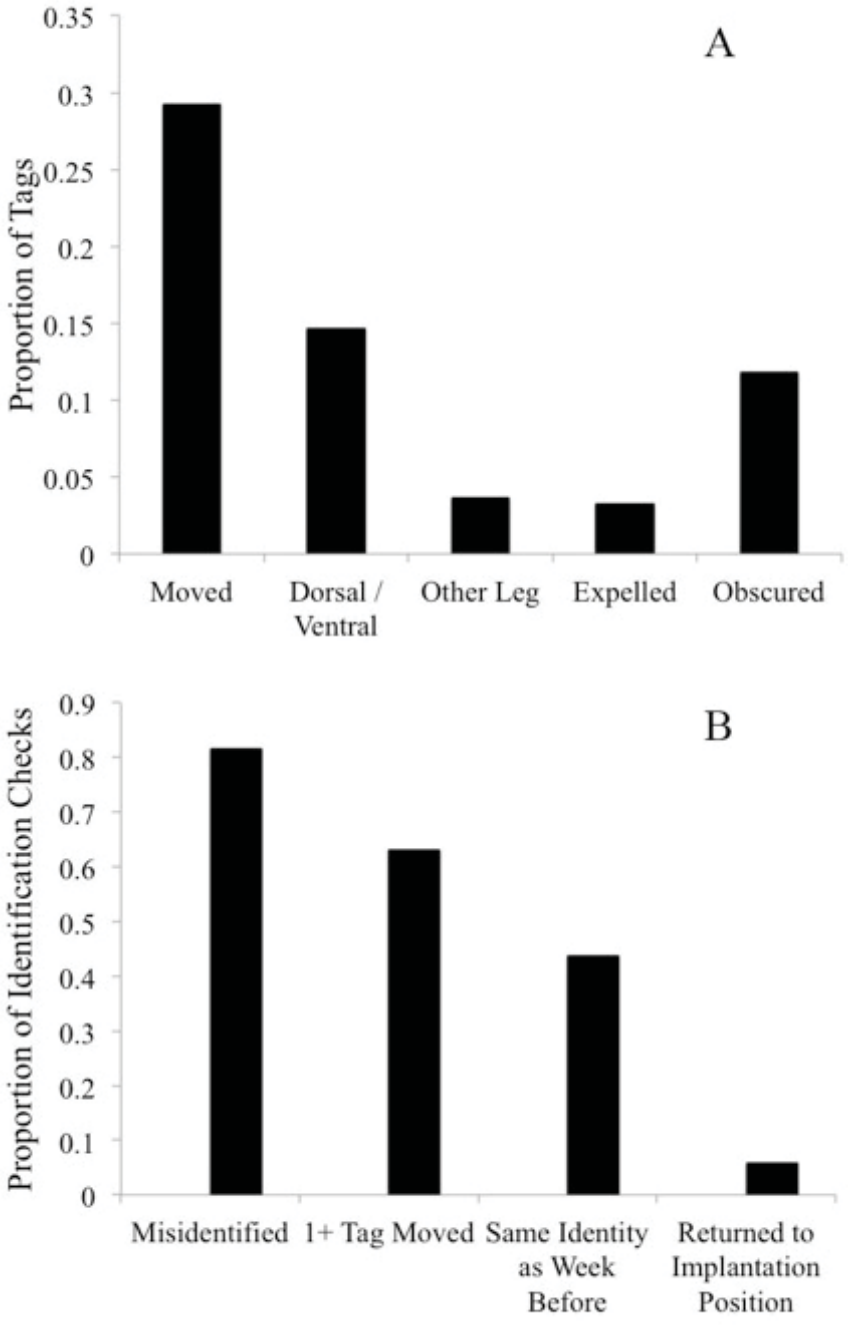

0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Proportion of tags expelled
0
10
20
30
40
50
60
Days after Implantation

Figure 3


A
Proportion of Tags
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Moved
Dorsal / Ventral
Other Leg
Expelled
Obscured
B
Proportion of Identification Checks
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Misidentified
1+ Tag Moved
Same Identity as Week Before
Returned to Implantation Position

CHAPTER 3

The effects of disease on reproduction

Introduction.

It is clear from the results of Chapter 2 that *L. v. alpina* are highly susceptible to *Bd* infection even after over 20 years of coexisting with the pathogen. Animals enter the breeding season *Bd*-negative, gain infection and succumb to disease by the end of the season. Because animals are gaining infection and succumbing to disease every breeding season, it appears that little disease resistance is evolving in the population. Therefore, another mechanism of population persistence must be present in this species. Because high recruitment is important for the persistence of the populations, reproduction is a mechanism that should be explored. Chapter 3 explores Aim 2 of this thesis: to investigate the effect of disease on reproduction.

In this chapter I explored the effect of disease on reproduction by using gametogenesis as a proxy for reproductive success. I explored spermatogenesis in two species, *P. corroboree,* and *L. v. alpina*, and oogenesis in *L. v. alpina*. The chapter consists of a paper entitled "Amphibians with infection disease increase their reproductive effort: evidence for the terminal investment hypothesis," which has been accepted to Open Biology and is in press at the time of thesis submission.

Paper 1

Citation: Brannelly LA, Webb R, Skerratt LF, Berger L. Amphibians with infections disease increase their reproductive effort: evidence of the terminal investment hypothesis. Open Biology. In Press.

Amphibians with infectious disease increase their reproductive effort: evidence for the terminal investment hypothesis

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Abstract

Mounting an immune response to fight disease is costly for an organism, and can reduce investment in another life history trait, like reproduction. The terminal investment hypothesis predicts that an organism will increase reproductive effort when threatened by disease. The reproductive fitness of amphibians infected with the deadly fungal pathogen, *Batrachochytrium dendrobatidis, Bd*, is largely unknown. In this study, we explored gametogenesis in two endangered and susceptible frog species, *Pseudophryne corroboree* and *Litoria verreauxii alpina*. Gametogenesis, both oogenesis and spermatogenesis, increased when animals were experimentally infected with *Bd*. In *P. corroboree*, infected males have thicker germinal epithelium, and a larger proportion of spermatocytes. In *L. v. alpina*, infected males had more spermatic cell bundles in total, and a larger proportion of spermatozoa bundles. In female *L. v. alpina*, ovaries and oviducts were larger in infected animals, and there were more cells present within the ovaries. Terminal investment has consequences for the evolution of disease resistance in declining species. If infected animals are increasing reproductive efforts and producing more offspring before succumbing to disease, it is possible that population level selection for disease resistance will be minimized.

Key Words

Chytridiomycosis, Oogenesis, Reproduction, Spermatogenesis, Terminal Investment, Wildlife Disease

Introduction

Overall fitness of an individual is controlled and restricted by finite levels of energy allocation. Therefore, life-history trade-offs in energy allocation between physiological processes such as reproduction and fighting infectious disease are fundamental to fitness [1]. Mounting an immune response is costly, and to effectively fight infection, a trade-off of resources occurs [2]. Many studies have characterised infections that lead to a reduction in various reproductive measurements, like gametogenesis (gamete development), fertility and parental care [3]. Decreased gametogenesis occurred in the northern cricket frog after antigenic stimulation [4], in mice exposed to *Toxoplasma gondii* [5], in cattle with viral diarrhoea [6] and pneumonia [7], in dogs with canine leishmaniasis [8], and in humans with *Helicobacter pylori* infection [9] and AIDS [10,11]. Decreased overall fertility has been reported in the insect *Rhodnius prolixus* when infected with *Trypanosoma rangeli* [12], and decreased parental care effort has been observed in blue tits [13], and in the house sparrow as they combat infection [14]. However, in other cases there is no loss in reproductive effort, for example gametogenesis is unaffected by asymptomatic HIV infection in humans [15]. Subclinical infections of *Cowdria ruminantium* in ewes did not affect female fertility [16], and rate of conception was unaffected in humans with Behçet's Syndrome (a rare autoimmune disorder that causes inflammation of the blood vessels) [17].

Alternatively, disease can lead to an enhanced reproductive effort, in accordance with the terminal investment hypothesis. Organisms are expected to increase reproductive investment when reproductive value declines. For example, when an organism is unlikely to reproduce in the future, it is in its interest to invest much of its energy into the current progeny [18,19]. Terminal investment is well documented with senescence in iteroparous animals; the young prioritize self-maintenance, while the old prioritize reproduction [20,21].

In the face of infection, illness or immune challenge, animals can increase their reproductive investment, which is one outcome of terminal investment. Reproductive investment can be measured through efforts in mating, parental care, and gametogenesis. For example, when male mealworms are immunocompromised or under stress, they produce more sex pheromone and so increase mating opportunities [22–24]. Parental efforts are increased in older male blue footed boobies' when an immune response is elicited [20]. In immune challenged house sparrows, females increase their reproductive

effort by laying replacement clutches more often than control animals [21], and in Arctic breeding common eiders, females that laid larger clutches had lower immune responses to avian cholera [25]. When snails are exposed to a trematode, they compensate by increasing egg laying soon after exposure, regardless of whether they become infected [26]. In trematode infected limpets, sexual maturity is reached earlier and gonads are much larger [27]. Investing in reproduction rather than fighting disease can be important for organisms to pass on their genes, a tenet of evolutionary theory.

Little is known about how infection affects reproduction in amphibians. The amphibian chytrid fungus, *Batrachochytrium dendrobatidis, Bd,* has caused amphibian declines globally [28]. *Bd* is an epidermal pathogen, which causes chytridiomycosis characterised by hyperkeratosis, electrolyte loss and ultimately death by cardiac arrest [29]. There has been much research devoted to understanding the immune system of amphibians and identifying resistance mechanisms in various species [30–32], but mitigating mortality through increased resistance is not the only mechanism by which a species can respond to infection. A shift in life history, such as reproductive fitness, can also explain population persistence in the face of large disease induced mortality. To date, only two studies have explored reproductive fitness in animals with *Bd* infection: 1) infected *Rana (Lithobates) pipiens* males had larger testicular size with more mature sperm, which suggests that exposed animals invest in more spermatogenesis [33] and 2) infected wild *Litoria rheocola* in good body condition were more likely to be found calling than uninfected males [34]. Female reproductive investment has not been explored in this system.

For this study we explored gametogenesis in two critically endangered species to assess the impacts of disease. The alpine tree frog, *Litoria verreauxii alpina*, and the southern corroboree frog, *Pseudophryne corroboree*, are endangered amphibians endemic to the Australian alpine regions of New South Wales and Victoria. Both species are highly susceptible and have significantly declined due to chytridiomycosis, with *P. corroboree* now functionally extinct in the wild [35–38]. Infection prevalence is highest during the breeding season, but while *Bd* infection prevalence in *P. corroboree* was typically about 30% in a few extant populations over the last 10 years, in *L. v. alpina* infection levels reach 100% causing near complete population turnover each year [39,40]. *Pseudophryne corroboree* are long-lived (>12 yrs), late to sexually mature (about 3yrs) and have low

fecundity, producing approximately 30 eggs per clutch [41]. *Litoria v. alpina* historically reach sexual maturity at two years, and aggregately breed [42].

Understanding the impact of *Bd* infection on the individual is needed to improve management efforts for both these species. Since the introduction of *Bd*, individuals are maturing faster but disease resistance may not be evolving at a population level [37,40]. In an infection experiment with *L. v. alpina*, susceptibility in frogs sourced from long exposed sites was not consistently lower than frogs from a naïve population, even though susceptibility varied among individuals and clutches [37,43]. As this species can breed before succumbing to infection [39,40], the opportunity to evolve disease resistance is dampened [44]. Annual recruitment success, characterised by high population turnover, has become key to this species persisting [49]. As many amphibian species become infected during the breeding season [39,45–48], and the incubation period for severe chytridiomycosis can be up to about four months, this provides an opportunity for susceptible frogs to breed prior to death, and therefore produce susceptible offspring. Furthermore, if infected and susceptible animals are stimulated to spawn more offspring, as will occur with terminal investment, selection for resistance is even less likely to occur. Understanding how *Bd* affects reproduction as well as mortality may identify new options for conservation management.

In this study we measured reproductive effort in *P. corroboree* and *L. v. alpina* experimentally infected with *Bd.* In males we measured germinal epithelium depth, counted spermatogenesis stages, and width, length and mass of the testis, number of spermatic cysts and cyst area as a proxy for reproductive effort [4,5,33]. In female *L. v. alpina* we measured mass of the gonads, and counted developed eggs and total grossly visible cells inside the ovaries. The effects of disease on reproduction are rarely studied in wildlife, especially in amphibians, and this is one of the first to explore *Bd* and reproduction in multiple species and to include effects on females and males.

Methods

Animal husbandry

Southern corroboree frogs (*Pseudophryne corroboree*) that were sexually mature and excess to breeding programs were delivered to James Cook University from the Amphibian Research Centre. They had been captive raised and ranged in age from five to

eight years old. *Pseudophryne corroboree* reach sexual maturity at 4-6 years, and the oldest animal found in the wild was nine years old but this species is known to survive much longer in captivity [41]. Animals were housed individually in 300 x 195 x 205 mm terraria with a damp and crumpled paper towel substrate, at a room temperature of 18-20°C. They were fed *ad libitum* three times weekly pinhead (5 – 10 mm) crickets (*Acheta domestica*). Animals were misted twice daily for 60 seconds with reverse osmosis water, and not artificial pond water because *P. corroboree* inhabit pristine habitats and are sensitive to water quality (MS McFadden pers comm, 2014). Temperature and humidity were monitored daily. Terraria were cleaned fortnightly by replacing the paper towel substrate.

Litoria verreauxii alpina that were excess to a reintroduction trial were delivered to James Cook University from Taronga Zoo. They had been captive raised from wild collected egg masses in spring 2011 and ranged from two (for the male trial) to three years old (for the female trial) over the course of these experiments. In the wild, *L. v. alpina* survive only one breeding season before succumbing to *Bd*, but before the introduction of the pathogen, animals could survive up to seven years of age [40,49]. They were housed individually under the same conditions as above, but with gravel substrate, which was replaced every three months.

Both *P. corroboree* and *L. v. alpina* breed seasonally following snow melt in the spring [36,39] after a few months of overwintering at low temperatures. In this study, animals were housed under consistent temperatures and daylight lengths that did not mirror breeding season regimes, and the gametogenesis observed represented activity outside peak breeding season. While animals were sampled at different times throughout the experiment (see data collection below), we do not expect confounding from any temporal variations.

Despite the experiments not being undertaken during peak breeding, *L. v. alpina* females were gravid at time of infection, and males were sexually mature, with observable nuptial pads and darkened throat patches. Secondary sexual characteristics are difficult to observe in *P. corroboree* because of their dark colouration. Males of both species were heard calling over the course of the experiments, but call details or secondary sexual characteristics were not measured.

Inoculation

Animals were allowed to acclimate to their new environment for seven days. We used two different isolates and protocols for inoculating the animals of two different species. *Pseudophryne corroboree* males were inoculated with a known virulent isolate of *Batrachochytrium dendrobatidis* from New South Wales (AbercrombieR-L.booroologensis-2009-LB1, Passage number 11) in March 2013 [38,50]. *Bd* was harvested from agar and tryptone, gelatin hydrolysate, lactose (TGhL) petri plates which had been incubated at 23°C for 5 days. Plates were flooded with 3mL of artificial spring water for 10 minutes to allow zoospores to release from zoosporangia. Inoculum was poured off the plates and zoospores were counted using a hemocytometer. *Pseudophryne corroboree* males (n = 17) were inoculated with $1x10^6$ zoospores by applying 3mL of inoculum dripped onto the venter over their individual 40mL inoculation container. Animals were kept in these containers for 6 hours, and then transferred back into their terraria. This method of inoculation has been successfully used for terrestrial amphibians [38,50,51].

Litoria v. alpina were inoculated using two different methods. *Litoria v. alpina* females were inoculated in the same manner as *P. corroboree* in February 2015 (n = 7). But *L. v. alpina* males were inoculated in February 2014 with a different *Bd* strain from the same region and isolated from clinically infected *L. v alpina* just prior to the start of this trial (WastePoint-L.v.alpina-2013-LB2, Passage number 1). This is the first experiment testing virulence of this strain of *Bd*. *Litoria v. alpina* males (n = 10) were inoculated with $5x10^5$ zoospores in 10mL of inoculum dripped onto their venter and allowed to runoff into their individual inoculation containers. The animals were held in inoculation containers for 24hr before returned to their individual terraria.

The change in methods was necessary due to the initial low proportion of infected *L. v. alpina* (see results). To overcome this variation between the species, we used an isolate of *Bd* cultured from *L. v. alpina* in 2013, with a larger volume of inoculum - adapted from a successful method used in other hylid frogs [37,47,51]. We do not expect the differences in protocols to confound the results, because gametogenesis was analysed in all frogs at a similar late stage of infection when effects of chytridiomycosis will be similar.

Bd negative control animals were mock-inoculated using uninfected petri plates (*P. corroboree* n = 10; *L. v. alpina* males n = 7, females n = 8).

Data collection

Animals were swabbed for *Bd* presence (see below), weighed to the nearest 0.01g, and measured snout to venter (SVL) to the nearest 0.1mm weekly. Animals were euthanized with an overdose of tricaine mesylate (MS-222) when clinical signs of chytridiomycosis (inappetence, irregular skin sloughing, cutaneous erythema, splayed legs) were displayed and righting reflex was abolished in accordance with animal ethics. The experiment ended when the last infected animals succumbed to disease or 13 weeks after inoculation, which ever was earlier. All animals remaining (controls and one female *L. v. alpina* that survived but maintained a high infection load) were euthanized at the end of the experiment (day 90-100). Both left and right testes were dissected from animals within thirty minutes after euthanasia. Oviducts and ovaries of *L. v. alpina* were weighted separately to the nearest 0.001g.

***Testing for* Bd**

We tested for *Bd* infection by using qPCR on skin swabs [52]. The swabbing protocol is standardized by performing 45 strokes with a sterile rayon-tipped swab (MW-113, Medical Wire & Equipment) per animal, five on the middle of the venter, five on each side of the venter, five on each thigh, and five each limb. The swab was gently rotated during and between strokes to ensure the greatest amount of DNA was gathered on the swab. Genomic DNA is extracted from the swabs using the Prepman Ultra kit and 2 minutes of bead beating to break apart the fungal cell walls. The extract was analysed using quantitative PCR following Boyle et al. [52], with a positive and negative control, and a series of dilution standards (100, 10, 1 and 0.1 zoospore equivalents, ZE, made in house) to estimate zoospore load. After inoculation animals were tested once a week until succumbing to disease or the experiment ended.

Testis histology

Testes were fixed in 4% phosphate buffered formaldehyde for at least 2 weeks; the left testis was sectioned in *P. corroboree* and the right testis was sectioned in *L. v. alpina.* Routine histological techniques were used to prepare the testes for light microscopy following standard methods [4]. Testes were dehydrated in a graded series of ethanol, cleared with xylene, and embedded in paraffin. They were serially sectioned at

5µm, affixed to glass slides and stained with hematoxylin followed by eosin counterstaining (H&E), and mounted with coverslips. Four randomly selected histosections were analysed for each animal.

All measurements were made using the computer software Image J to the nearest 0.0001mm. The area of the histosection, area of the three largest circular seminiferous tubules, and number of tubules per histosections were measured. In the largest circular tubules per histosection, maximum germinal epithelium was measured. In instances where tubules had no luminal space, the germinal epithelium depth was estimated as half the diameter. It must be noted that these histological measurements produce a relative indication rather than accurate depths because it is not possible to ensure sections pass through the centre perpendicularly to the tubule.

One field of view per histosections that included the largest seminiferous tubule was used to quantify spermatogenesis stages (with guidance from de Oliveira *et al.* 2002) (Fig. 1). Within the seminiferous tubules, spermatogenesis stages can be quantified by number of cell layers in mammal and reptile testes [54,55], but in amphibians spermatogenetic cells occur in spermatic cysts rather than layers [53,56]; therefore groups of cells were counted for each stage per field of view, and spermatic cyst (a group of cells or cell bundle) typically has only one spermatogenesis stage [57]. The four main stages of spermatogenesis were counted: spermatogonia, spermatocytes (primary and secondary combined), spermatids (primary and secondary combined) and spermatozoa bundles (Fig 1).

Female gonad histology

The left ovary and oviducts were preserved in 4% buffered formaldehyde and sectioned following the procedure above. Sections were analysed for pathology. To count eggs and total cells within the ovary, the right ovary was fixed in ethanol, the ovary membrane was separated and all cells within the ovary were counted grossly under 100x magnification for each animal. Cells within the ovary were grouped within two types: 1) developed ovum (with black yolk forming) and 2) all other cells within the ovary, which includes all other stages of oogenesis such as oogonium and oocytes

Statistical analysis

Bd *infection*

Infection load was represented as median and interquartile range (IQR) of the zoospore equivalents (ZE), which was calculated in SPSS v21. Infection loads are highly variable, but all animals included in the infected group had clinical chytridiomycosis, and died with high infection loads.

Spermatogenesis

Distribution of all stages of spermatogenesis were analysed using Pearson's Chi-Squared test on total cyst counts per spermatogenesis stage per individual. Significant results were further explored by calculating the mean proportion of each spermatogenesis stage cysts per individual in order to determine how each spermatogenesis stage differed between the uninfected and infected individuals. We compared these means of each spermatogenesis stage using independent two tailed t-tests after normal distribution of the data was determined. Normal distribution of the data was determined using four measures: the distribution of the histogram, the ratio of mean to median, the ratio of mean to standard deviation, and the Shapiro-Wilk test of normality.

Number of tubules per histosection, area of the histosections, area of the largest tubule per histosection and germinal epithelium depths were averaged for each individual and compared using independent t-tests, after normal distribution was determined. Only animals that succumbed to *Bd* during the experiment were included in the *Bd*+ group for analysis.

Oogenesis

Number of cells within the ovaries and proportion of developed eggs compared with total cells within the ovary were analysed using Mann-Whitney nonparametric tests. Wet gonad mass was analysed for female *L. v. alpina* using Mann-Whitney nonparametric test. Individual size was controlled for by analysing gonad mass/animal mass, and gonad length/SVL. Only animals that succumbed to *Bd* during the experiment were included in the *Bd*+ group for analysis.

All statistical analyses were conducted in SPSS (v. 21). Effect size was determined using Cohen's *d* statistic in Microsoft excel.

Animal ethics

Animal ethics was approved by James Cook University in applications A1875 for the corroboree frogs, and A1897 and A2171 for the alpine tree frogs.

Results

Bd *infection*

All uninfected control animals from both species, *P. corroboree* (n = 10) and *L. v. alpina* (n = 15), remained *Bd*- throughout the study. All 17 *Bd*+ *P. corroboree* died between days 29 and 81 (mean 45.94 ± 14.87 days) post exposure. Median infection load at date of death was 124,317 ZE (IQR 126,851). Four of seven *L. v. alpina* females became infected with *Bd* after inoculation, and three died due to chytridiomycosis 58 – 60 days post exposure, while one survived with a heavy infection until the end of the experiment (day 92). Median infection load in females at date of death or week 13 was 35,8001 ZE (IQR 354,736). Six of the 10 inoculated *L. v. alpina* males developed chytridiomycosis and died between 39 and 63 days post exposure (mean 52.67 ± 8.68 days). Infection load at date of death was 66,407 ZE (IQR 135,555).

Spermatogenesis

Pseudophryne corroboree

In male *P. corroboree*, overall proportions of spermatogenesis stages were significantly different between *Bd*+ and *Bd*- animals (Chi-squared: $\chi^2_3 = 374.802$; $p < 0.001$) (Fig 2a). There was no difference in number of spermatogenesis stage cysts per animal (t-test: $t_{24} = 0.538$, $p = 0.596$), but there were 116.9% more spermatocytes in the *Bd*+ animals (t-test: $t_{24} = -2.813$, $p < 0.01$, $d = 1.32$). The *Bd*- animals had a 32.9% higher proportion of spermatogonia (t-test, equal variances not assumed: $t_{8.76} = 2.32$, $p = 0.046$, $d = 1.05$).

Bd+ animals had a 46.5% larger germinal epithelium depth (*Bd*+ = 0.104mm ± 0.035mm; *Bd*- = 0.071mm ± 0.035mm; t-test: $t_{25} = -2.556$, $p = 0.017$, $d = 0.94$). There was no difference between *Bd*- and *Bd*+ number of seminiferous tubules (t-test: $t_{24} = -1.575$, $p = 0.128$), area of histosections of testis (t-test: $t_{24} = -0.34$, $p = 0.737$) or area of tubules (t-test: $t_{24} = 2.027$, $p = 0.053$).

There was no difference in any measure between *Bd*+ animals that died before day 37 post exposure and those that died between day 48 and 81 post exposure.

Litoria verreauxii alpina

In male *L. v. alpina*, overall proportions of spermatogenesis stages were significantly different between *Bd*- and *Bd*+ animals (Chi-Squared: $\chi^2_3 = 445.52$, $p < 0.001$) (Fig. 2b). There were 82.3% more spermatic cysts in the *Bd*+ animals (t-test: $t_{11} = -3.746$, $p = 0.003$, $d = 2.02$). There was 27.2% higher proportion of spermatozoa bundles in the *Bd*+ animals (t-test: $t_{11} = -2.421$, $p = 0.034$, $d = 1.36$), but 49.5% fewer spermatogonia (t-test: $t_{11} = 3.302$, $p = 0.007$, $d = 1.85$). There was no difference in the number of spermatocytes between the two groups (t-test: $t_{11} = -0.532$, $p = 0.605$).

No differences were found between the *Bd*+ and *Bd*- male *L. v. alpina* in germinal epithelium depth (t-test: $t_{11} = 0.523$, $p = 0.612$), seminiferous tubule number (t-test: $t_{11} = -0.394$, $p = 0.702$), seminiferous tubule size (t-test: $t_{11} = 0.352$, $p = 0.731$), or total histosection area (t-test: $t_{11} = 0.393$, $p = 0.702$).

Oogenesis

Counts of grossly visible eggs in female *L. v. alpina*, revealed infected animals had 59.1% more cells inside the egg masses compared with uninfected (Mann-Whitney: $Z = -2.084$, $p = 0.037$) and 67.2% more developed eggs present in the ovaries (Mann-Whitney: $Z = -2.079$, $p = 0.038$) but there was no difference in proportion of developed eggs to other cell types within the masses between *Bd*+ and *Bd*- animals (Mann-Whitney: $Z = 0$, $p = 1$) (Fig. 3a).

Ovaries of infected animals were 2.15x bigger as a proportion of body size than ovaries of uninfected animals (Mann-Whitney: $Z = -2.548$, $p = 0.011$). Oviducts of infected animals were 1.56x bigger as a proportion of body size than oviducts of uninfected animals (Mann-Whitney: $Z = -2.717$, $p < 0.01$) (Fig. 3b).

Ovary pathology

There was a wide range of stage of development of eggs within an individual and among individuals. Development ranged from early egg stages to fully developed and full of yolk platelets to atresia of developed eggs that were being reabsorbed. Oviducts looked

normal. There were no obvious differences in the ovaries and oviducts between the infected and uninfected animals.

Discussion

Our study shows that more gametogenesis occurred in male and female frogs experimentally infected with the fungus *Bd.* Increased gametogenesis is a proxy for increased reproductive effort [4,5,7,33], which may result in more offspring. Mounting an immune response to fight disease represents an energy cost for the host. Some organisms may prioritise reproduction over investing in immunity, a hypothesis known as terminal investment. Terminal investment in species susceptible to the deadly amphibian chytrid fungus may have enabled populations to persist but have resulted in them not evolving disease resistance.

Spermatogenesis

In *P. corroboree* males, there were a greater proportion of spermatocytes and a deeper germinal epithelium, suggesting higher production of spermatozoa after infection. There were more spermatogenesis cell bundles, and more spermatozoa bundles present in infected *L. v. alpina,* consistent with greater sperm production and storage. Higher proportions of spermatocytes and spermatozoa is correlated with higher reproductive success in other taxa [58], suggesting a similar pattern of increased reproduction in these frog species.

Both species had a lower proportion of spermatogonia in the *Bd+* animals. Spermatogonia are the early stages of spermatogenesis, the stock stem cells [57]. When the spermatogonia cells divide, some become committed cells that differentiate into spermatozoa, while a subset remain stem cell spermatogonia. Therefore, the number of spermatogonia present in the testis does not change [59]. However, spermatocytes (the first stage of sperm development that undergo mitosis), spermatids (later stages where meiosis is undertaken) and spermatozoa bundles (the mature sperm cells) increase with more sperm production. Therefore, a lower proportion of spermatogonia is consistent with a phase of active spermatogenesis.

While timing of spermatogenesis varies among species and has never been explicitly studied in the species studied here, the entire process of spermatogenesis in amphibians can range from approximately 30 days to four months [57]. The long

incubation period of chytridiomycosis as seen here of one or more months enables frogs to appear unaffected and in good body condition, only showing clinical signs in the last few days when severe disease manifests. Therefore, increased reproduction and even life history shifts toward increased reproduction is feasible during the subclinical phase of chytridiomycosis.

Oogenesis

In the female *L. v. alpina*, the ovaries and oviducts were much larger in infected animals. In addition, there were more cells present in the ovaries of infected females, demonstrating a functional increase rather than pathological swelling. This finding is surprising because all females were gravid at the time of initial exposure, and oogenesis is more time intensive and energy consuming than spermatogenesis [60]. Oogenesis and female investment is more logistically difficult to quantify than male investment; therefore, is less often studied. In female amphibians, exact length of time for the full oogenesis cycle is unknown, except that females appear to lay eggs either once or twice a year [57]; suggesting that oogenesis is a much longer cycle than spermatogenesis. Therefore, we recommend further work in this area to test our initial findings which were based on a few animals and breeding outside of the peak season.

***Gametogenesis and* Bd**

Our results extend previous findings from two species suggesting frogs increase reproductive investment via mating displays or gametogenesis when infected with *Bd*. Wild *Litoria rheocola* males were found calling more often upon capture when infected with *Bd* [34], suggesting an increased mating effort, while *Rana pipiens* had longer testes with a higher proportion of mature spermatozoa when experimentally infected with *Bd* [33], suggesting increased gamete production. Our study explored reproduction in both males and females by assessing gametogenesis and quantifying the differences in gamete production. This reproductive response in four frog species may be *Bd* specific, due to the immunosuppressive effects of the fungus or due more generally to subclinical disease, because antigenic stimulation (a non-specific substitute for the immune response aspect of disease) in another frog species decreased reproductive investment [4].

We used histological measurements as a proxy for sperm production, but gamete viability, mating success, normal embryo development, offspring survival, and overall

reproductive fitness cannot be determined using this method. While spermatogenesis is often used as a proxy for reproductive investment [4,5,7,33], we do not know how the increases in gametogenesis that we observed translate into mating success or increased reproductive fecundity. Our study is the first step in understanding how disease impacts reproduction, but more research is needed to fully understand the phenomenon. At this stage the mechanism of increased gametogenesis is not known, and could be a result of resource partitioning by the animal as per terminal investment, or a hormone-like chemical produced by the pathogen.

Population persistence

Bd infects over 600 amphibian species globally [28,61] but while many species are currently declining some populations appear to be rebounding since the original epidemic [62,63]. Population rebound may point to natural selection for disease resistance or decreased virulence of the pathogen, as occurred after introduction of myxomatosis to rabbits [64]. Evolution of resistance should occur if animals preferentially breed after surviving exposure, and this may explain the pattern of recovery in some species where longevity is increasing (e.g. [62]) However, with *Bd* infection, some species may lack an effective innate immune response, and the adaptive immune response may be suppressed [65–67], and while different strains of *Bd* differ in virulence [47,68] there is no clear evidence of decreasing pathogen virulence over time [69]. Therefore, another mechanism of population level persistence, such as increased reproduction, might explain the lack of widespread resistance within a population with endemic disease.

The terminal investment hypothesis refers to the trade-off between investing in one large but final reproductive event versus investing in survival and future breeding. Here we propose that a population threatened by chytridiomycosis adopts the terminal investment strategy, and that higher reproductive output (whether innate or stimulated by infection) will dampen the population level evolution of disease resistance. For *L. v. alpina*, progeny survival has enabled populations to survive so far, but appears a precarious strategy as it is dependent on uninterrupted breeding seasons [40]. One generation of failed recruitment will lead to population extirpation.

Pseudophryne corroboree is a low fecundity, long-lived species that declined gradually after *Bd* introduction. Even if there has been an increase in reproduction after infection it has not been enough for populations to survive and the species is now

functionally extinct in the wild [70]. For both species, perhaps an increase in reproduction and resistance to infection would help avoid population extirpation and species extinction.

Conclusions

Our results suggest that increased reproductive investment might be more widespread than previously thought, adding amphibians and fungi to the list of host and pathogens that are involved in this response. Terminal investment of infected animals has consequences for conservation management of declining species. With an increase of infected and susceptible animals reproducing, population level selection for disease resistance or tolerance is likely to be minimized. Artificial selection for resistance has been proposed as a management technique for mitigating *Bd* [71,72], but if natural selection in wild persisting populations has led to other outcomes such as increased reproduction associated with terminal investment, then which direction should interventions take? Management of the habitat [71] to support recruitment appears critical in the short-term for the two species investigated here, but is also applicable to a broader range of species. However, a concurrent approach of understanding and promoting genes for innate resistance factors is also likely to be useful to increase individual longevity and therefore population security.

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Conflict of Interest

Authors declare no conflict of interest.

Statement on Authorship

LAB, LB and LFS designed the experiment, LAB and RW collected the data, LAB analysed the data. LFS and LB contributed reagents/materials to project. LAB wrote the manuscript, LAB, RW, LB and LFS commented on the manuscript, and all authors approve the final version of this manuscript.

Data Accessibility

Data is supplemental file not included in this thesis.

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Figure legend

***Figure 1*. Spermatogenesis stages.**
A)*Pseudophryne corroboree* and B) *Litoria verreauxii alpina.* 1) Locular Wall, 2) Interlocular Tissue, 3) Primary Spermatogonia, 4) Secondary Spermatogonia, 5) Primary Spermatocyte, 6) Secondary Spermatocyte, 7) Primary Spermatid 8) Secondary Spermatids, 9) Spermatozoa Bundle 10) Sertoli Cells. Line indicates germinal epithelium depth. Magnification is 400x.

***Figure 2.* Spermatogenesis stage proportions found in the testes.**

Pseudophryne corroboree (*Bd*- n = 10; *Bd*+ n = 17) (a) and *Litoria verreauxii alpina* (*Bd*- n = 7, *Bd*+ n = 6) (b). Mean proportions of each spermatogenesis stage are graphed for *Bd* infected (light grey boxes) and *Bd* negative (dark grey boxes) individuals to represent the total cell bundles present in the testes. Error bars indicate standard error. (*) indicates a significant difference when *Bd*+ and *Bd*- were compared using a t-test. Only animals that succumbed to disease were included in the *Bd*+ group.

***Figure 3.* Oogenesis comparisons between infected and uninfected female.**

Litoria verreauxii alpina (*Bd*- n = 7, *Bd*+ n = 4). (a) Number or cells counted in the ovary per animal. (b) Gonad proportion of ovary and oviduct compared to the body mass of the individual. Central tendency is presented as the median where *Bd* infected (light grey boxes) and *Bd* negative (dark grey boxes) individuals. Error bars indicate interquartile range. (*) indicates a significant difference when *Bd*+ and *Bd*- were compared using a Mann-Whitney U test. Only animals that succumbed to disease were included in the *Bd*+ group.

Figures

Figure 1

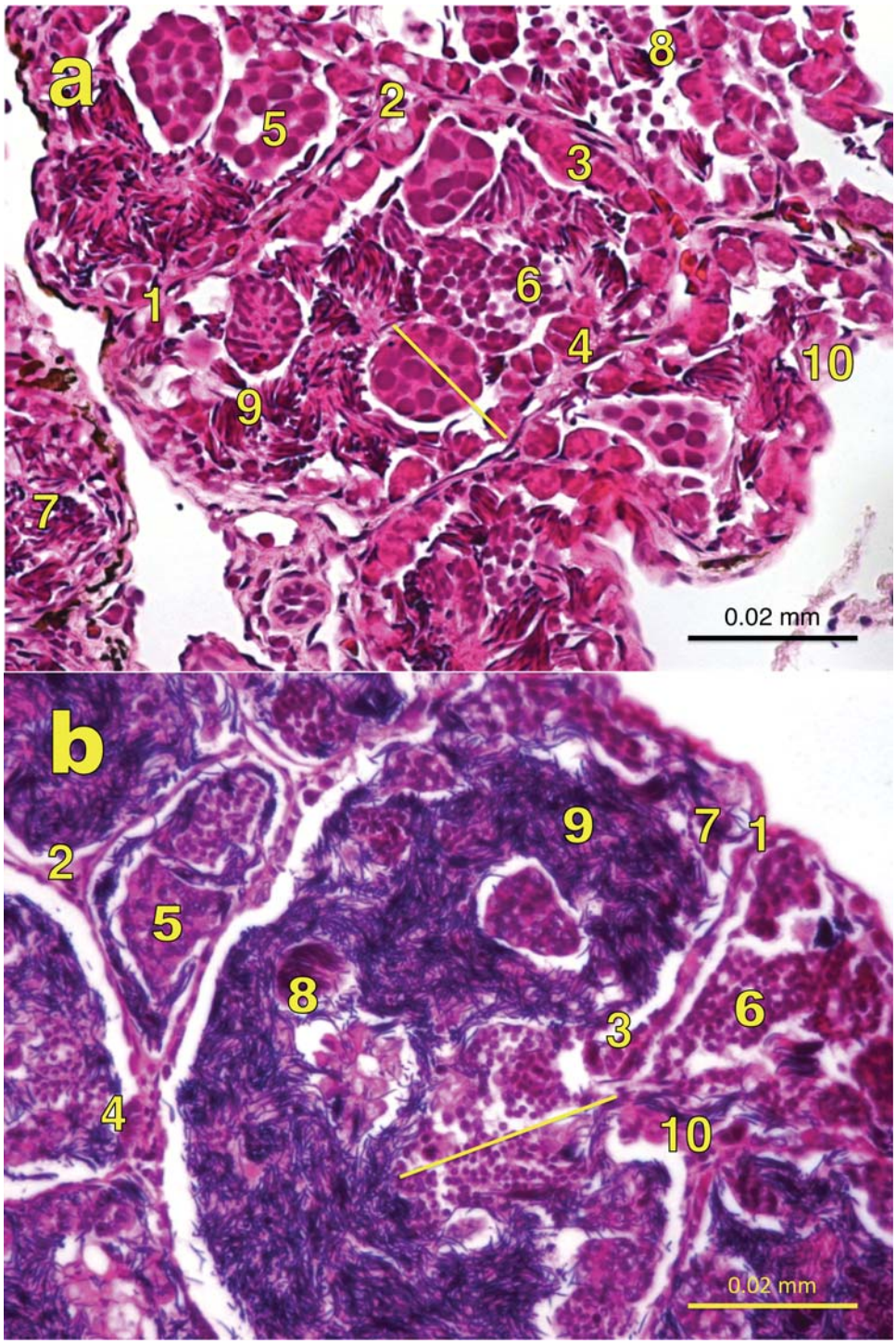
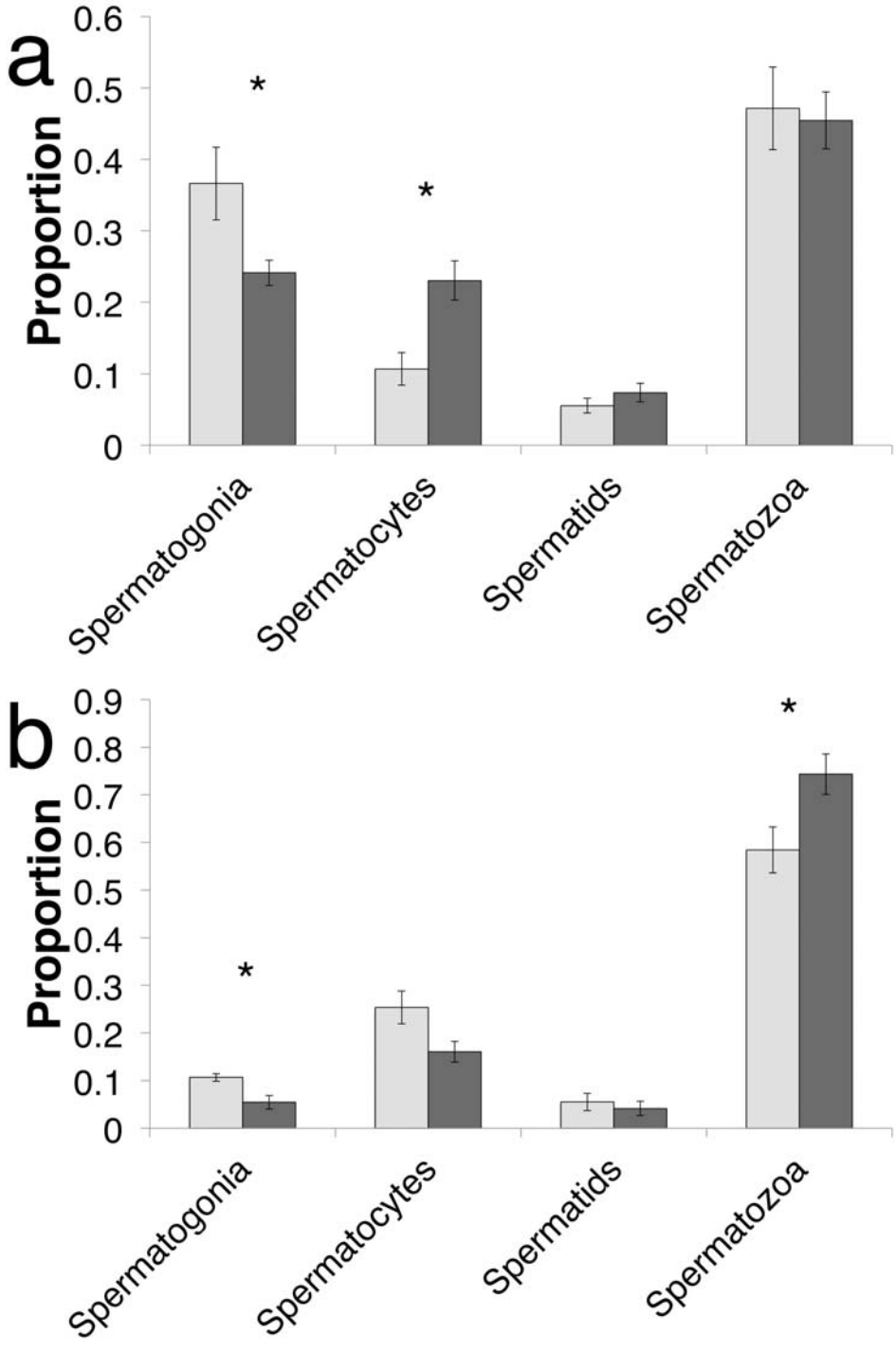


Figure 2.


a
Proportion
0.6
0.5
0.4
0.3
0.2
0.1
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*
*
Spermatogonia
Spermatocytes
Spermatids
Spermatozoa
b
Proportion
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
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*
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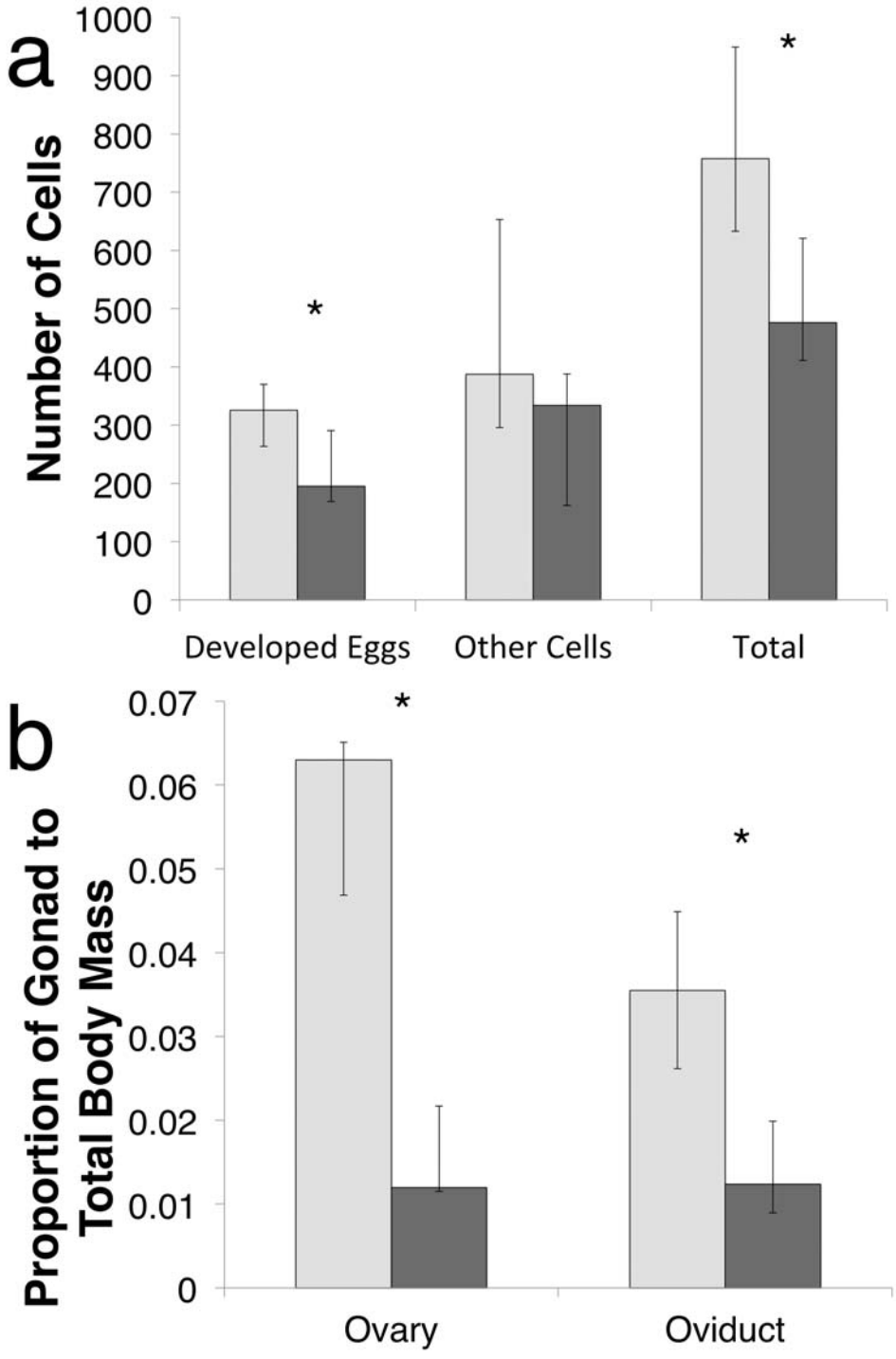
Spermatogonia
Spermatocytes
Spermatids
Spermatozoa

Figure 3.


a
Number of Cells
1000
900
800
700
600
500
400
300
200
100
0
*
*
Developed Eggs
Other Cells
Total
b
Proportion of Gonad to Total Body Mass
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
*
*
Ovary
Oviduct

CHAPTER 4

Pathogenesis of disease: changes in hematopoietic tissue

Introduction.

Exploring pathogenesis and mechanisms of immunity of declining and susceptible species is a key step in understanding how to minimise disease impacts. In Chapter 4 and Chapter 5, I investigate aim 3 of this thesis: to investigate underexplored mechanisms of pathogenesis of disease in susceptible species.

Previous results that suggested *Batrachochytrium dendrobatidis*, *Bd*, causes immunosuppression include 1) the lack of cutaneous inflammation, 2) ability of *Bd* to suppress lymphocyte proliferation in vitro, and 3) reduced general host immune activity. Here I measured lymphocyte depletion within host tissues of infected frogs using histology, and explored the differences in hematopoietic tissues within and between species. As a comparison to the declining species, I also included the invasive cane toad (*Rhinella marina*). This study is presented as a manuscript entitled “Effects of chytridiomycosis on hematopoietic tissue in the spleen, kidney and bone marrow in three diverse amphibian species,” which has been submitted and is under review at the time this thesis was submitted.

Paper 1

Citation: Brannelly LA, Webb R, Skerratt LF, Berger L. Effects of chytridiomycosis on hematopoietic tissue in the spleen, kidney and bone marrow in three diverse amphibian species. In Review.

Effects of chytridiomycosis on hematopoietic tissue in the spleen, kidney and bone marrow in three diverse amphibian species

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Key Words

Amphibian, Chytridiomycosis, Haematopoiesis, Hematopoietic Tissue, Histology, Wildlife Disease

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Abstract.

One of the major causes of amphibian population decline is the deadly fungal pathogen *Batrachochytrium dendrobatidis, Bd*. Research on pathogenesis and host immunity aims to inform development of targeted conservation interventions. Studies examining global host immune responses as well as effects on lymphocytes *in vitro* suggest that *Bd* infection causes immunosuppression. However, it is unknown which hematopoietic tissues are affected and if these effects differ among host species. We investigated the effect of experimental *Bd* infection on three different amphibian species by characterizing the hematopoietic tissue in the spleen, bone marrow and kidney. Upon *Bd* infection hematopoietic tissue in the kidney tended to be depleted, while the spleen appeared unaffected. The bone marrow in highly susceptible species was depleted, whereas an increase in hematopoietic tissue was observed in the more resistant species. Our study demonstrates that species and hematopoietic tissues behave differently in response to *Bd* infection, and may be related to the species' susceptibility to infection.

Introduction.

Amphibians are currently experiencing the greatest loss in global biodiversity of all vertebrate taxa (Stuart *et al.* 2004) and in many declining species this is due to spread of the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), the cause of chytridiomycosis (Skerratt *et al.* 2007). Chytridiomycosis is an infection of the epidermis, leading to disruption of cutaneous ion channels, low plasma electrolytes and eventually death by cardiac arrest (Voyles, Rosenblum and Berger 2011). *Bd* was discovered in 1998 (Berger *et al.* 1998; Longcore, Pessier and Nichols 1999), but has been causing major declines since the 1970s in Australia, and North and South America (Weldon *et al.* 2004).

Despite nearly two decades of research on chytridiomycosis, the impacts on individual infected animals are not fully understood. Infiltration of inflammatory cells is negligible at infected sites, unlike the granulomatous response that occurs with some other fungal infections in amphibians (Speare *et al.* 1997; Berger *et al.* 2009). Also, strong acquired immunity has not been detected in treated then reinfected frogs (Cashins *et al.* 2013; McMahon *et al.* 2014). It has been suggested the chytrid fungus suppresses immunity or evades immune detection once encysted within the epidermal cells of the host (Berger, Speare and Hyatt 1999), and *in vitro* studies showed cultured *Bd* causes apoptosis of lymphocytes from the spleen and reduced proliferation (Fites *et al.* 2013). Further, experimentally infected frogs *in vivo* had a reduced immunoglobulin and splenic lymphocyte response to antigenic stimulation with sheep red blood cells, and lower blood lymphocyte counts (Young *et al.* 2014), and a reduced response to PHA injection (Fites *et al.* 2014); however, in another study, animals that overcame infection had increased splenic lymphocytes (McMahon *et al.* 2014).

A systematic assessment of the effect of infection on amphibians on different hematopoietic tissues have not been conducted *in vivo*. To date, the general importance of each hematopoietic organ in amphibians is largely unknown, and has not been assessed in different species in recent years (Jordan 1933; Kanesada 1956). According to the generalized amphibian species studies published to date, the spleen of anurans is the primary site of lymphocyte production including both B and T cells, and is important in

the adaptive immune response (Jordan 1933; Kanesada 1956; Press 1999; Du Pasquier *et al.* 2000; Robert and Ohta 2009; Rollins-Smith *et al.* 2009). Hematopoetic activity in anurans is concentrated in the bone marrow and kidney, where the hematopoetic stem cells serve as precursors to lymphocytes, monocytes, erythrocytes, basophils, heterophils and eosinophils (Grayfer and Robert, in press). The bone marrow is seen as an important site for erythrocytopoiesis and granulocytopoiesis and has greater function for the innate immune response (Jordan 1933; Kanesada 1956; Robert and Ohta 2009; Rollins-Smith *et al.* 2009). The bone marrow is also an important site for macrophage precursor populations (Grayfer and Robert, in press). The function of kidney as hematopoietic tissue is unknown for most anuran species, but can be a site of erythrocyte and granulocyte production and may represent a compensatory mechanism for depleted bone marrow (Kanesada 1956; Grace and Manning 1980; Press 1999). However, there might be more species variation in hematopoietic tissue and cell production than we fully understand.

However, much of the research focus has been on commercially available species, like *Xenopus laevis, X. tropicalis, Rana pipiens, Lithobates catesbeianus* (previously *R. catesbeiana*), *Litoria caerulea* and other species that are often tolerant of infection and have not experienced declines in the wild due to *Bd* infection (Voyles *et al.* 2009; Baitchman and Pessier 2013; Peterson *et al.* 2013). There appears to be high interspecies variation in pathogenesis of *Bd* and yet susceptible and declining species, like *Litoria verreauxii alpina* and *Pseudophryne corroboree* remain understudied. These target species require adequate research to help inform management and improve the understanding of these highly endangered/functionally extinct species. Therefore, in this study we have explored the hematopoietic tissue in critically endangered species.

To investigate how *Bd* infection affects the cellular structure of hematopoietic tissues, we examined immune tissues in organs of three amphibian species that differ phylogenetically and in their conservation status: the functionally extinct and susceptible southern corroboree frog, *Pseudophryne corroboree*, the critically endangered and susceptible alpine tree frog, *Litoria verreauxii alpina*, and the invasive, abundant and less susceptible cane toad, *Rhinella marina* [previously *Bufo marinus*]. From experimentally infected animals we collected three organs (bone marrow, kidney [mesonephros] and the

spleen) and characterised each by histology to determine hematopoietic tissue coverage and cell density.

Hematopoietic tissues examined were at morbidity in *Bd*+ animals. We chose to investigate *Bd*+ animals at morbidity because we expected that at this point the largest changes would be observed between the infected and healthy animals, because this is the point where the animals begin to show signs of disease. Because the effect chytridiomycosis on hematopoietic tissue *in vivo* has never been attempted on multiple species and organs, information gathered from this study will serve to inform other studies.

Materials and Methods.

Study species

Three species were investigated in this study: *Pseudophryne corroboree*, *Litoria verreauxii alpina* and *Rhinella marina.* The first two species occur in alpine areas of southern Australia and are highly susceptible to *Bd*, which has caused their populations to decline severely (Hunter *et al.* 2010a, 2010b; Brannelly *et al.* 2015a, 2015b, 2015c; Brannelly, Skerratt and Berger 2015; Scheele *et al.* 2015). *Rhinella marina* is native to Latin America and is invasive in many parts of the world including Australia. Early life stages of *R. marina* are known to be *Bd* susceptible (Berger *et al.* 1998; Daszak *et al.* 1999) but have not been experimentally infected with *Bd*, and there is very little field data on disease prevalence in the wild, either in the native or introduced ranges.

Animal husbandry

Animal history

Pseudophryne corroboree had been captive raised and ranged in age from five to eight years old, under strict quarantine protocol and had never been exposed to *Bd*. *Litoria v. alpina* had been captive raised and ranged from two to three years old under strict quarantine protocol and had never been exposed to *Bd*.

Rhinella marina were wild caught from various locations around Townsville, Queensland, Australia. These animals were recent metamorphs, under 30mm in length (Cohen and Alford 1993). Townsville, Queensland is too hot and dry for *Bd*, and it has

never been observed inside the city limits, so it is unlikely that the animals were ever exposed to the pathogen. Juveniles were chosen for this species because only juveniles are thought to be susceptible to chytridiomycosis (Berger *et al.* 1998).

Captive environment

All animals were housed individually in a room temperature of 18-20°C. They were fed *ad libitum* three times weekly pinhead (5-10 mm) crickets (*Acheta domestica*) (dusted with amphibians vitamins and gut-loaded). Animals were misted once daily for 60 seconds with reverse osmosis water. Temperature and humidity were monitored daily. Animal enclosures were designed based on recommendations from husbandry experts and varied between species due on their natural habitats and ammonia production. For *P. corroboree* animals were housed in 300 x 195 x 205 mm terrarium with a damp and crumpled paper towel substrate (Earthcare, ABC Tissue, Wetherill Park, New South Wales, Australia). Terraria were cleaned fortnightly by replacing the paper towel substrate (Brannelly *et al.* 2015a). *Litoria v. alpina* were housed in 300 x 195 x 205 mm terrarium with gravel substrate, which was replaced every three months (Grogan 2015). *Rhinella marina* were housed in terraria 100 x 60 x 60 mm terraria with a paper towel substrate that was changed twice weekly: more often because of the smaller container and increased waste production

Inoculation

Animals were allowed to acclimate to their new environment for at least seven days, and all animals were tested for *Bd* infection prior to the start of the experiment (see methods below) and all were found to be negative for *Bd.* All animals were inoculated with a New South Wales strain of *Batrachochytrium dendrobatidis* (AbercrombieR-L.booroologensis-2009-LB1, Passage number 11). *Bd* was harvested from agar and tryptone, gelatin hydrolysate, lactose (TGhL) petri plates after incubation at 23°C for 5 days. Plates were flooded with 3mL of artificial spring water and allowed to sit for 10 minutes to allow zoospores to be released from zoosporangia. Inoculum was poured off the plates and zoospores were counted using a hemocytometer. Animals were inoculated with $1x10^6$ zoospores by applying 3mL of inoculum onto the venter. Animals were placed in individual 40mL containers for 6 hours, and then transferred back into their

terraria (*P. corroboree* n = 16, March 2013; *L. v. alpina* n = 27, February 2015; *R. marina* n = 19, February 2015). *Bd* negative control animals were mock-inoculated using uninfected petri plates (*P. corroboree* n = 9; *L. v. alpina* n = 9; *R. marina* n = 9).

Only five *L. v. alpina* successfully gained *Bd* infection from the inoculation, so a subset of animals were taken from a larger infection experiment (Brannelly et al. Chapter 3) in order to increase the sample size of this species at morbidity caused by chytridiomycosis. Animals were inoculated using a different *Bd* strain from the same region (WastePoint-L.v.alpina-2013-LB2, Passage number 1) and inoculated with $5x10^5$ zoospores in 10mL of inoculum and held in inoculation containers for 24hr (n = 5, February 2014). Infection rate was much higher (80%) in this inoculation, which may be due to the different inoculation protocol.

Data collection

Each week animals were swabbed for *Bd* presence (see below), weighed to the nearest 0.01g, and measured snout to venter (SVL) to the nearest 0.1mm as part of our standard data collection protocol. Throughout the experiment, animals were euthanized with an overdose of MS-222 when clinical signs of chytridiomycosis were displayed and righting reflex was abolished in accordance with animal ethics. The experiment ended after the last infected animal from each species succumbed to disease, or 12 weeks after inoculation (*P. corroboree*, day 94; *L. v. alpina*, day 91; *R. marina*, day 93). All control and exposed animals were euthanized at the end of the experiment.

***Testing for* Bd**

We tested for *Bd* infection by using qPCR on skin swabs (Boyle *et al.* 2004). The standard protocol involves 45 strokes with a sterile rayon-tipped swab (MW-113, Medical Wire & Equipment, Corsham, Wiltshire, United Kingdom) per animal, five on the middle of the venter, five on each side of the venter, five on each thigh, and five each limb. The swab was gently rotated during and between strokes to ensure the greatest amount of DNA was gathered on the swab. Genomic DNA was extracted from the swabs (PrepMan® Ultra, ThermoFisher Scientific, Waltham, Massachusetts, United States) and 2 minutes of bead beating to break apart the fungal cell walls. The extract was analysed using quantitative PCR following Boyle et al. (Boyle *et al.* 2004), in singlicate (Kriger, Hero and Ashton 2006; Brannelly *et al.* 2015b) with a positive and negative control, and

a series of dilution standards. After inoculation animals were tested once a week until succumbing to disease or the experiment concluded.

Histological procedure

Hematopoietic tissues chosen

Spleen, kidney and bone marrow was chosen for hematopoietic tissue analysis. The whole kidney and spleen were sectioned, and two bone marrow segments were chosen in order to determine the highest hematopoietic tissue density sites: the metatarsals in the foot, and the distal femur. The kidney, spleen and bone marrow in all three species had hematopoietic tissue present in a large proportion of the organ, in nearly all histosections. Because of the high proportion of hematopoietic tissue within each organ, randomisation of the histosections was possible for analysis. While liver and gut are important hematopoietic tissue sites in some amphibian species (Grayfer and Robert, 2016), these organs were not examined in this study. Australian species appear to have less hematopoietic tissue in liver and gut and we found only small unevenly distributed clusters (Brannelly unpublished data), which made randomisation of histosections difficult and counts inaccurate.

Histological preparation

Upon euthanasia, animals were dissected. Kidneys, spleen, and bone from the foot and knee were removed. These tissues were not weighed or measured directly after dissection because of concerns of inaccuracy due to their small size. After removal tissues were fixed in 4% phosphate buffered formaldehyde for 2 hrs, and then transferred to 80% ethanol prior to embedding in paraffin wax for standard histological preparation (Woods and Ellis 1994). Tissues with bone were decalcified in 10% formic acid for 24 hours, rinsed in water and transferred to 80% ethanol. Tissues were dehydrated in a graded series of ethanol, cleared with xylene, and embedded in paraffin. For tissue orientation, the kidneys and bones were embedded to enable cross-sections and longitudinal sections respectively. Because the spleens were small and circular, they were not orientated in a particular way. All tissues were serially sectioned in order at 5μm, affixed to glass slides and stained. For this study we primarily stained with hematoxylin followed by eosin counterstaining (H&E), and mounted with coverslips. However, for the

P. corroboree sections we instead used Masson's trichome stain (Jones, Bancroft and Gamble 2008) for the spleen sections and half of the kidney and bone sections for clearer differentiation between cells and connective tissue.

Histological measurements

Hematopoietic tissue was quantified differently in each organ. Different organs were not compared to each other, and each organ had different important characteristics that could be measured.

Bone marrow

Bone marrow cell density was assessed in longitudinal sections of the distal femur and talus, choosing the section with the largest bone width. Hematopoietic tissue was determined as nucleated non-adipose tissue cells within the bone cavity. In each field of view, magnification of 200x in the foot (field of view = 0.45 x 0.34 mm), and 100x in the femur (field of view = 0.66 x 0.88 mm) we measured density of nuclei (proportion of nuclei area covered compared to total area of hematopoietic tissue), proportion of hematopoietic tissue and proportion of adipose tissue within the bone marrow. Four sections were randomly chosen using a random number generator for each animal for each tissue, and one field of view was chosen per section and measurements were then averaged per animal.

Kidney

Following a similar method as the bone marrow, hematopoietic tissue cell density was assessed by percent area of the kidney, excluding the anterior and posterior ends. Four sections were randomly chosen to assess tissue coverage in a field of view under 100x magnification (0.66 x 0.88 mm). One field of view per section was chosen along the medial edge of the kidney where hematopoietic tissue is denser. White space within the hematopoietic tissue was assessed to estimate cell density. White space within renal parenchyma was analysed to assess fluid retention, a typical terminal change that could confound the hematopoietic tissue comparisons. Number of tubules was counted per field of view, in order to account for potential swelling upon morbidity in *Bd+* animals.

Spleen

Because the whole spleen is hematopoietic tissue, and white and red cells are distinguishable under traditional histological methods, white and red cells were counted in the spleens in two randomly selected sections per animal with one field of view per section in the middle of the section to ensure that at least 500 cells were counted per individual. Cells were counted at 400x magnification (field of view = 0.17 x 0.23 mm), where red, white, total counts were assessed, in addition to proportions of red to white, and white to total cells per field of view.

Health assessment

Health assessment is important for captive raised animals, as problems are common in amphibians despite quality husbandry. Bone density and structure is an important indicator of health, and was assessed after the completion of the experiment. For this study we investigated trabecular bone in the bone marrow histosections. Trabecular bone is a sign of metabolic bone disease (Shaw *et al.* 2012), and was present in all *L. v. alpina*. In *P. corroboree* there was no evidence of trabecular bone, so as a second measure of bone integrity, x-rays for bone fractures, were taken at 45Kv and 14mAS for a subset of individuals; 8 *Bd*- and 12 *Bd*+ animals. All x-rays appeared normal with no signs of fractures. *Rhinella marina* were wild caught, therefore captive raised health was not a primary concern, and no trabecular bone was observed.

Statistical analyses

For the histological measurements an average measurement of each organ per individual was calculated. All counts were done visually and measurements, both distance and percent coverage, were made in ImageJ software (Wayne Rasband, NIMH). We assessed total tissue coverage and density of hematopoietic tissue within the kidney and bone marrow, and cell counts in the spleen. We did not identify leucocyte populations because immunohistochemical stains for these cell types are not available in these frog species, and using nuclear morphology to differentiate mononuclear cells is not reliable in these samples because cells were in various stages of development (as per recommendation by veterinary pathologists). A small proportion (<20%) of various mature leucocyte cell types were seen in each tissue type, but the majority of cells were

unidentifiable immature leucocytes; therefore cell population proportions were not quantified

In *P. corroboree* and *L. v. alpina* comparisons between the *Bd*+ and *Bd*- animals were assessed using independent t-tests in SPSS (IBM, v21) and Cohen's d statistic to estimate effect size. While some *L. v. alpina* were not infected at the end of the experiment, it is unclear whether they never gained an infection, or cleared it early, and were therefore removed from the study. As many *R. marina* survived exposure, a comparison between *Bd*+ animals that died (*Bd*+), and *Bd* inoculated animals that cleared and survived (cleared) and *Bd*- control animals (*Bd*-) was made using ANOVAs in SPSS v21 and Bonferroni's post-hoc tests, and Cohen's d statistic. All animals classified at *Bd*+ displayed clinical signs of chytridiomycosis, which include irregular skin slough, change in posture, splayed legs, inappetance and loss of righting reflex. It is important to note that zoospore infection load is highly variable, and therefore the Log_{10}(ZE) +/- standard deviation are reported. A comparison of infection loads comparing species were not conducted because the experiments for each species took place at different times, and inoculation protocols varied. However, the endpoint of morbidity due to chytridiomycosis was the same for each species, and therefore comparable. Survival analysis was conducted using Kaplan-Meier comparing only the animals that became infected after exposure.

Animal ethics

Animal ethics was approved by James Cook University in applications A1875 for *P. corroboree*, and A1897 and A2171 for *L. v. alpina* and *R. marina*.

Results

Bd *infection*

In *P. corroboree*, all 16 inoculated animals became infected with *Bd*, and all succumbed to chytridiomycosis between 21 and 94 days post inoculation (average 52.08). At death the mean infection load was 4.66 Log_{10}(ZE) +/- 1.00. A total of 10 *L. v. alpina* became infected with *Bd* and succumbed between 39 and 70 days (average 57.4). At

death the average infection load was 4.27 Log_{10}(ZE) +/- 0.99. Of the 19 inoculated *R. marina*, eight became infected and succumbed to *Bd* between 65 and 90 days post inoculation (average of 83.37), and four had heavy infections but survived the 13 week experiment. The average *Bd* load at death was 4.35 Log_{10}(ZE) +/- 1.62. Seven *R. marina* successfully cleared infection, and the last animal cleared infection at 12 weeks post inoculation (Fig. 1). Survival differed between the species (Kaplan-Meier, Generalised Wilcoxon: $\chi^2_2 = 19.63$, $p < 0.01$) where *R. marina* had the highest survival and *P. corroborree* and *L. v. alpina* had similar survival once infected (Kaplan-Meier, Generalised Wilcoxon: $\chi^2_1 = 0.31$, $p = 0.58$).

Pseudophryne corroboree *histology*

Bone marrow

There was significantly greater cell density in the *Bd*- animals in both the foot and the femur. In the foot, the bone marrow was 33.86% less dense (proportion of the marrow surface covered by nuclei compared to the proportion covered by cytoplasm) in *Bd*+ animals than in *Bd*- animals (t-test: $t_{17} = 2.703$, $p = 0.015$; $d = 1.22$) (Fig. 2A). There was no difference in the proportion of hematopoietic tissue relative to bone cavity in the marrow between the two disease status groups (*Bd*+ and *Bd*-) (t-test: $t_{17} = 0.277$, $p = 0.785$) (Fig. 2B). In the distal femur, the bone marrow was 26.21% less dense (proportion of the marrow that was nuclei compared to the proportion that was cytoplasm) in *Bd*+ animals than in *Bd*- animals (t-test: $t_{17} = 2.322$, $p = 0.033$; $d = 1.08$) (Figs 2A, 3A,B). There was no difference in percent cover of hematopoietic tissue in the marrow between the two groups (t-test: $t_{17} = 1.17$, $p = 0.258$) (Fig. 2B).

Spleen

There was no difference in total number of cells per field of view between the two disease status groups, *Bd*+ and *Bd*- animals (t-test: $t_{23} = 1.791$, $p = 0.086$), and no difference in the number of white cells between the two groups (t-test: $t_{23} = 0.125$, $p = 0.902$). However, there were 66.3% fewer red cells in the *Bd*+ animals than in the *Bd*- animals (t-test, equal variances not assumed: $t_{9.024} = 5.739$, $p < 0.01$; $d = 1.87$), and lower proportion of red cells to white cells in the *Bd*+ animals than in the *Bd*- animals (t-test, equal variances not assumed: $t_{8.875} = 5.131$, $p < 0.01$; $d = 1.68$) (Figs 2C, 4A,B).

Kidney

There was 63.0% less hematopoietic tissue in the *Bd*+ animals than the *Bd*- animals (t-test: $t_{22} = 9.68$, $p < 0.01$; $d = 2.79$) (Fig. 2D). The density of cells within the hematopoietic tissue was 66.3% less than *Bd*+ animals (as a proportion of hematopoietic cell space to white space with the tissue (t-test, equal variances not assumed: $t_{10.64} = 4.40$, $p < 0.01$; $d = 1.40$) (Figs 2E, 5A,B). There was no difference in number of tubules per field of view between the *Bd*- and *Bd*+ animals (t-test, equal variances not assumed: $t_{9.978} = 1.991$, $p = 0.075$).

Litoria verreauxii alpina *histology*

Bone marrow

There was no difference in either the foot or the distal femur in hematopoietic tissue proportion (Foot, t-test: $t_{14} = 1.604$, $p = 0.131$; Femur, t-test: $t_{14} = 0.981$, $p = 0.343$) or cell density in the hematopoietic tissue (Foot, t-test: $t_{14} = 1.746$, $p = 0.103$; Femur, t-test: $t_{14} = 0.688$, $p = 0.503$) (Figs 2, 3E). We also noted trabecular bone near the proximal diaphysis of the distal femur in every animal.

Spleen

There was no difference in total number of cells per field of view between the *Bd*+ and *Bd*- animals (t-test: $t_{16} = 1.51$, $p = 0.151$), and no difference in the number of white cells between the two groups (t-test: $t_{16} = 0.89$, $p = 0.387$). However, there were 36.5% fewer red cells in the *Bd*+ animals than in the *Bd*- animals (t-test, equal variances not assumed: $t_{12.512} = 2.757$, $p = 0.017$; $d = 0.919$), and a significantly lower proportion of red cells to white cells in the *Bd*+ animals than in the *Bd*- animals (t-test: $t_{16} = 2.233$, $p = 0.04$; $d = 0.745$) (Figs 2C, 4C,D).

Kidney

There was no difference in percent area of hematopoietic tissue per field of view between the *Bd*+ and *Bd*- animals (t-test: $t_{16} = 0.188$, $p = 0.853$), the density of cells within the hematopoietic tissue (t-test: $t_{16} = 0.334$, $p = 0.743$), and number of tubules per field of view (t-test: $t_{16} = 0.289$, $p = 0.777$) (Fig. 2).

Rhinella marina *histology*

Bone marrow

There was significantly more hematopoietic tissue relative to cavity area in bone marrow of both the distal femur and the foot of the *Bd*+ animals than in both the *Bd*- animals (Bonferroni post hoc: Foot, 14.5% more, $p = 0.049$, $d = 0.764$; Femur, 17.89% more, $p = 0.01$, $d = 1.283$) and the *Bd*-cleared animals (Bonferroni post hoc: Foot, 20.41% more, $p < 0.01$, $d = 1.300$; Femur, 29.63% more, $p < 0.01$, $d = 1.436$) (ANOVA: Foot, $F_{2,23} = 5.477$, $p < 0.01$; Femur, $F_{2,23} = 11.913$, $p < 0.01$) (Figs 2B, 3C,D). There was no difference between the groups in density of nuclei within the hematopoietic tissue inside the bone marrow of either the foot or the distal femur (ANOVA: Foot, $F_{2,23} = 0.386$, $p = 0.684$; Femur, $F_{2,23} = 0.831$, $p = 0.448$) (Fig. 2A).

Spleen

There was no difference in total number of cells per field of view between the *Bd*+, *Bd*-, and *Bd*-cleared animals (ANOVA: $F_{2,19} = 3.061$, $p = 0.07$), and no difference in the number of white cells between the two groups (ANOVA: $F_{2,19} = 1.145$, $p = 0.339$). However, there were significantly fewer (41.76% fewer) red cells in the *Bd*+ animals than in the *Bd*- animals (ANOVA: $F_{2,19} = 3.763$, $p = 0.042$, $d = 0.926$; Bonferroni: $p = 0.39$) but there was no difference between the *Bd*+ and the *Bd*-cleared (Bonferroni: $p = 0.535$) or the *Bd*-cleared and the *Bd*- animals (Bonferroni: $p = 0.628$). There was no difference in proportion of red cells to white cells within the field of view in the spleen between the three groups of animals (ANOVA: $F_{2,19} = 1.184$, $p = 0.140$) (Figs 2C, 4E,F).

Kidney

There was no difference between the three disease status groups, *Bd*-, *Bd*+ and the *Bd*-cleared, in area of hematopoietic tissue per field of view (ANOVA: $F_{2,21} = 0.062$, $p = 0.940$), but the *Bd*+ hematopoietic tissue was significantly less dense than the *Bd*- and *Bd*-cleared groups (ANOVA: $F_{2,21} = 7.391$, $p < 0.01$) with 13.03% less cell density within the hematopoietic tissue than *Bd*- animals (Bonferroni: $p < 0.01$; $d = 1.116$) and 11.06% less cell density than the *Bd*-cleared animals (Bonferroni: $p = 0.029$; $d = 1.057$) (Figs 2 D,E, 5E,F). There was no difference between the groups in number of tubules per field of view (ANOVA: $F_{2,21} = 0.227$, $p = 0.227$).

Discussion

Activation of immune system by infections is often observed as stimulation within immune organs (Day and Schultz 2012) but in our study the only sign of increased immune activity was in the bone marrow of *R. marina*. In the highly susceptible *P. corroboree*, infection led to depletion of hematopoietic tissue in the kidney and bone marrow. Although hematopoietic tissue within bone marrow covered the same area, the lower cell density suggests hematopoietic depletion. Depletion can occur due to cell death, lack of production (Shibahara *et al.* 2000; McCune 2001) or alternatively, the response becomes is exhausted after chronic proliferation and cannot keep up with cell emigration (Hazenberg *et al.* 2000a, 2000b). However the lack of necrosis observed in bone marrow and lack of cellular inflammation at infected skin sites (even at moderate incubation times) suggests reduced production.

The importance of each hematopoietic organ in amphibians is largely unknown, and has not been assessed in different species in recent years (Jordan 1933; Kanesada 1956), but in some species of anuran, the bone marrow is a site of development of granulocytes (predominantly neutrophils), and important in the innate immune response (Kanesada 1956; Rollins-Smith *et al.* 2009). The area of hematopoietic tissue in kidney sections was reduced in infected frogs despite signs of edema in this group, which would have enlarged kidney tissue. The importance of the kidney in hematopoiesis has not been studied recently, but it can act as a site of erythrocyte and granulocyte production, and may represent a compensatory mechanism for depleted bone marrow (Kanesada 1956; Grace and Manning 1980; Press 1999). In *P. corroboree*, both the kidney and the bone marrow had evidence of depleted hematopoietic tissue, consistent with an active but exhausted innate immune response, or immunosuppression caused by *Bd*. There was no evidence of immune depletion in spleen: the number of white cells did not differ between infected and uninfected frogs, nor did the total number of cells, but number of red cells was lower in infected frogs. This result may be related to altered blood pressure and flow in terminal stages of disease. In clinically ill animals, osmotic pressure in blood was reduced due to loss of electrolytes, but packed cell volume remained the same (Voyles *et al.* 2007, 2009). The spleen is the major secondary lymphoid organ where antigens from the blood, peritoneum, and tissue fluids are degraded and come into contact with T and B

cells for development of an adaptive immune response (Press 1999; Du Pasquier *et al.* 2000; Rollins-Smith *et al.* 2009). We observed no change in white cells of the spleen suggesting that if there is an adaptive response to chytridiomycosis such a response is not observable in the spleen.

The highly susceptible *L. v. alpina,* a species that undergoes near complete population turnover each year due to chytridiomycosis (Brannelly *et al.* 2015b; Scheele *et al.* 2015), did not show any hematopoietic tissue depletion after infection with *Bd* in the spleen, bone marrow and kidney. While there was no evidence of depletion in the spleen, the number of red blood cells was significantly lower per field of view in the *Bd*+ animals, and a lower proportion of red cells to white within the spleen. Similarly to the *P. corroboree* results, this difference between the two groups may be due to low blood pressure or flow in animals clinically ill with chytridiomycosis. The hematopoietic tissue in the spleen, kidney and bone marrow were not depleted by *Bd* infection, nor was there activation by infection in this species. However, there was evidence of metabolic bone disease in all *L. v. alpina,* with prominent trabeculae near the proximal diaphysis (Shaw *et al.* 2012), especially in the distal femur. These animals were captive raised as eggs through maturity, and were 3 – 4 years old when the experiment was completed. While they were maintained under suitable conditions (vitamin dusted crickets, UV light exposure, and de-chlorinated and de-fluorinated water via reverse osmosis), unnatural diets for long periods can result in health problems, particularly metabolic bone disease (Shaw *et al.* 2012).

Rhinella marina has been suggested as a reservoir species for *Bd* persistence and spread in Australia (Daszak *et al.* 2003; Johnson and Speare 2005), however there are no reports of clinically infected adults in Australia, with only one field study ever testing for *Bd* presence (Lettoof *et al.* 2013). The one published account of clinical *Bd* infection and *R. marina* describes an outbreak where tadpoles were infected and juveniles died, but adults were unaffected and may be resistant to infection (Berger *et al.* 1998; Hyatt 1998). In this study we infected recent metamorphs in order to investigate a susceptible life stage from a resistant species, and they reached a much higher infection burden than the other two species before becoming ill. The spleen demonstrated few differences between the disease-status groups (*Bd*+, *Bd*- and *Bd*-cleared), but *Bd*+ animals had more red cells than *Bd*- or *Bd*-cleared animals, suggesting altered blood pressure or congestion at time of death. The kidneys demonstrated lower hematopoietic density in the *Bd*+ animals,

consistent with immunosuppression. But interestingly, the bone marrow hematopoietic tissue appeared stimulated: it covered a larger area, although cell density was the same as the *Bd*- and the *Bd*-cleared animals. Hematopoietic tissue of the kidney may compensate for depleted bone marrow in some amphibians (Kanesada 1956; Grace and Manning 1980; Press 1999), but in these *R. marina,* kidney hematopoietic tissue seemed to be less resilient. Although metamorphic *R. marina* appear to have an active immune response to *Bd* in bone marrow, most still developed high infection intensities and succumbed to chytridiomycosis; suggesting the hematopoietic activity did not result in ability for the animals to recover from high infection. Toads that cleared infection were similar to *Bd*- animals in all parameters measured. These animals were histologically examined at one time point (90 days post inoculation), which was at least a week since the last animal cleared infection. While a residual cost of successfully fighting *Bd* was not observed, in contrast to tree frogs that cleared infection (Young *et al.* 2014), we do not know what the effective immune response was in these individual species.

As we only examined frogs with late stage disease, it is unclear whether these changes are specific to chytridiomycosis or reflect general deterioration in health that may occur with other diseases. Our results show that future studies on this topic are warranted and should aim to determine the effect of the disease on hematopoietic tissue throughout the infection period.

Conclusion

This study represents a descriptive account of the changes that occur in *Bd* infected individuals in different hematopoietic tissues in different species. This study suggests that *Bd* negatively affects immune organs, but this effect was not consistent between species or tissues. Kidney and bone marrow (site of innate immune responses) (Kanesada 1956; Grace and Manning 1980; Press 1999; Du Pasquier *et al.* 2000; Rollins-Smith *et al.* 2009; Hofmann *et al.* 2010) were depleted in the susceptible *P. corroboree*. Bone marrow in *R. marina* had increased hematopoietic tissue suggesting that the innate immune response is the most active in combating *Bd*. We detected little change in the spleen, which is considered an important site of adaptive immune response development. The little change within the spleen may be consistent with results from a reinfection trial showing adaptive immunity has little efficacy against *Bd* (Cashins *et al.* 2013). Further

work should 1) identify which leucocyte cell types are activated or depleted within the tissues, and this requires development of specific tests for each species, 2) determine at what stage of infection these changes are occurring, and 3) include assessment of leucocyte changes in blood and skin. More detailed characterisation of these changes would indicate whether the depletion we observed is due to immunosuppression. While causal mechanisms of hematopoietic tissue depletion have not been investigated in this study, it presents the preliminary knowledge to direct future investigations. Knowledge on the mechanisms of hematopoietic depletion by *Bd* and why species differ may lead to targeted interventions, such as a vaccine or treatment or selection of resistance genes that overcomes depletion.

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Conflict of interest

The authors declare no conflicts of interest.

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Figure legend

***Figure 1*. Infection load and survivorship over the experiment.**

Litoria verreauxii alpina, (n = 10): *Pseudophryne corroboree*, (n = 16): *Rhinella marina*, (n = 19 total; cleared n = 7, infected n = 12). a) Infection load over time, weeks post inoculation. Zoospore equivalents were calculated as mean log base 10, and error bars indicate standard error. b) Survival over the course of the experiment (12 weeks post inoculation). Only infected *L. v. alpina* were included in this survivorship graph.

Figure 2. Comparison of hematopoietic tissue over the three tissues in the three species.

Pseudophryne corroboree (*Bd*-, n = 9 ; *Bd*+, n = 16), *Litoria verreauxii alpina* (*Bd*-, n = 9; *Bd*+, n = 10), and *Rhinella marina* (*Bd*-, n = 9; *Bd*+, n = 12, cleared, n = 7). Measurements were taken comparing the *Bd*-negative (*Bd*-) controls to the *Bd*-infected (*Bd*+) animals upon morbidity. In *R. marina*, a proportion of animals cleared infection (Cleared) and were compared separately. A) The density of hematopoietic cells within the bone marrow of both the foot and the femur. Density was measured as the nuclei coverage compared to the hematopoietic tissue coverage within the bone cavity (*P. corroboree*: t-test: Foot, $t_{17} = 2.703$, $p = 0.015$; $d = 1.22$; Femur: $t_{17} = 2.322$, $p = 0.033$; $d = 1.08$). B) The proportion of hematopoietic tissue (HT) coverage within the bone marrow cavity (*R. marina*: ANOVA: Foot, $F_{2,23} = 5.477$, $p < 0.01$; Femur, $F_{2,23} = 11.913$, $p < 0.01$). C) Cell counts of both the white cells and the red cells within the splenic tissue. (Red cells *P. corroboree*, t-test, equal variances not assumed: $t_{9.024} = 5.739$, $p < 0.01$; $d = 1.87$; *L. v. alpina*, t-test, equal variances not assumed: $t_{12.512} = 2.757$, $p = 0.017$; $d = 0.919$; *R. marina*, ANOVA: $F_{2,19} = 3.763$, $p = 0.042$, $d = 0.926$) D) The density of hematopoietic cells within the kidney. Density was measured as the nuclei coverage compared to the hematopoietic tissue coverage within the kidney (*P. corroboree*, t-test, equal variances not assumed: $t_{10.64} = 4.40$, $p < 0.01$; $d = 1.40$; *R. marina*, ANOVA: $F_{2,21} = 7.391$, $p < 0.01$). E) The proportion of HT coverage within the kidney tissue (*P. corroboree*, t-test: $t_{22} = 9.68$, $p < 0.01$; $d = 2.79$). (*) Indicates a statistical difference between the *Bd*+ and *Bd*- or Cleared group.

Figure 3. Histosections of bone marrow demonstrating reduced haemopoeitic tissue area.

Pseudophryne corroboree bone marrow in the foot. Trichrome staining at 200x magnification. A) *Bd*- and B) *Bd*+. The scale bar is 0.1mm. *Rhinella marina* bone marrow in the femur, H&E staining at 100x magnification. C) *Bd*- and D) *Bd*+. E) *Litoria verreauxii alpina* bone marrow in the femur, at 100x magnification. Notice the trabecular bone near the proximal diaphysis (arrow).

Figure 4. Histosections of spleen demonstrating a decrease of erthrocytes in infected animals .

Pseudophryne corroboree spleen, trichrome staining at 400x magnification. A) *Bd*- and B) *Bd*+. *Litoria verreauxii alpina* spleen, H&E staining C) *Bd*- and D) *Bd*+. *Rhinella marina* spleen, H&E staining, E) *Bd*- and F) *Bd*+. The majority of the cells are leucocytes, and the white arrow points to erythrocytes. The scale bar is 0.05mm.

Figure 5. Histosections of kidney demonstrating reduced hematopoetic tissue area.

Pseudophryne corroboree. A) *Bd*- and B) *Bd*+. *Rhinella marina* C) *Bd*- and D) *Bd*+. The scale bar is 0.1mm.

Figures

Figure 1

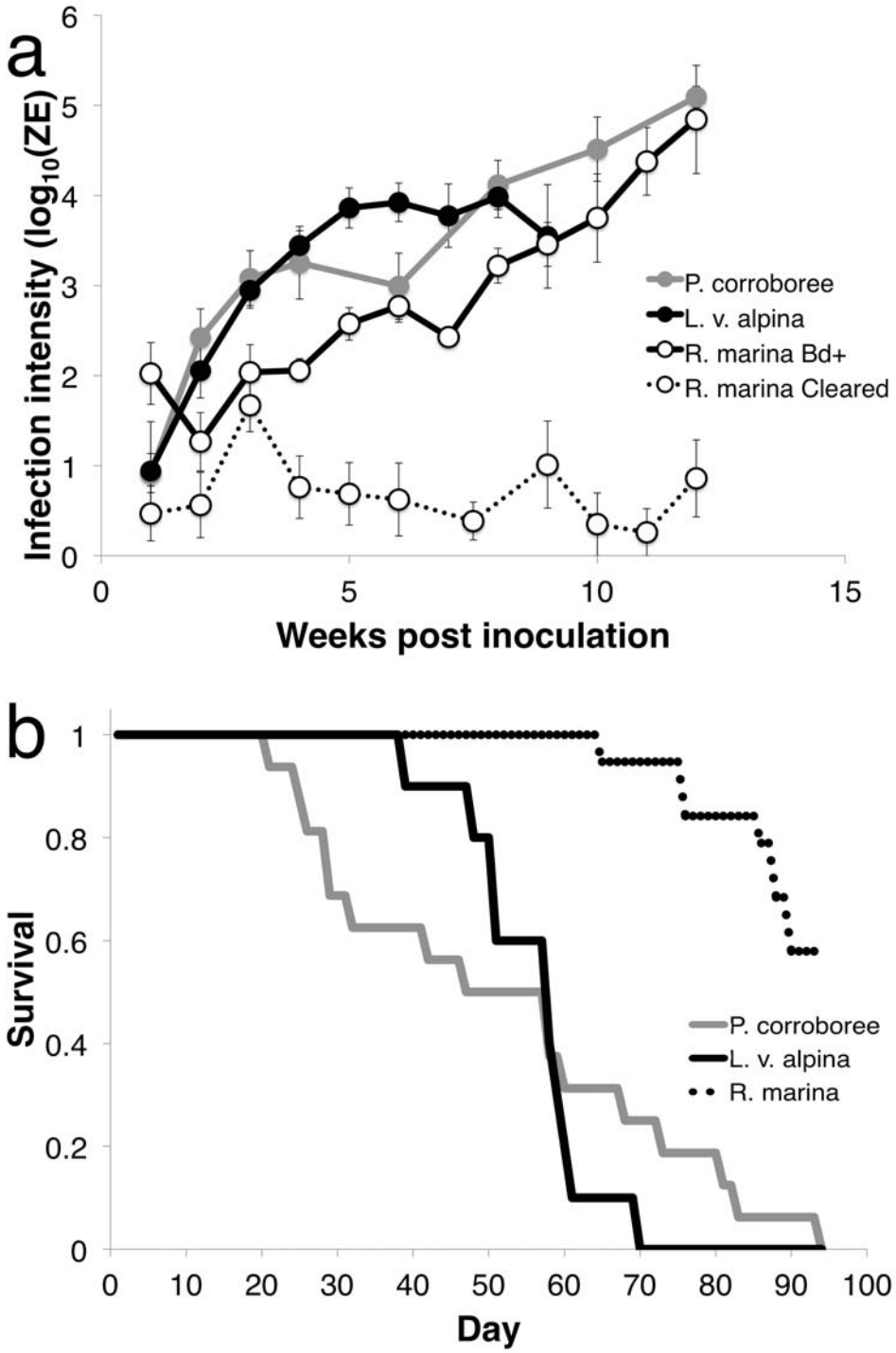


Figure 2

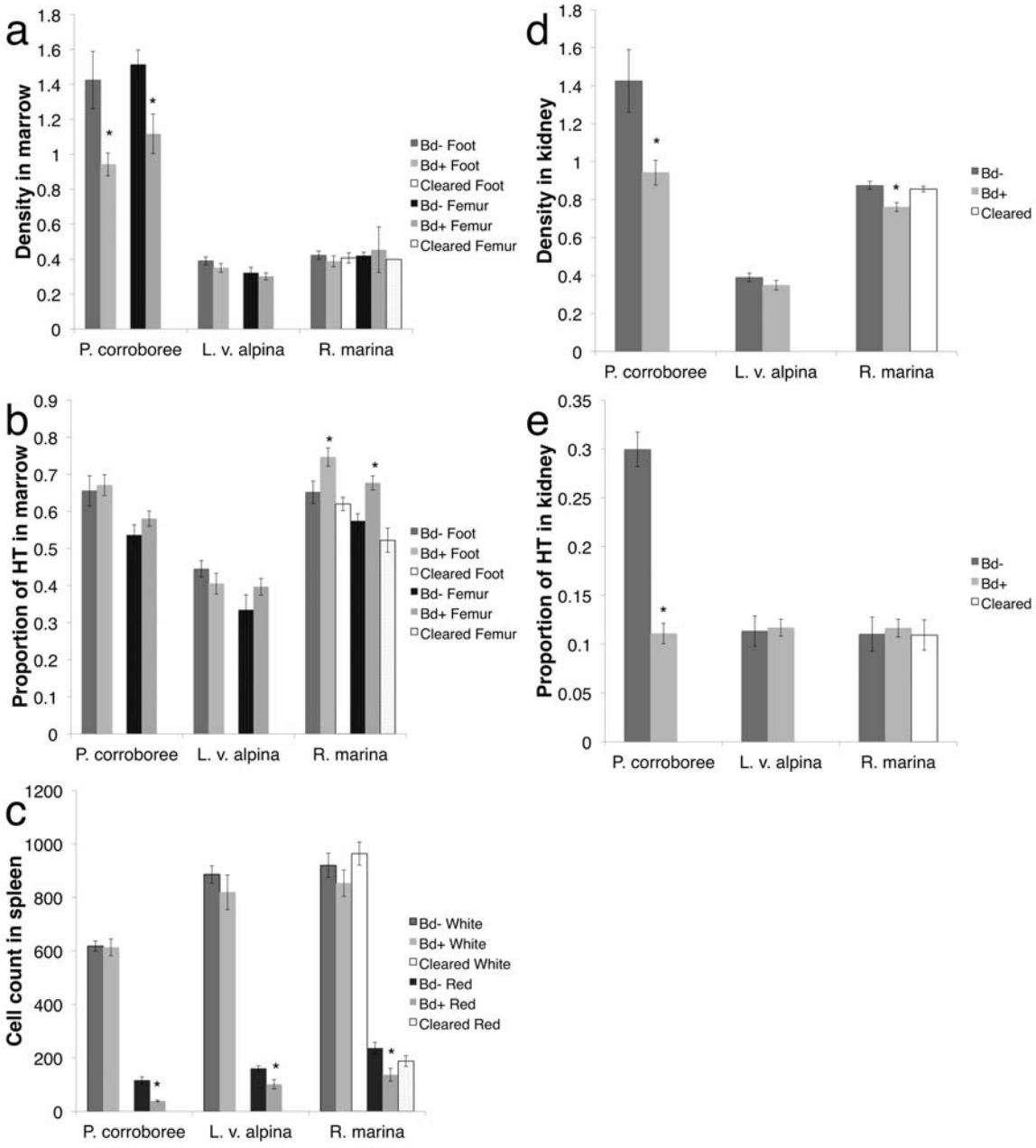

a
Density in marrow
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
P. corroboree
L. v. alpina
R. marina
Bd- Foot
Bd+ Foot
Cleared Foot
Bd- Femur
Bd+ Femur
Cleared Femur
d
Density in kidney
Bd-
Bd+
Cleared
b
Proportion of HT in marrow
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
e
Proportion of HT in kidney
0.35
0.3
0.25
0.2
0.15
0.1
0.05
c
Cell count in spleen
1200
1000
800
600
400
200
Bd- White
Bd+ White
Cleared White
Bd- Red
Bd+ Red
Cleared Red

Figure 3

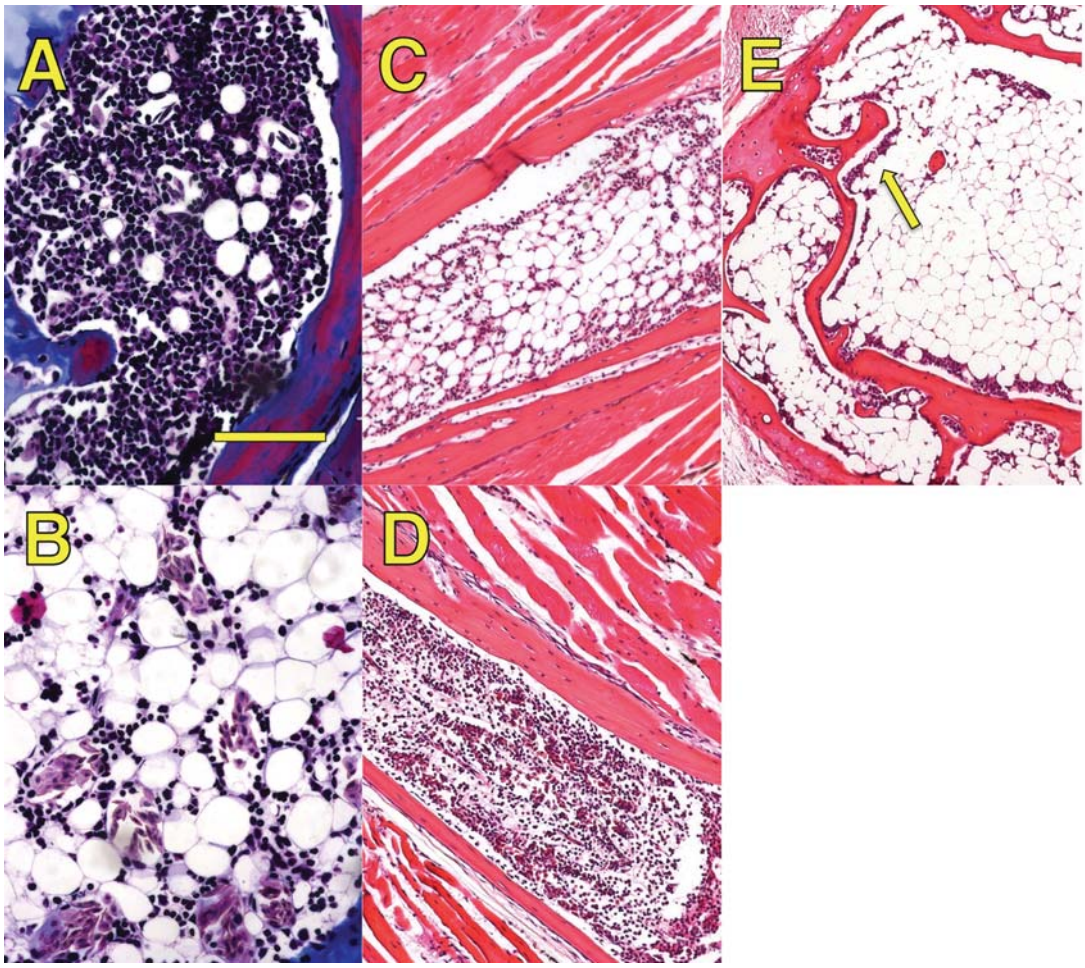


Figure 4

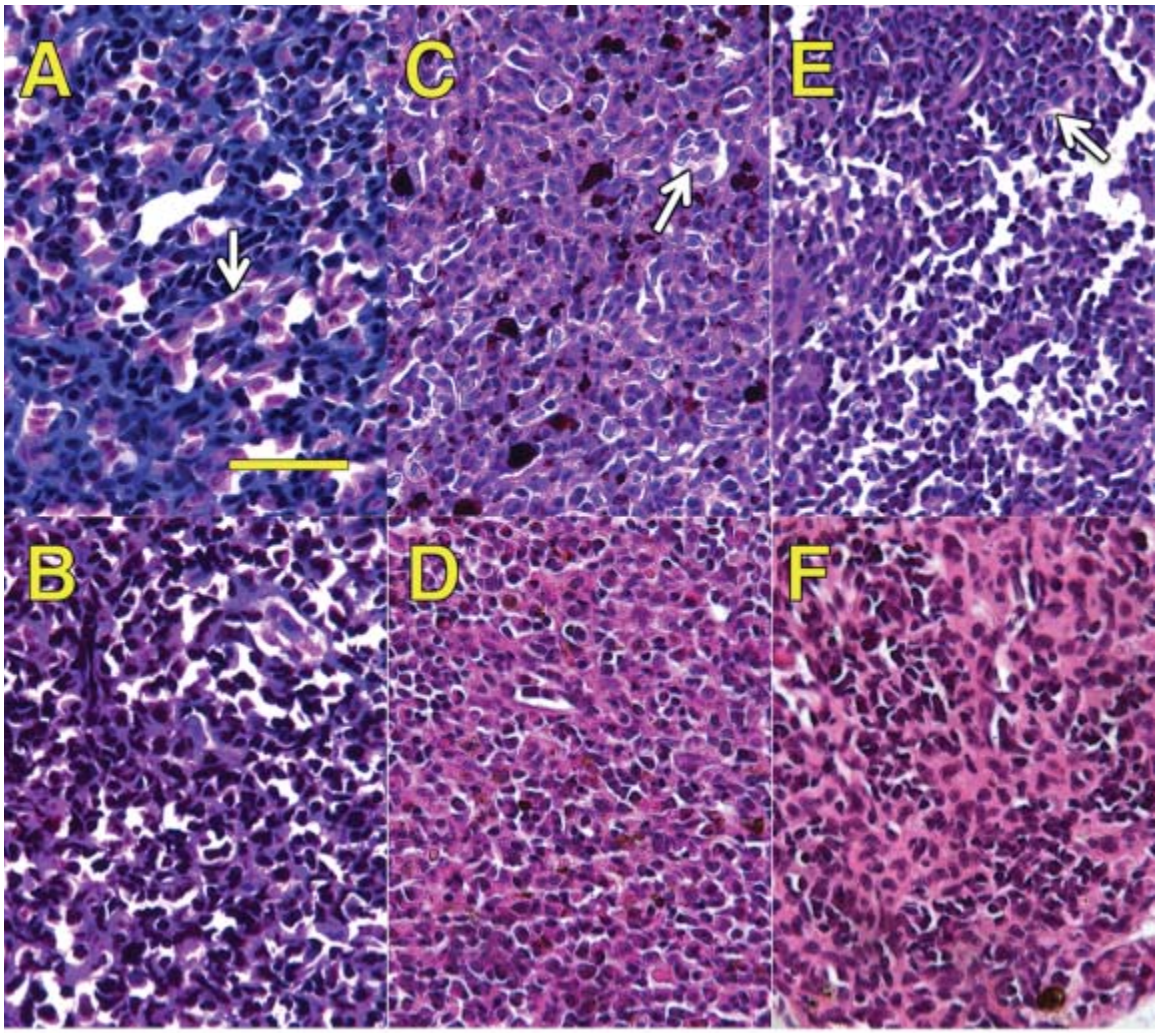
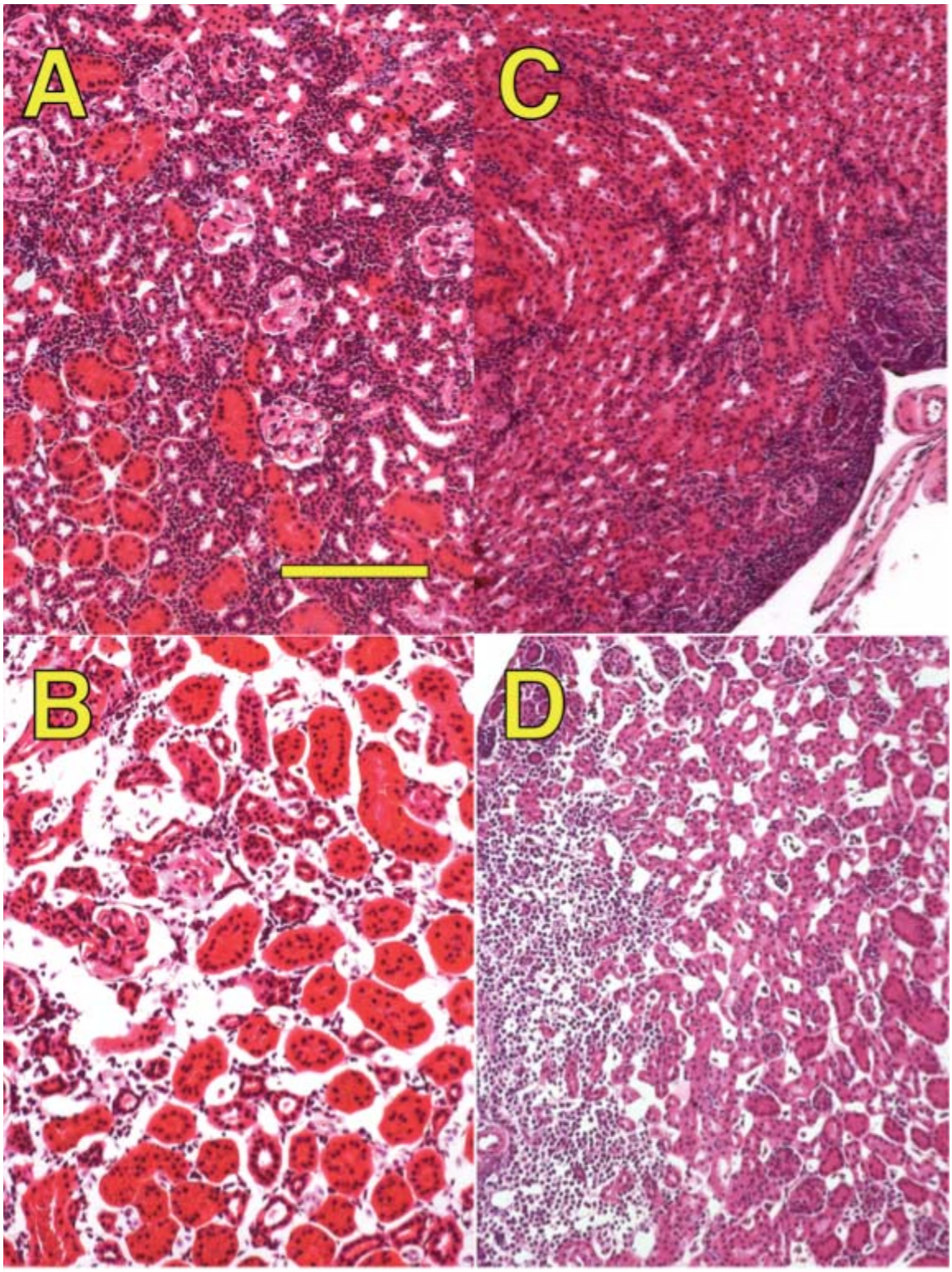


Figure 5



CHAPTER 5

Pathogenesis of disease: epidermal apoptosis

Introduction.

Chapter 5 continues to explore aim 3 of this thesis: to investigate underexplored mechanisms of pathogenesis of disease in susceptible species. In this chapter I explored epidermal apoptosis as a pathology of disease, and investigated the difference in apoptosis over the course of infection as well as between species: *P. corroboree* and *L. v. alpina*. This chapter consists of a manuscript entitled “Epidermal apoptosis in frogs with chytridiomycosis,” which has been submitted to Microbes and Infection and is under review at submission of this thesis.

Paper 1

Citation: Brannelly LA, Roberts AA, Skerratt LF, Berger L. Epidermal apoptosis in frogs with chytridiomycosis. In Review

Epidermal apoptosis in frogs with chytridiomycosis

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Abstract

Amphibians are declining globally, and one of the major causes of decline is the infectious disease chytridiomycosis. Fungal sporangia occur within epidermal cells causing epidermal disruption, but these changes have not been characterised. Apoptosis can be a damaging response to the host but may alternatively be a mechanism of removal for some intracellular infections. In this study we experimentally infected animals and observed apoptosis in the epidermis by TUNEL assays on histological sections and caspase 3/7 protein assays. Apoptosis was positively associated with infection load of clinically infected animals. Apoptotic cells were concentrated in epidermal layers, correlating to the localisation of infection within the skin. Interestingly, caspase activity was supressed in early infection, where pathogen loads were low. But, in amphibians that ultimately succumbed to disease, caspase activity increased dramatically when infection loads peaked. Our results suggest that increased apoptosis is a pathology of the fungal pathogen, likely contributing to loss of skin homeostatic functions, but also that apoptosis suppression may be used initially by the pathogen to help establish infection. The difference between host morbidity and pathogen clearance may therefore lie in the ability of the host to regulate apoptosis. Further research should explore the specific mechanisms of apoptosis regulation during fungal infection, and investigate antifungal chemotherapies that modulate apoptosis.

Key Words

Apoptosis, Caspases, Chytridiomycosis, TUNEL, Wildlife Disease

Introduction

Amphibians globally are experiencing the greatest loss in biodiversity of all vertebrate taxa [1]. One of the major causes of decline is disease, specifically a fungal pathogen, *Batrachochytrium dendrobatidis, Bd* [2], which causes the fatal skin disease chytridiomycosis. So far, no solutions have been developed and proven, and research devoted to improving survival rates within these declining populations will be key to conservation management.

Although chytridiomycosis is a superficial epidermal infection, disruption to skin function causes severe loss of electrolytes leading to cardiac failure. It is unknown if the damage to epidermal ion transport is primarily through physical damage to layers or via a fungal toxin. Global research into the host-pathogen dynamics has shown that resistance varies among species, populations and individuals. After demonstrating that vaccination is unlikely to be effective [3], the focus has been on identifying key immune factors which may then be selected for in breeding programs. While various potential immune mechanisms – such as antimicrobial peptides [4,5], cutaneous bacterial flora [6], MHC [7,8] and lymphocyte activity [9] – are currently being studied, few studies have explored apoptosis in the skin as an immune mechanism.

Apoptosis is a controlled process resulting in programmed non-inflammatory cell death via the action of caspase proteases and phagocytosis [10]. It is vital to tissue growth and differentiation and is associated with elimination of damaged cells and infectious agents, particularly intracellular pathogens [11–15]. Hosts utilise apoptosis of infected cells to block replication of pathogens, resulting in clearance of the infection. However, some pathogens can evade the immune system by hijacking the host's apoptotic machinery: they produce apoptosis inhibitors in order to aid in replication and survival, while others can induce apoptosis to facilitate dissemination or destroy host immune cells [15–19]. Therefore, the ability of the host and pathogen to control apoptosis can greatly influence disease severity and clinical outcomes.

Apoptosis may be a pathology of *Bd* infection in amphibians, as characteristic epidermal degenerative changes have been observed in *Bd*-infected frogs by electron microscopy [20,21]. Dissociation of epidermal intracellular junctions triggering detachment induced apoptosis was observed when skin explants were treated with zoospore supernatants in vitro [22]. Transcriptomic studies have shown apoptosis is

upregulated in skin of resistant and susceptible frog species [23]. Amphibian splenocytes also undergo apoptosis when treated with *Bd* sporangial supernatants in in vitro assays, associated with increased intrinsic, extrinsic and effector caspase activity in these immune cells [24]. Despite the evidence to suggest that *Bd* can induce apoptosis of specific cells in vitro, there are no studies that use direct and quantifiable assays, or that explore apoptosis mechanisms during progress of an infection in vivo. Furthermore, it is not known whether the host can also effectively use apoptosis as an immune defence against chytridiomycosis.

The aim of this study was to quantify rates of apoptosis in amphibian skin during an experimental infection. To ensure accurate measurement of the apoptotic effect, two methods of detection were utilised [25]: caspase 3/7 protein assay, and terminal transferase-mediated dUTP nick end-labeling (TUNEL) *in situ* assay. The caspase 3/7 assay quantifies the activity of effector caspases activated by both the intrinsic and extrinsic apoptosis pathways, while the TUNEL assay detects DNA fragmentation characteristically caused by apoptosis. Apoptosis rates were correlated with infection intensity and survival, to determine if apoptosis is a mechanism of disease resistance by the host or whether *Bd* infection is associated with a pathogen-induced modulation of apoptosis.

Materials and methods

Study organism

Litoria verreauxii alpina that were excess to a reintroduction trial were obtained from Taronga Zoo, Sydney. *Litoria v. alpina* are a declining anuran endemic to the Australian Alps in New South Wales and Victoria, Australia. The species is highly susceptible to *Bd*, which is the primary cause of decline [8,26,27]. The animals were captive raised and ranged from two to three years old under strict quarantine protocol and had never been exposed to *Bd*. Animals were housed individually in 300 x 195 x 205 mm terrarium with gravel substrate, at a room temperature of 18-20°C. They were fed *ad libitum* three times weekly with juvenile (10 mm) crickets (*Acheta domestica*) (dusted with amphibian vitamins and gut-loaded). Animals were misted twice daily for 60 seconds with reverse osmosis water. Temperature and humidity were monitored daily.

Pseudophryne corroboree were excess to a breeding program at the Amphibian Research Centre, Pearcedale, Victoria, Australia. These animals were 5 – 8 years old and part of a larger research experiment. *Pseudophryne corroboree* are functionally extinct in the wild, highly susceptible to *Bd* [28,29]. These animals were housed in the same conditions as above but on a paper towel substrate that was changed once a fortnight.

Inoculation

Animals were allowed to acclimate to their new environment for at least seven days. All animals were tested for *Bd* infection prior to the start of the experiment (see methods below) and all were found to be negative for *Bd. Bd* was harvested from tryptone, gelatin hydrolysate, lactose (TGhL) agar plates after incubation at 23°C for 5 days. Plates were flooded with 3 mL of artificial spring water for 10 minutes to allow zoospores to be released from zoosporangia. Inoculum was poured off the plates and zoospores were counted using a hemocytometer.

TUNEL assay

Litoria v. alpina used in the TUNEL assay (n = 6), were inoculated with a New South Wales strain of *Bd* (WastePoint-L.v.alpina-2013-LB2, Passage number 1). Animals were inoculated with $5x10^5$ zoospores in 10 mL of inoculum and held in inoculation containers for 24 h.

Pseudophryne corroboree (n = 12) were inoculated with a New South Wales strain of *Bd* (AbercrombieR-L.booroologensis-2009-LB1, Passage number 11). Animals were inoculated with $1x10^6$ zoospores by applying 3 mL of inoculum onto the venter. Animals were placed in individual 40 mL containers for 6 h, and then transferred back into their terraria.*Bd* negative control animals were mock-inoculated using uninfected petri plates (*P. corroboree* n = 6; *L. v. alpina* n = 7). These animals, both *L. v. alpina* and *P. corroboree,* were part of a larger experiment, described in Chapter 6 of this thesis.

Caspase assay

Litoria v. alpina used in the caspase 3/7 assay (n = 27) were inoculated following the same protocol as for *P. corroboree* above. *Bd* negative control animals were mock-inoculated using uninfected petri plates (n = 8). These animals were the same as described in Chapter 3&4 of this thesis.

Data collection

Each week animals were swabbed for *Bd* presence (see below), weighed to the nearest 0.01 g, and measured snout to venter (SVL) to the nearest 0.1 mm. Animals were euthanized with an overdose of MS-222 when clinical signs of chytridiomycosis were displayed and righting reflex was abolished in accordance with animal ethics. *Litoria v. alpina* succumbed to disease day 52-70; *P. corroboree* succumbed to disease day 45-83. Three *P. corroboree* were euthanized on day 21 post inoculation to explore TUNEL assay on animals with a light infection (Early *Bd*+). The experiment ended after the last infected animal succumbed to disease. All control and exposed animals were euthanized on day 90, at the end of the experiment.

For animals in the caspase 3/7 assay, toe clips were removed from each animal at the second phalange and immediately frozen at -80°C. Toe clips were removed weekly until week 3, and then fortnightly until the end of the experiment.

***Testing for* Bd**

We tested for *Bd* infection by using qPCR on skin swabs [30]. The standard protocol involves 45 strokes per animal with a sterile rayon-tipped swab (MW-113, Medical Wire & Equipment), five on the middle of the venter, five on each side of the venter, five on each thigh, and five each limb. The swab was gently rotated during and between strokes to ensure the greatest amount of DNA was gathered on the swab. Genomic DNA was extracted from the swabs using the Prepman Ultra kit and 2 minutes of bead beating to break apart the fungal cell walls. The extract was analysed using quantitative PCR following Boyle et al. (2004), in singlicate [27,31] with a positive and negative control, and a series of dilution standards. After inoculation animals were tested once a week until succumbing to disease.

TUNEL assay

Upon euthanasia, a subset of *L. v. alpina* animals (n = 2 control and n = 2 exposed), and *P. corroboree* (n = 5 *Bd*- control, n = 3 Early *Bd*+ animals with light *Bd* infection, and n = 10 Late *Bd*+ animals at morbidity) were dissected for skin samples (dorsum, venter and thigh). Skin was fixed in 4% phosphate buffered formaldehyde for 2

h, and tissues were transferred to 80% ethanol prior to embedding in paraffin wax for histological preparation. Routine histological techniques were used to prepare the tissues for light microscopy following standard methods. Tissues were dehydrated in a graded series of ethanol, cleared with xylene, and embedded in paraffin. Tissues were serially sectioned at 5 µm, affixed to hydrophilic glass slides. One slide per animal was stained with hematoxylin followed by eosin counterstaining (H&E), and mounted with coverslips. Two slides per animal were processed with TUNEL assay following manufacturer's instructions (ApopTag® Red In Situ Apoptosis Detection Kit, Merck Millipore), followed with a DAPI counterstain. Cells were counted using fluorescent microscopy at 200x magnification under DAPI fluorescence, then the same section counted for TUNEL positive staining, indicating apoptotic cells. At least 100 cells per animal were counted per skin section. Skin sections analysed with TUNEL fluorescence were paired with H&E stained sections to determine whether the site of infection correlated with sections used in the TUNEL assay.

Caspase 3/7 assay

Frozen toe clip samples were extracted in 100 µL Buffer A (25 mM HEPES pH 7, 5 mM $MgCl_2$) buffer with two 3.2 mm stainless steel beads in a 1.5 mL microtube. Samples were lysed by four cycles of 1 min bead beating and 3 min ice. Samples were then centrifuged at 4°C for 5 min at 12,000 x *g*. Supernatant was collected and used in the assay. As toe samples were very small, reaction volumes were kept to a minimum. Protein concentration of each toe was determined to standardise the sample sizes for the caspase assay. Concentration was quantified using the Bradford assay, with a reaction volume of 10 µL Coomassie Bradford reagent (Pierce) and 10 µL of protein extract at room temperature. The samples and BSA standards were run in duplicate or triplicate in a 384 well plate, and the absorbance was measured after 2 minutes at 595 nm (POLARstar Omega, BMG Labtech). Caspase 3/7 assay (Caspase Glo® 3/7, Promega) was performed in triplicate in a 384 well plate with a reaction volume of 10 µL Caspase Glo reagent and 10 µL protein extract. After mixing, the reactions were incubated in the dark at room temperature for 30 min, after which luminescence was measured (POLARstar Omega, BMG Labtech). All *Bd*+ samples of animals that succumbed to chytridiomycosis (n = 4), all *Bd*- control samples (n = 8), and a randomised subset of animals that cleared infection (n = 11) were analysed.

Statistical analysis

Infection loads of animals were log base 10 transformed. Individuals that succumbed to infection were compared to animals that cleared infection by week 12 post inoculation in infection intensity using Mixed Models; where individual was repeated, and fixed effects were week, infection status and week*infection status. Overall infection difference between the *Bd*-cleared, and *Bd*+ groups were determined by averaging all infection data and performing a student's t-test, followed by determining the effect size using Cohen's *d* statistic [32] in Microsoft Excel. These analyses only included animals from the caspase 3/7 assay trial.

Apoptotic cells from the TUNEL assay were used to compare rates of apoptosis in infected animals compared to *Bd*-control animals using Pearson's Chi-Squared test for association; where the number apoptotic cells per group were compared to non-apoptotic cells for each tissue type (dorsal, ventral and thigh skin). Following the association test, the strength of association was determined by odds ratio analysis performed in Microsoft Excel [32].

Caspase 3/7 activity was calculated as caspase activity over protein concentration per sample, and then log base 10 transformed. Weeks 1, 2, 3, 5 and 7 were analysed for *Bd*-, *Bd*+ and *Bd*-cleared animals. Caspase activity was assessed using Mixed Models; where individual was repeated, and week, infection status and week*infection status were fixed effects. To determine in which weeks caspase activity varied between the groups, one-way ANOVAs were performed using Bonferroni's post hoc test. To determine the change in caspase activity each week, Mixed Models were used with the same parameters as above. To determine which weeks change in caspase activity varied between groups, one-way ANOVAs were performed using Bonferroni's post hoc test. The association between caspase activity and infection intensity was performed using a pearson's correlation and logistic regression in *Bd* inoculated animals only. In order to determine if week or status has an effect on the linear regression a general linear model was performed; where $\log_{10}$(Caspase) was compared with status, week and $\log_{10}$(ZE). All analyses were performed using SPSS (v21) unless otherwise stated.

Animal ethics

Animal ethics was approved by James Cook University in applications A1897 and A2171 for *L. v. alpina* and A1875 for *P. corroboree.*

Results

Bd *infection*

Of the 27 *L. v. alpina* inoculated in the caspase infection experiment, all became infected, but only four succumbed to chytridiomycosis. The first animal died on day 58 after inoculation and the last on day 71. All animals that did not succumb to chytridiomycosis cleared infection after week 12 post-inoculation. The factors that influenced infection load were week, disease status and week*status (Mixed Model: week, $F_{11} = 5.425$, $p < 0.01$; status, $F_1 = 23.763$, $p < 0.01$; week*status, $F_9 = 3.071$, $p < 0.01$) (Fig.1). Animals that succumbed to chytridiomycosis had, on average 74.3% higher infection load (*Bd*+ = 3.09 +/- 1.22 Log_{10}(ZE); Cleared = 0.80 +/- 1.28 Log_{10}(ZE)) than animals that cleared infection (t-test: $t_{190} = 9.659$, $p < 0.01$, $d = 1.30$).

For the TUNEL assay samples, average infection load of *Bd*+ *L. v. alpina* at date of death was 3.57 Log_{10}(ZE) +/- 0.75. The average infection load of *Bd*+ animals that succumbed to disease (Late *Bd*+) at date of death was 1.2 times higher than the average infection load of *P. corroboree* with light infection loads (Early *Bd*+) (*Bd*+ = 5.19 Log_{10}(ZE) +/- 0.34; *Bd*- = 2.29 Log_{10}(ZE) +/- 0.89; d = 3.04).

TUNEL assay

There was significantly more apoptosis in infected animals compared to uninfected animals in both *L. v. alpina* and *P. corroboree*. The location of apoptotic cells *in situ* differed in *Bd*+ and *Bd*- animals of both species. In *Bd*- animals, background apoptosis was evenly distributed throughout the epidermal and dermal layers of the skin (Fig. 2A), but in the *Bd*+ animals, the apoptotic cells were concentrated in the layers of the epidermis (Fig. 2B). There was *Bd* infection throughout the ventral and thigh skin, but apoptosis in the epidermis was more widespread than just the localised sites of infection (Fig. 2C). While all *Bd*+ *L. v. alpina* and Late *Bd*+ *P. corroboree* animals had histologically visible *Bd* infection in the thigh and venter skin; there was no histologically visible *Bd* infection in the dorsum of these animals, or in the *P. corroboree* with light infections (Early *Bd*+).

In *L. v. alpina*, the venter skin of infected animals had 2.60 (95% CI: 1.13 – 6.01) times more apoptotic cells than uninfected animals (Odds Ratio: Z = 2.24, p = 0.51; Pearson's Chi Squared: $\chi^2_1 = 5.38$, p = 0.02), and the thigh skin of infected animals had 2.82 (95% CI: 1.40 – 5.66) times more apoptotic cells than uninfected animals (Odds ratio: Z = 2.91, p <0.01; Pearson's Chi Squared: $\chi^2_1 = 9.198$, p <0.01) (Fig. 3A). However, there was no observable increase in apoptotic cells in the dorsal skin of infected animals (Pearson's Chi-Squared: $\chi^2_1 = 1.694$, p = 0.19) (Fig. 2).

In *P. corroboree*, all three skin types showed an increase in apoptotic cells when infected with *Bd* in both early infection and late infection (Fig. 3B). In the thigh skin, Late *Bd*+ animals had 12.01 (95% CI: 4.92 – 26.30) times more apoptotic cells than *Bd*- animals (Odds Ratio: Z = 5.46, p < 0.01; Pearson's Chi Squared: $\chi^2_1 = 44.30$, p < 0.01). In the venter skin, Late *Bd*+ animals had 22.31 (95% CI 5.25 – 94.82) times more apoptotic cells than *Bd*- animals (Odds Ratio: Z = 4.21, p < 0.01) and 2.16 (95% CI 1.15 – 4.03) times more apoptotic cells than Early *Bd*+ animals (Odds Ratio: Z = 4.21, p < 0.01). The Early *Bd*+ animals had 10.33 (95% CI 2.37 – 45.067) times more apoptotic cells than *Bd*- animals (Odds Ratio: Z = 3.11, p < 0.01; Pearson's Chi Squared: $\chi^2_2 = 33.45$, p < 0.01). In the dorsal skin, the Late *Bd*+ animals had 14.38 (95% CI 3.32 – 62.24) times more apoptotic cells than *Bd*- animals (Odds Ratio: Z = 3.57, p < 0.01) and Early *Bd*+ animals had 19.88 (95% CI 4.67 – 84.20) times more apoptotic cells than *Bd*- animals (Odds Ratio: Z = 4.05, p < 0.01; Pearson's Chi Squared: $\chi^2_2 = 29.45$, p < 0.01) but there was no difference observed in apoptotic cells of the dorsum in Early and Late *Bd*+ animals (Fig. 3B).

Caspase 3/7 assay

Caspase activity was positively correlated with infection load in inoculated animals (Pearson's Correlation: $R_{64} = 0.463$, p<0.01; Linear Regression: $F_{1,62} = 16.943$, p<0.01) (Fig.3). However, there was no difference between the animals that cleared infection and those that succumbed to chytridiomycosis (GLM: $F_1 = 0.079$, p = 0.685), or between weeks (GLM: $F_4 = 0.226$, p = 0.717).

There was a difference in total caspase activity over time between the three groups (*Bd*-, *Bd*+ and *Bd*-cleared). The three groups differedy over week and week*disease status (Mixed Model: week, $F_4 = 11.974$, p<0.01; week*status, $F_8 = 2.139$, p = 0.037). There was no effect of week on the *Bd*- control group (Mixed model: $F_4 =$

2.463, p = 0.069) (Fig. 5A). At week 3, there was 48.36% less $\log_{10}$ caspase activity in the *Bd*+ compared to the *Bd*- animals, and 41.63% less activity in the cleared animals compared to the *Bd*- animals (ANOVA: $F_{2,18} = 5.512$, p = 0.014; Bonferroni Post-Hoc: *Bd*- v *Bd*+, p = 0.046, d = 1.408; *Bd*- v Cleared, p = 0.028, d = 0.923).

When investigating the change in caspase activity each week over the first seven weeks post inoculation, there was a difference between the three groups, with week and week*disease status as important factors (Mixed Model: week, $F_3 = 5.764$, p<0.01; week*status, $F_6 = 3.044$, p = 0.01), but there was no effect of week on the *Bd*- control group (Mixed model: $F_3 = 20.004$, p = 0.371) (Fig. 5B). The change in caspase activity between weeks 3 and 5 differed significantly between the three groups, with the *Bd*+ animals increasing in caspase 3/7 activity 15.35 times the change in *Bd*- animals, and 2.162 times the change in cleared animals (ANOVA: $F_{2,25} = 10.65$, p<0.01; Bonferroni Post-Hoc: *Bd*- v *Bd*+, p<0.01, d = 2.519; *Bd*+ v Cleared, p<0.01, d = 1.241).

Discussion

We found an increase in apoptotic cells in the epidermis of infected *P. corroboree* and *L. v. alpina* upon morbidity, as well as in early infection, using *in situ* TUNEL assay, confirming the apoptosis suggested by microscopy [20–22]. Apoptotic cells were located near the site of infection, and occurred on the ventral surface of the animal (thigh and venter skin); but not on the dorsum in *L. v. alpina*. However, *Pseudophryne corroboree* demonstrated increased apoptosis as infection progressed on all skin tissues. The location of apoptotic cells within the epidermal layers is consistent with infection of *Bd* being the cause of epidermal apoptosis. Through this evidence, it is clear that *Bd* infection leads to increased apoptosis in the epidermis.

To determine how rates of apoptosis change over infection, caspase 3/7 (effector) activity was quantified through time in *L. v. alpina*. Early in infection (weeks 1-3), animals that were inoculated with *Bd* initially demonstrated suppression of apoptosis (Fig.4A). There was then a rapid and sustained increase of caspase activity over weeks 3-5 in animals that succumb to chytridiomycosis (Fig.4B), which correlates with high pathogen burden (Fig.1). This rapid increase is not observed for animals that clear infection, suggesting that rapidly increasing apoptosis is a mechanism of pathogenesis by *Bd*.

These delayed apoptotic response suggest that *Bd* may initially suppress apoptosis in order to establish infection. If the host is able to overcome infection, the apoptosis suppression is overcome, and caspase 3/7 levels rise.. At high infection loads, however, the apoptosis-inducing effect of *Bd* may result in cellular damage and morbidity for the host. It is apparent that there is a critical mid-infection stage (approximately week 3 in this experiment), where either apoptosis is not stimulated or *Bd* controls apoptosis in susceptible individuals, while resistant hosts are able to slowly regain control of apoptosis. The host and pathogen mechanisms for this phenomenon are still unclear. The pathogen *Shigella flexneri*, uses a dual cell death control strategy by producing cytoprotective factors early in infection to aid in replication, followed by necrotic cell death signals later in the infection to enable transmission and host tissue damage [33].

The hypothesised dual strategy of apoptosis suppression and induction in *Bd* could be partially explained by its complex life cycle. Amphibians are infected by unicellular, motile zoospores that invade the epidermal cells, and which later develop into mature sporangia to complete the life cycle [34,35]. In in vitro studies, only sporangial, and not zoospore, supernatants suppressed amphibian lymphocytes [24]. In addition, there may be cell-specific apoptotic effects by *Bd*, such as apoptotic effects towards lymphocytes, but suppressive effects towards epidermal cells. A similar cell-specific effect is seen in other pathogens, for example, *Salmonella enterica* induces cell death in macrophages [36], but suppresses apoptosis in epithelial cells [37]. While the apoptotic effect on lymphocytes is known [9], further experiments are required to confirm whether *Bd* can exert a cell-specific suppressive effect on epithelial cells.

Interestingly, while there is evidence of more apoptotic cells in infected animals at morbidity through the TUNEL endpoint assay, we observed no difference in caspase 3/7 activities between *Bd*+ and *Bd*- animals at week 7. This discrepancy may be explained by the two different measures of apoptosis, which may suggest that other mechanisms in addition to the regulation of caspase 3/7 enzymes are involved in *Bd* induction of apoptosis. Other caspase enzymes may be better at predicting apoptosis in the epidermis. Caspase 8 (extrinsic pathway) and 9 (intrinsic pathway) are both known to be active in the induction of apoptosis in response to *Bd* in lymphocytes in vitro [24], and further work should explore this pathogen-induced apoptosis pathway. In addition, the last caspase data point was measured at week 7, which is 1-3 weeks prior to the frogs becoming moribund. Therefore, the caspase activity may have increased later in the

infection if samples were taken until the time of death. Finally, the high TUNEL positive cell proportion in the *Bd*+ moribund animals compared to the week 7 *Bd*+ caspase activity may be explained by reduced cell clearance in *L. v. alpina*. Cell clearance is an important step in the apoptosis pathway, and a mismatching of the two processes may result in higher pathogenicity of a disease caused by a down-regulation of CED genes resulting in decreased macrophages participating in phagocytosis of dead cells [38].

It must also be noted that while the TUNEL assay is most often used to explore apoptotic cells, it is an assay to determine DNA damage, which can be caused by other cell death mechanism like necrosis and pyroptosis [39]. Therefore, the increase in assay positive cells at morbidity may be caused by non-apoptotic cells death pathways that do not involve caspase enzymes. Non-caspase mediated cell death might explain the pattern of increased positive TUNEL assay in the Early *Bd*+ *P.* corroboree, which was not mirrored in the caspase assay of *L. v. alpina*. Alternatively the two species might exhibit different host-pathogen interactions.

While more research into the mechanisms of apoptosis control need to be explored within the amphibian-*Bd* system, it is evident that apoptosis plays an important role in the pathogenesis of chytridiomycosis. Therefore, early treatments of infection might aim to induce apoptosis within the epidermis, while apoptosis inhibitors may be effective in later treatments. There are many successful anti-fungal, anti-bacterial and anti-viral chemotherapies that increase apoptosis [38,40–42], but few target apoptosis suppression or increased phagocytosis of apoptotic cells [38]. These treatments can be used to assess the role of apoptosis and cell death in pathogenesis. We suggest that future studies focus on exploring the mechanisms of *Bd*- and host-induced apoptosis, and investigating chemotherapies that control apoptosis for use in conjunction with antifungals.

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Conflicts of interest

The authors declare no conflicts of interest.

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Figure legend

Figure 1. Infection intensity over the course of the experiment.

Infection in animals that succumbed to chytridiomycosis (*Bd*+) and those that cleared infection (Cleared) after week 12 for the *Litoria verreauxii alpina* in the caspase trial. Infection intensity is $\log_{10}$(ZE), and error bars indicate standard error.

Figure 2. Apoptosis increased in *Bd* infected animals.

Detected by the terminal transferase-mediated dUTP nick end-labelling (TUNEL) assay. A) *Bd*- control ventral skin section of *Litoria verreauxii alpina,* B) *Bd*+ ventral skin section of *L. v. alpina,* C) *Bd*- control ventral skin section of *Pseudophryne corroboree,* and D) *Bd*+ ventral skin section of *P. corroboree* stained by *in situ* TUNEL assay. The blue is DAPI staining indicating nuclei of the cells, and the red is the rhodamine stain, which indicates DNA fragmentation; characteristically caused by apoptosis. Rhodamine staining is occurring in epidermal cell layers 2-3 cells deep. 400x magnification and the scale bar indicates 0.03mm. E) The proportion of TUNEL positive apoptotic cells per skin type in the *Bd*+ and *Bd*- animals analyses in *L. v. alpina*. F) The proportion of TUNEL positive apoptotic cells per skin type in *P. corroboree* with light infection intensity (Early *Bd*+), animals that succumbed to disease (Late *Bd*+) and *Bd*- animals. Error bars indicate 95% confidence intervals of a proportion, and * indicates a significant increase in apoptotic cell proportions where ($*^a$) indicates a difference between *Bd*- and Late *Bd*+, ($*^b$) indicates a difference between *Bd*- and Early *Bd*+, and ($*^c$) indicates a difference between Early *Bd*+ and Late *Bd*+ skin samples.

Figure 3. The correlation between infection intensity, Log_{10}(ZE), and caspase 3/7, Log_{10}(Caspase) of inoculated animals over the course of the experiment.

The correlation between infection intensity and caspase activity is 0.463, and the trend line has an equation of $y = (0.229)x + 0.939$. There is no difference between *Bd*+

animals that succumbed to *Bd* infection and animals that were inoculated and then cleared infection, or between weeks of infection.

Figure 4. Caspase 3/7 activity through week 7 for each group.

Bd+ that succumbed (*Bd*+), *Bd*- controls (*Bd*-) and *Bd* inoculated that cleared infection (Cleared). A) The caspase activity (Log_{10} transformed) for each group per week. B) The weekly change in caspase activity for each group. The change is in log base 10. Error bars indicate standard error. $*^a$ indicates the *Bd*+ group differed significantly from the *Bd*- group at that week, $*^b$ indicates the Cleared group differed from the *Bd*- group, and $*^c$ indicates that the *Bd*+ group differed from the Cleared group.

Figures

Figure 1

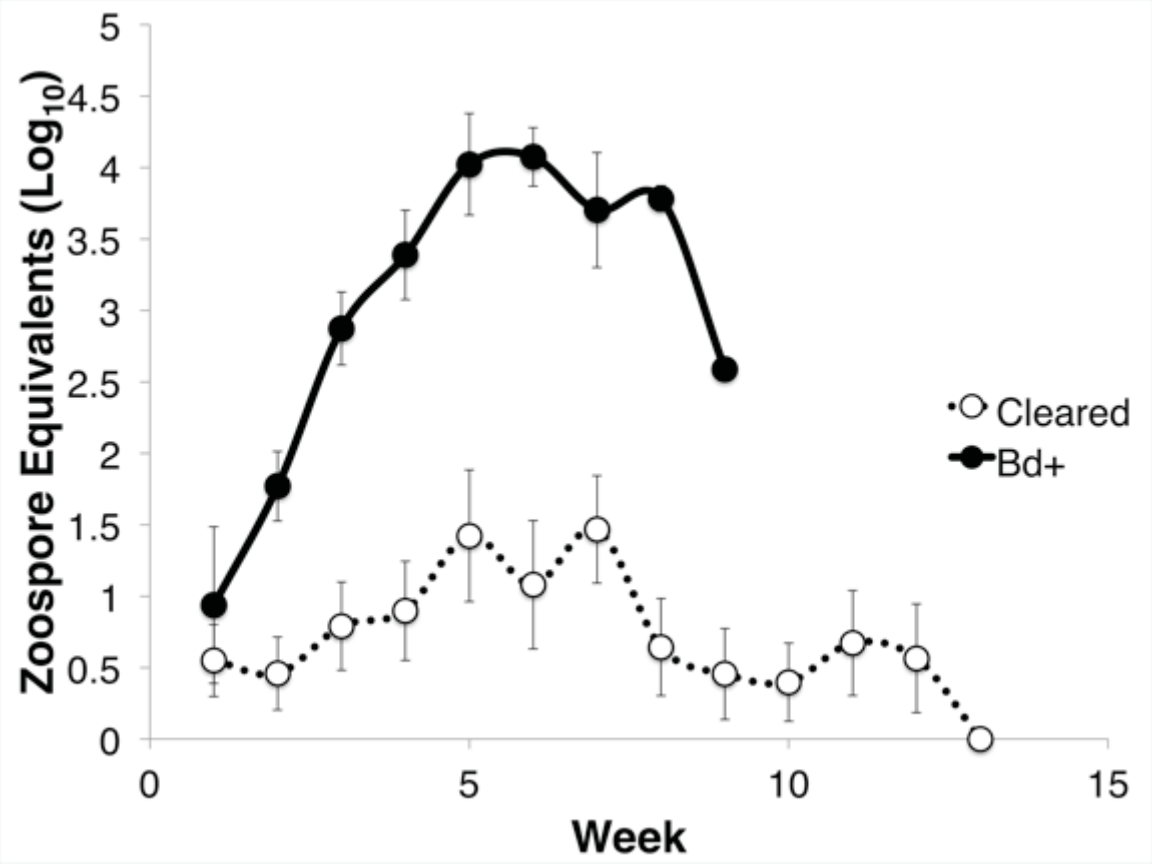


Figure 2

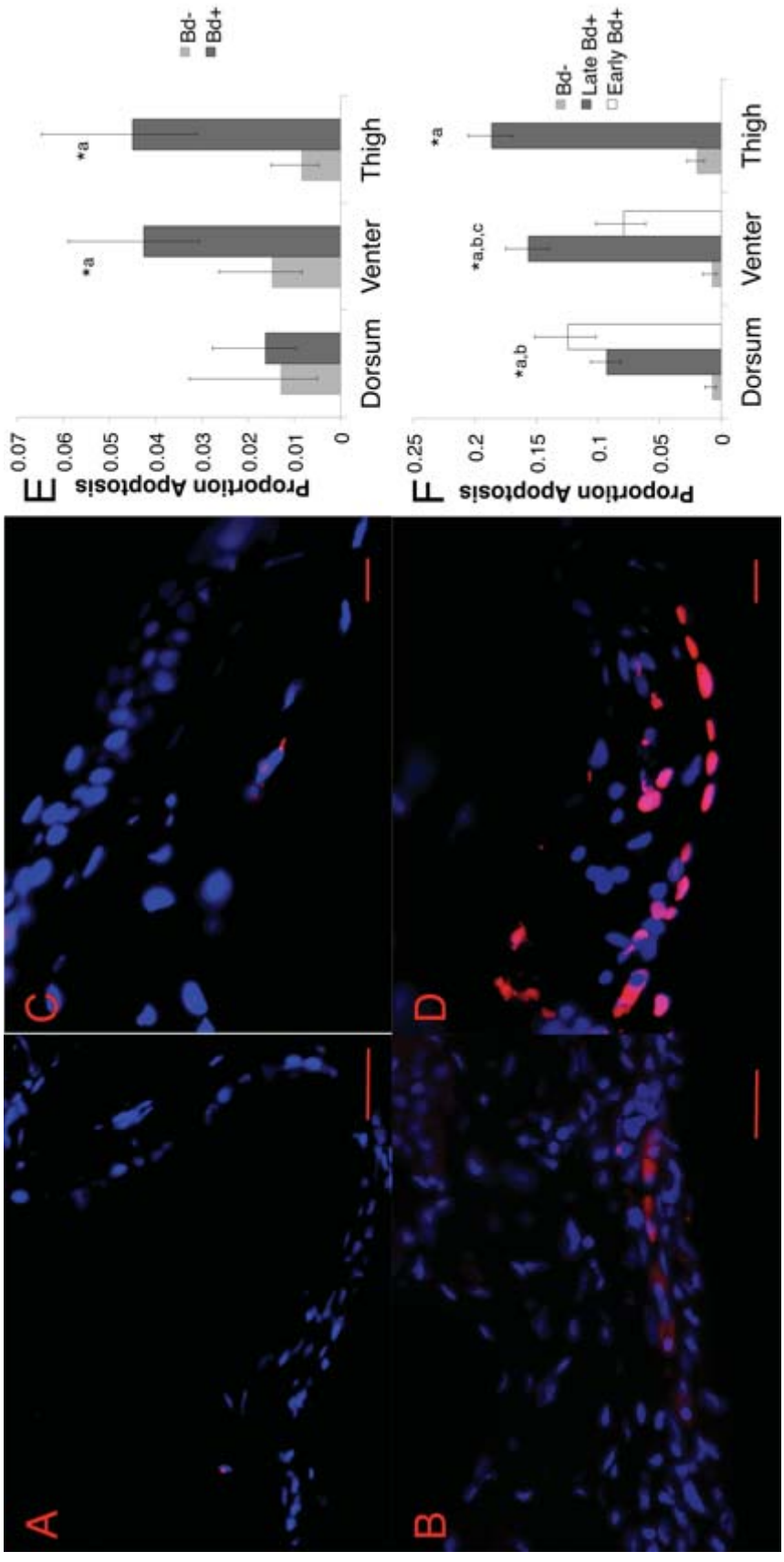

A
B
C
D
E
F
Proportion Apoptosis
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Bd-
Bd+
*a
*a
Dorsum
Venter
Thigh
Proportion Apoptosis
0.25
0.2
0.15
0.1
0.05
0
Bd-
Late Bd+
Early Bd+
*a
*a,b,c
*a,b
Dorsum
Venter
Thigh

Figure 3

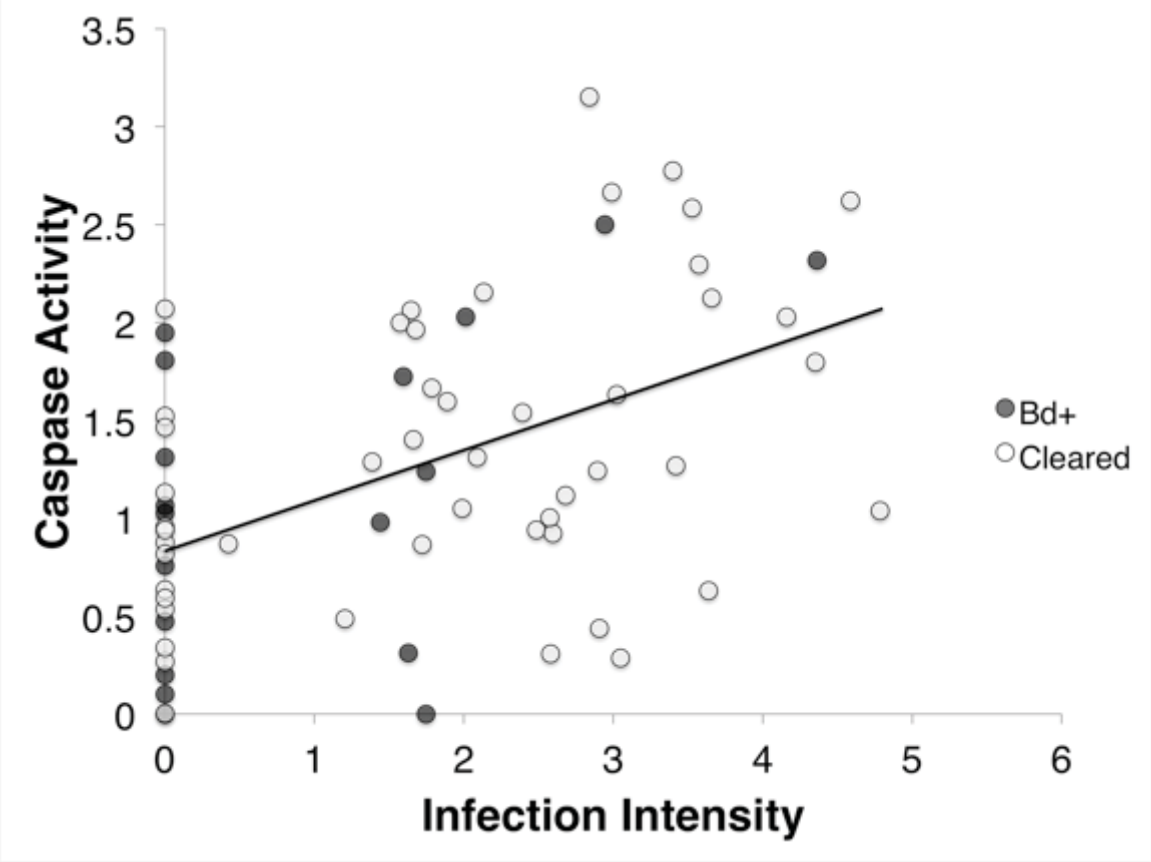
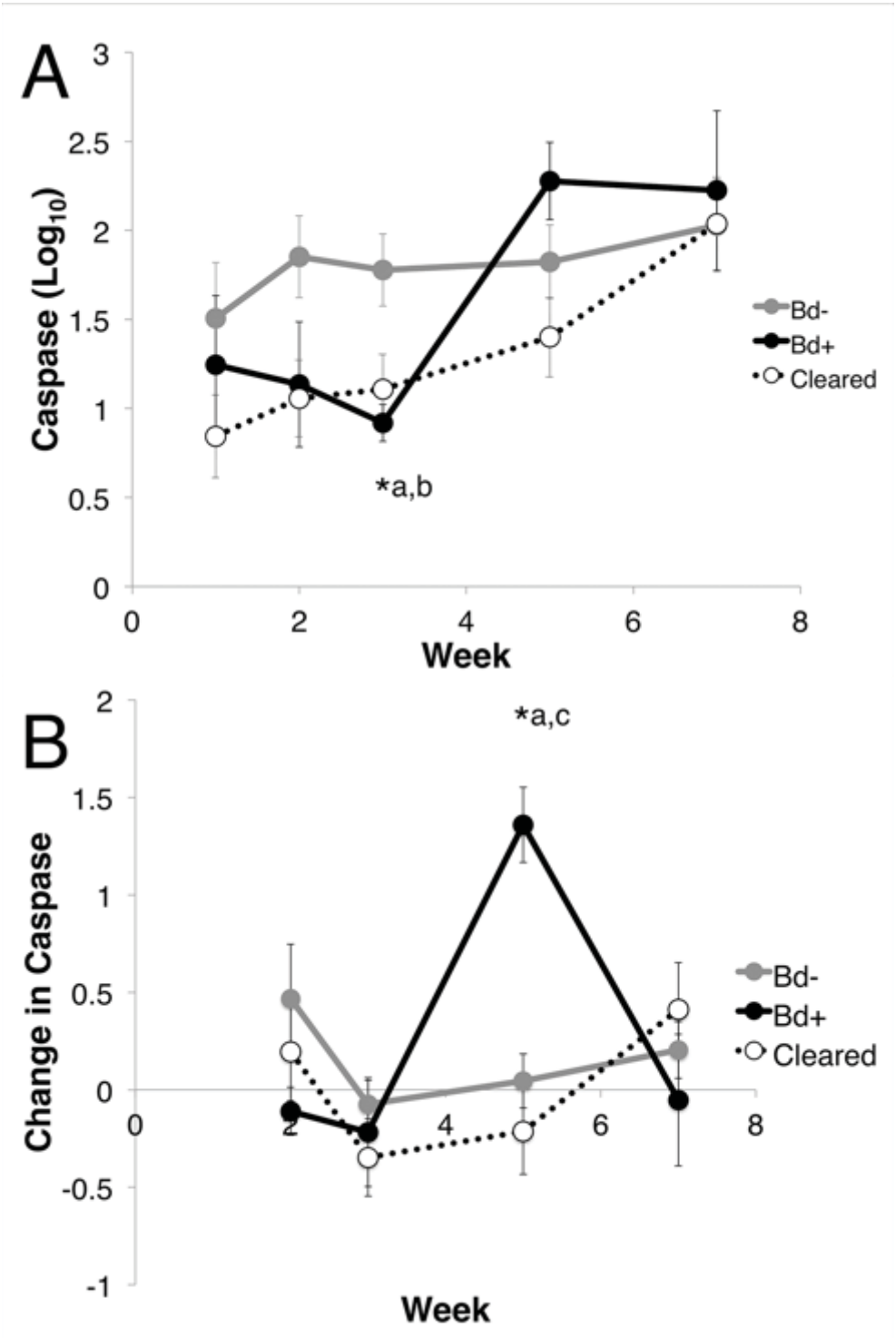

Caspase Activity
3.5
3
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
6
Infection Intensity
Bd+
Cleared

Figure 4


A
Caspase (Log10)
3
2.5
2
1.5
1
0.5
0
0
2
4
6
8
Week
*a,b
Bd-
Bd+
Cleared
B
Change in Caspase
2
1.5
1
0.5
0
-0.5
-1
0
2
4
6
8
Week
*a,c
Bd-
Bd+
Cleared

CHAPTER 6

Differences in susceptibility to disease between populations

Introduction

In Chapter 6 I explore aim 4: to investigate population differences in susceptibility to infection in a susceptible and endangered species. I investigated the susceptibility of *P. corroboree*, a functionally extinct amphibian whose key threatening process is the disease chytridiomycosis. Populations of this species began declining upon the arrival of *Bd* to the Mt Koscuisko region in the 1980's. Intensive management efforts involving captive breeding, reintroduction and monitoring have saved the species from extinction and have contributed to detailed knowledge of disease ecology. However, little is known about the effects of disease on the individual or the mortality rates in this species, as there have been no exposure experiments conducted. Determining if there is a difference in susceptibility between populations and individuals is important evidence regarding the feasibility of selecting for resistance in captive populations prior to reintroduction. A 50 year breeding plan has been devised for the species involving four institutes, and should be informed by knowledge of innate resistance.

This chapter is organised into one manuscript, which represents preliminary results and will be combined with further genetic and immune mechanism analysis conducted on the samples collected from this study. The follow up work is currently underway by post-doctoral research Tiffany Kosch. The manuscript that makes up Chapter 6 is entitled "Population differences in survivorship after disease of a functionally extinct amphibian species," and is written in the style of a stand-alone manuscript.

Paper 1

Citation: Brannelly LA, Hunter DA, Marrentelli G, Skerratt LF, Berger L. Population differences in survivorship after disease of a functionally extinct amphibian species. In Preperation

Population differences in survivorship after disease of a functionally extinct amphibian species.

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Abstract

Amphibians are currently experiencing the greatest loss in biodiversity of any vertebrate taxa, and disease is a major culprit in causing those declines. One disease in particular, chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis*, *Bd*, has caused amphibian declines and extinctions globally. In Australia, the southern corroboree frog, *Pseudophryne corroboree*, is functionally extinct in the wild and being maintained in captive colonies with the goal of reintroduction. There is an extensive effort into managing this species, but little is known about the susceptibility of the animals to *Bd* at an individual or population level. In this study we experimentally infected animals from four distinct meta-populations and monitored susceptibility through infection intensity, survivorship and body condition estimates. We found that of the four populations, one was more susceptible and had increased infection loads, decreased survivorship and decreased body condition, and one was less susceptible with decreased infection loads and increased survivorship. Our results suggest that there are differences between populations in susceptibility to infection, and reintroduction efforts should focus on sourcing frogs from more resistant populations. Also, these differences can be exploited to understand effective immune mechanisms for example to identify genetic markers of resistance that can be utilised by captive breeding programs.

Key Words

Chytridiomycosis, Endangered Species, Population, *Pseudophryne corroboree*, Selective Breeding, Susceptibility.

Introduction

Amphibians are currently the most threatened vertebrate taxa for biodiversity decline (Stuart et al. 2004), and one of the major culprets for causing these declines is wildlife disease, specifically chytridiomycosis. Chytridiomycosis, a skin disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), is the worst pathogen in recorded history for causing biodiversity loss (Skerratt et al. 2007). *Bd* infects over 600 species, caused declines in over 300 species of which about 200 species have become critically endangered or extinct (Wake & Vredenburg 2008). One such species driven to extinction by *Bd* in Australia is the southern corroboree frog, *Pseudophryne corroboree*, once endemic to the alpine regions of New South Wales (Hunter et al. 2009b, 2010a, 2010b). *Bd* entered the system in the 1980's and began causing declines. Currently, the species is being sustained by captive colonies with the goal of reintroduction.

Extensive management and reintroduction efforts are currently underway to release this species back into the wild. Managers of New South Wales in collaboration with zoos and other animal sanctuaries across New South Wales, Victoria and the Australian National Territory are breeding animals in captivity, and releasing eggs and adults into previously occupied habitat, as well as creating disease-free enclosures, and disease free tadpole rearing pools within the larger habitat (Hunter et al. 2009b). Because most frogs die from *Bd* after release, reintroductions need to be on going to maintain wild populations. Research is needed to develop strategies for releasing animals that are less susceptible to infection, so that reintroductions can be more successful and efficient.

As there have been no exposure experiments in this species, little is known about differences in susceptibility between populations and on the effect of disease and mortality rates in *P. corroboree*. Identifying individual and population level variation in susceptibility is the first step in improving survival of reintroduced animals in the presence of *Bd*, because *Bd* cannot be eradicated from these sites, and vaccination is likely ineffective (Cashins et al. 2013; McMahon et al. 2014). Knowledge of the variation in susceptibility between meta-populations is useful for captive managers because release efforts can be focused on breeding and releasing animals from the least susceptible populations in order to bolster survivorship.

In this study we aim to investigate the differences between populations of *P. corroboree* in susceptibility to *Bd* infection. We explored infection intensity, survivorship

and the effect of disease on body condition as a measure of susceptibility to disease. We investigated the susceptibility of four meta-populations, with animals that were collected from the sites as eggs during the decline. This is the first comprehensive study to determine susceptibility of *P. corroboree* to *Bd* infection.

Methods

Animal husbandry

Ninety-eight southern corroboree frogs (*Pseudophryne corroboree*) that were excess to breeding programs were delivered to James Cook University from the Amphibian Research Centre. They had been captive raised, ranged in age from five to eight years old, ranged in snout to vent length 23.63 – 30.8 mm and mass 0.88 – 2.54 g. Animals came from four separate and distinct meta-populations (Fig. 1): Jagumba (J) (n = 19), Snakey Plains (S) (n = 20), Cool Plains (C) (n = 19), Manjar (M) (n = 20), and *Bd*-control group consisting of a combination of individuals from these four meta-populations (n = 20). The individuals used in this experiment were collected from the wild as eggs when the populations were on the brink of collapse, and raised in captivity until adulthood. Once at James Cook University, animals were housed individually in 300 x 195 x 205 mm terraria with a damp and crumpled paper towel substrate (Earthcare®, ABC Tissue), at a room temperature of 18-20°C. They were fed *ad libitum* three times weekly with 5 – 10 mm crickets (*Acheta domestica*). Animals were misted twice daily for 60 seconds with reverse osmosis water. Temperature and humidity were monitored daily. Terraria were cleaned fortnightly by replacing the paper towel substrate.

Inoculation

Animals were allowed to acclimate to their new environment for seven days. The animals were inoculated with a New South Wales strain of *Batrachochytrium dendrobatidis* (AbercrombieR-L.booroologensis-2009-LB1 passage number 11). *Bd* was harvested from agar and tryptone, gelatin hydrolysate, lactose (TGhL) petri plates after incubation at 23°C for 5 days. Plates were flooded with 3mL of artificial spring water and allowed to sit for 10 minutes to allow zoospores to be released from zoosporangia. Inoculum was poured off the plates and zoospores were counted using a hemocytometer. Animals were inoculated with $1x10^6$ zoospores by applying 3mL of inoculum onto the

venter (n = 78). Animals were placed in individual 40mL containers for 6 hours, and then transferred back into their terraria. *Bd* negative control animals were mock-inoculated using *Bd* negative petri plates (n = 20).

Testing for Bd

We tested for *Bd* infection by using qPCR analysis of skin swabs (Boyle et al. 2004), which is standard practice for *Bd* diagnosis. The technique has been well validated and has high accuracy for quantifying heavy infections (Hyatt *et al.* 2007; Skerratt *et al.* 2011). The swabbing protocol was standardised by performing 45 strokes with a sterile rayon-tipped swab (MW-113, Medical Wire & Equipment) per animal, five on the middle of the venter, five on each side of the venter, five on each thigh, and five each limb. The swab was gently rotated during and between strokes to ensure the greatest amount of DNA was gathered on the swab. Genomic DNA was extracted from the swabs by bead beating each sample for two minutes to disrupt the fungal cell wall and using the Prepman Ultra kit, and analysed using quantitative PCR following Boyle et al. (2004), with a positive and negative control, and a series of dilution standards in singlicate (Kriger et al. 2006; Brannelly et al. 2015b).

Disease monitoring

Animals were checked daily for general health by monitoring mobility, sloughing patterns, food consumption, and alertness. Clinical signs of chytridiomycosis were monitored using three steps. The first step was checking for movement when the terrarium was tilted. If the animal moved (head bob, adjustment of limb), they were considered alert. The second step involved touching the back of the animal and checking for attempts to escape. Thirdly, the righting reflex was assessed by positioning the frog on its back. If the animal failed all three alertness steps it was considered moribund (Brannelly et al. 2015a, 2015d), and euthanized with an overdose of MS-222 in accordance with animal ethics guidelines.

Once each week for the duration of the experiment (103 days), animals were swabbed for *Bd* presence, weighed to the nearest 0.01g and measured snout to vent (SVL) length to the nearest 0.1mm. Any animal that cleared infection and survived by the end of the experiment were returned to the Amphibian Research Centre with the intention of

conducting a breeding experiment in order to assess whether their resistance to infection is heritable.

Data analysis

Survival data was analysed using Cox Regression analysis by comparing the different populations. Infection loads Log base 10 transformed and analysed using mixed models where week, population and week*population were compared as factors. Bonferroni's post hoc tests were used to determine differences within the factors analysed. Body condition was estimated as $Log_{10}(Mass+1)/Log_{10}(SLV+1)$ analysed using mixed models where week, population and week*population interactions were compared as factors. Bonferroni's post hoc tests were used to determine differences within the factors analyzed. All statistics were performed in SPSS (v21). Cohen's d statistic was used to determine effect size and calculated in Microsoft Excel.

Animal ethics

Ethical approval was granted by James Cook University for this study under application A1875 entitled "Innate and adaptive immune mechanisms against amphibians chytrid fungus and non-chemotherapeutic treatment methods."

Results

Infection intensity

All animals inoculated were *Bd*+ for at least two weeks during the experiment. Five animals (6.4%, 95% CI 2.7% - 14.1%) successfully cleared infection by week 12. Infection intensity increased with time (Mixed models: $F_{11,407.1} = 63.56$, $p < 0.01$), and infection intensity differed between the populations (Mixed models: $F_{3,121.3} = 5.089$, $p < 0.01$), but there was no impact of week*population (Mixed models: $F_{32,406.6} = 1.148$, $p = 0.27$). Infection load was 35.7% higher in the J population compared to the M population (Bonferroni's post hoc: $p < 0.01$; $d = 0.37$) but overall infection loads were not different between the other populations (Fig. 2).

Survival

The five animals (6.4%) that cleared infection survived. Three animals were from population M (15% of M survived, 95% CI = 5.2% - 36%), one from population S (5%, 95% CI = 0.8% - 23.6%), and one from population C (5.3%, 95% CI = 0.9% - 24.6%). No animal in the *Bd*- control group died during the experiment, and survival was significantly decreased when infected with *Bd* (Cox regression: $\chi^2_4 = 48.64$, $p < 0.01$). The first animal succumbed on day 21 post inoculation, and the last succumbed on day 94. When only inoculated animals were considered, there was no difference in survival between populations overall (Cox regression: $\chi^2_3 = 6.91$, $p = 0.073$), but the J population had 2.38x higher mortality than the M population (Cox Regression Variables: Wald = 5.85, $p = 0.016$, exp(B) = 2.38), while there was no difference in survival between the other populations (Fig. 3). The average time until death for animals in population J was 43 days, while population M average time until death was 60 days: 17 days longer survival.

Body condition

Inoculated animals had significantly lower overall body condition (10.1% lower body condition) than the uninfected controls (Mixed Models: $F_{1,96} = 7.74$, $p < 0.01$). Week, population and week*population all significantly affected body condition (Mixed Models; Week: $F_{13,709.2} = 521.2$, $p < 0.01$, Population: $F_{4,101.2} = 5.089$, $p < 0.01$, Week*Population: $F_{51,709.2} = 3.197$, $p < 0.01$). Animals from population J had the lowest body condition over the course of the experiment. J animals had 20.0% lower body condition than the *Bd*- animals (Bonferroni's post hoc: $p < 0.01$), 17.1% lower body concentration than the M population (Bonferroni's post hoc: $p < 0.01$), and 14.7% lower body condition than the S population (Bonferroni's post hoc: $p < 0.01$). Body condition of animals from population C and J did not differ (Bonferroni's post hoc: $p = 1$) and animals from population C had 10.9% lower body condition than the *Bd*- animals (Bonferroni's post hoc: $p = 0.04$). There was no difference in body condition between the other populations (Fig. 4).

Discussion

The purpose of this experiment was to determine if there was a difference in susceptibility to *Bd* infection between four meta-populations of the functionally extinct

species, *P. corroboree*. Susceptibility was determined by infection load, survivorship and body condition. Of the four meta-populations examined, one population was substantially more susceptible to *Bd* infection – population J. Population M had the highest survival with the least impact of disease on individuals.

Animals from population J succumbed more quickly to disease with no recovery, surviving an average of 17 days less than animals from population M. The body condition of population J animals was lower than the others and decreased over time. Infection load was 35.7% higher than animals from population M. These results combined suggest that infection affects animals from this population more quickly, with more negative impacts than those from the other populations.

In contrast, Population M appears to be the least susceptible. Infection load throughout the course of the experiment was lower, survival was higher, and body condition was the same as the *Bd*- control animals. In addition, 15% of the animals cleared infection and survived. Based on the evidence of this infection trial, we suggest that reintroduction efforts focus on breeding and releasing animals from population M, and reduce production of animals from population J.

The mechanisms of resistance in *P. corroboree* are unknown at this time, and the variation in susceptibility that we have uncovered will be key to determining protective immune mechanisms. Research can exploit differences between these meta-populations as well as between individuals. Individual resistance/tolerance of infection appears to be species dependent, and an overall immune mechanism that is highly protective has not been identified (Grogan 2015a). Some potential immune mechanisms against *Bd* that have been explored in other species and show promise are secreted anti-microbial peptides from skin glands (Woodhams et al. 2007), cutaneous bacterial communities with anti-fungal properties (Woodhams et al. 2014), splenic lymphocyte activity (McMahon et al. 2014), and major histo-compatibility (MHC) complex alleles (Savage & Zamudio 2011; Bataille et al. 2015). The next steps in researching the susceptibility of *P. corroboree* is to begin exploring immune mechanisms that have a genetic marker, so that captive colonies can be screened for breeding purposes. We are currently using samples from these experimental animals to detect MHC alleles linked to resistance.

While exploring immune mechanisms is important for improving reintroduction success and selective breeding for disease resistance in highly threatened species (Scheele et al. 2014b), identifying immune mechanisms is time consuming and may take

years. In the mean time, it is important to trial management options based on current knowledge. For *P. corroboree*, we recommend preferentially breeding animals from population M, and reducing production of individuals from population J. However, this approach must be conducted as a trial initially and genetic diversity should be retained in the captive stock. This is because *Bd* resistance is just one aspect of fitness, and other traits such as reproduction are also likely to be important in population sustainability. However, lower susceptibility to *Bd* in the laboratory should translate into higher survivorship upon release, as mortality from chytridiomycosis is a key limiting factor, but survivorship in the wild results from an interplay of many factors. While trialling targeted release efforts is important, cohesive and detailed monitoring of releases with appropriate control and trial groups is important to understanding factors that influence survivorship (Brannelly et al. 2015c).

Acknowledgements

We would like to thank R. Webb and S. Bell for help testing the animals for *Bd* infection, M. McFadden for his contribution to the ecological and husbandry knowledge on *P. corroboree*, and our volunteers J. Hawkes, K. Fossen and C. De Jong for help with the husbandry.

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Figure legend

Figure 1. Map of Kosciuszko National Park, New South Wales, Australia.

Labelled with sites of the meta-populations used in this study. The white lines indicate state borders, Victoria to the west and Australian Capital Territory to the northeast. Scale bar = 10km.

Figure 2. Infection intensity over the course of the experiment through 12 weeks post inoculation.

Infection intensity is $\log_{10}(ZE+1)$. Error bars indicate standard error. Each line represents a population: J – Jugumba, S – Snakey Plains, C – Cool Plain, M – Manjar.

Figure 3. Survivorship over the experiment.

Each line represents a different population. J – Jugumba, S – Snakey Plains, C – Cool Plain, M – Manjar. Controls are the *Bd*- control animals, and all survived through to the end of the experiment.

Figure 4. Body condition over the course of the experiment.

Body condition was calculated as $Log_{10}(Mass+1)/Log_{10}(SVL+1)$. Error bars indicate standard error. Each line represents a population: J – Jugumba, S – Snakey Plains, C – Cool Plain, M – Manjar.

Figures

Figure 1

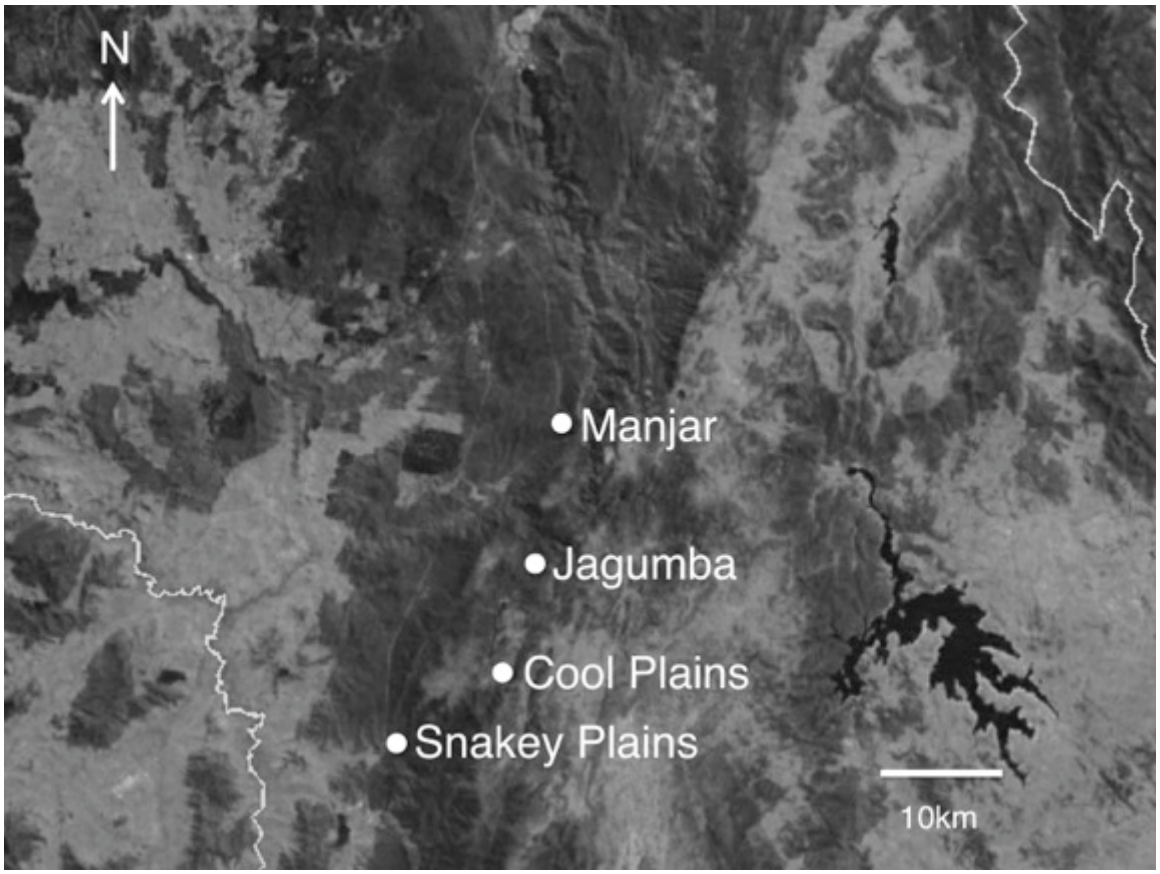


Figure 2

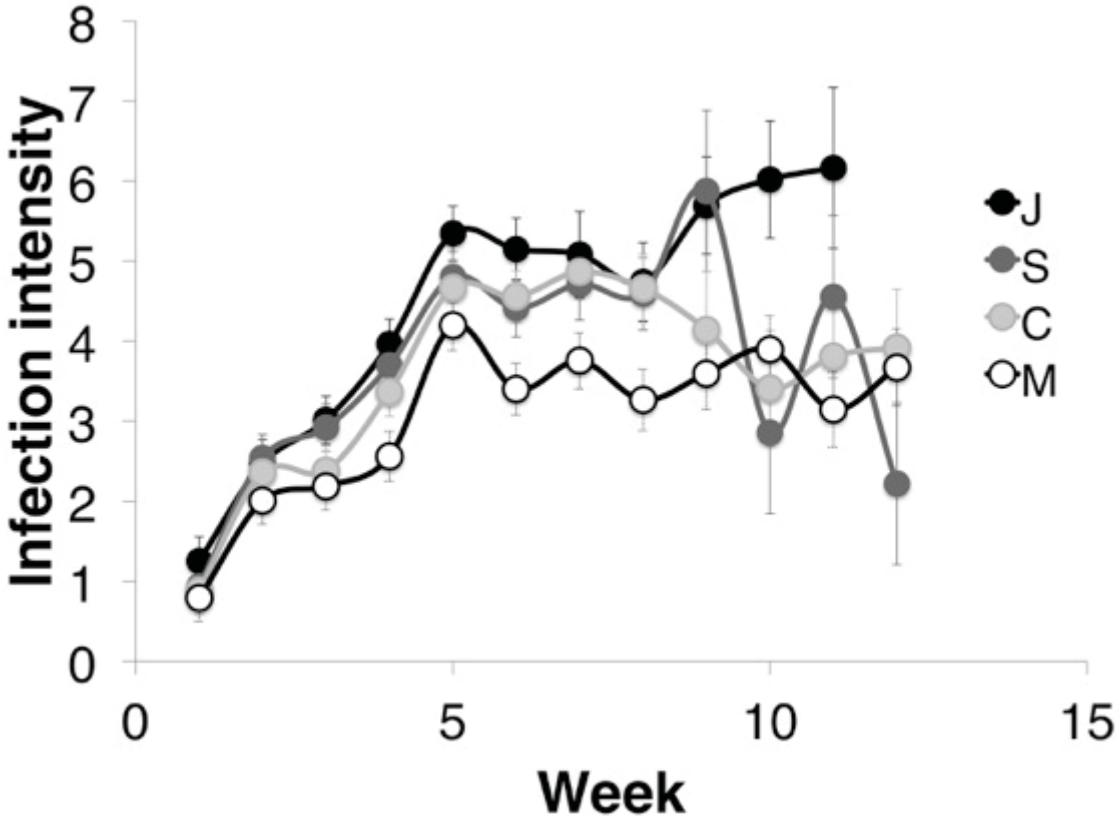


Figure 3

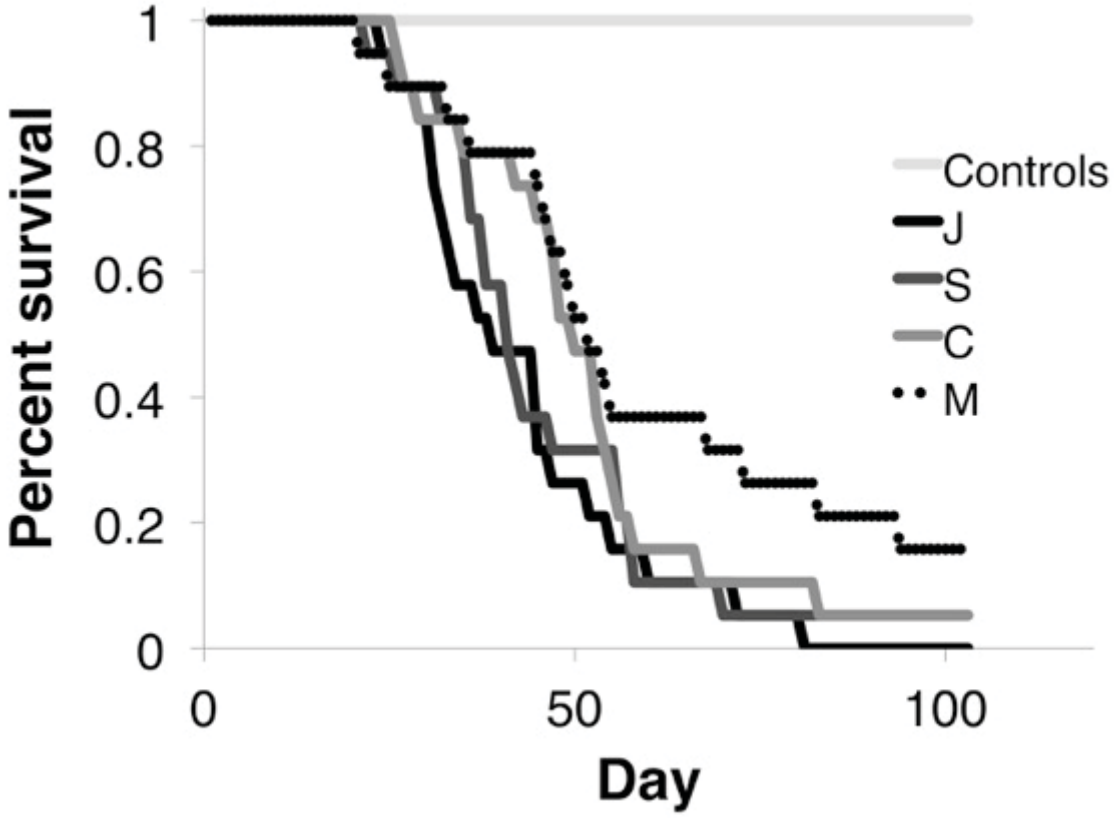
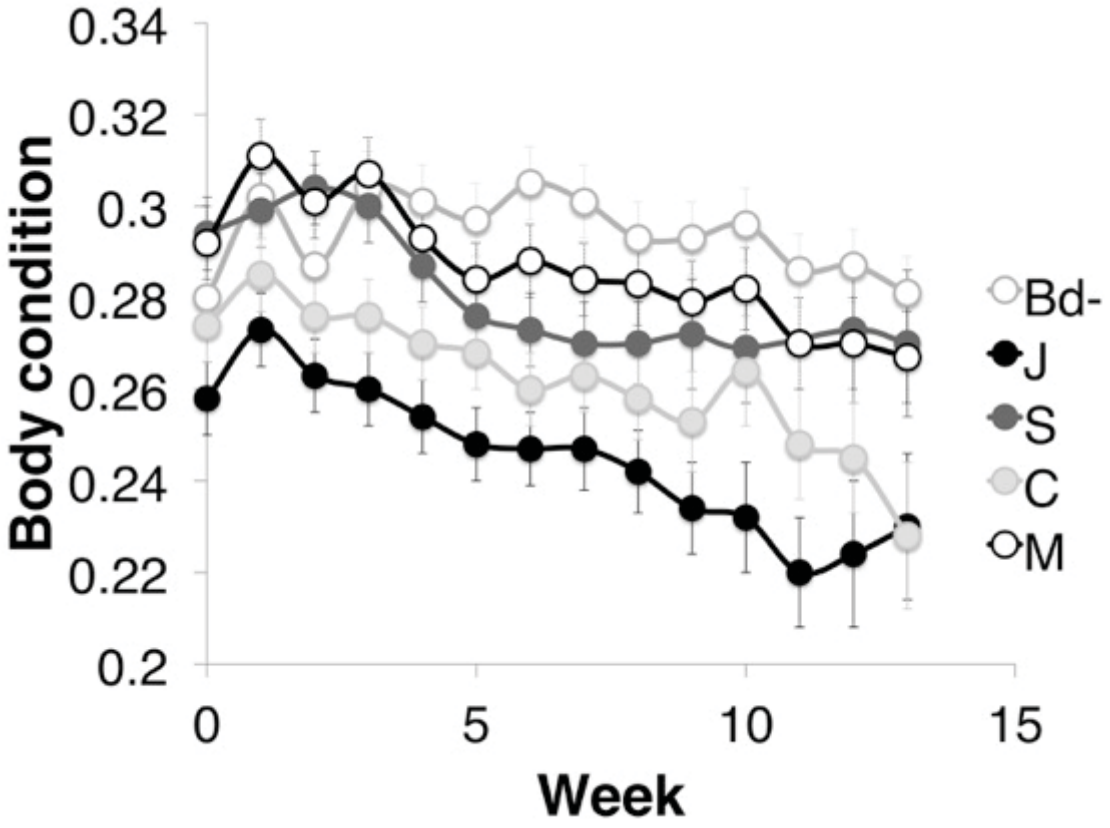


Figure 4


Body condition
0.34
0.32
0.3
0.28
0.26
0.24
0.22
0.2
0
5
10
15
Week
Bd-
J
S
C
M

CHAPTER 7

Alternative treatment options for endangered alpine species in captivity

Introduction

Throughout this thesis I have explored the disease dynamics and impacts of disease on two critically endangered/extinct alpine species, highlighting the importance of understanding the impacts of disease on the individual, population and ecology because the knowledge will assist in informing management decisions. It has been clear that critically endangered and extinct species require many management resources, including captive breeding colonies. A major priority of captive breeding colonies is to maintain the health of the populations, and effectively treat animals if a disease breakout occurs. In Chapter 7 I explore my final aim: to explore new potential treatment options for captive colonies. These treatment trials were conducted concurrently with the experiment in chapter 6, by using frogs that were excess to requirements or to utilise frogs that were moribund and would have otherwise been euthanased.

In this chapter I trialled two novel treatment options. The first treatment is a drying regimen, which has been published in Wildlife Research as Paper 1: "Low humidity is a failed treatment option for chytridiomycosis in the critically endangered southern corroboree frog."

The second treatment trialled is an intensive treatment of clinically ill frogs utilising both antifungals and electrolyte therapy. Paper 2 was published in Veterinary Research and Communication and entitled "Treatment trial of clinically ill corroboree frogs with chytridiomycosis with two triazole antifungals and electrolyte therapy."

Paper 1

Citation: Brannelly LA, Berger L, Marrantelli G, Skerratt LF. Low humidity is a failed treatment option for chytridiomycosis in the critically endangered southern corroboree frog. Wildlife Research. 42:44-49.

Low humidity is a failed treatment option for chytridiomycosis in the critically endangered southern corroboree frog

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Abstract

Context: One of the major drivers of the current worldwide amphibian decline and extinction crisis is the spread of the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*). Captive assurance colonies may be the only lifeline for some species. Current antifungal chemotherapies can be effective for clearing infection but may have detrimental side effects. The only non-chemotherapeutic treatment that has been clinically tested is heat, which is impractical for cold-adapted species. The fungus does not survive desiccation, and therefore a plausible alternative non-chemotherapeutic treatment would be a low-humidity regimen.

Aims: We tested the efficacy of a low-humidity treatment regimen for treating *Bd*-infected animals of the critically endangered species *Pseudophryne corroboree*. This species has high tolerance of dry environments, and is thus an ideal candidate for this treatment trial.

Methods: Forty frogs were exposed to 1×10^6 zoospores. At three weeks after exposure access to water was reduced to once daily for 10 days for 20 animals. Ten of these animals were also rinsed daily with the aim of removing zoospores.

Key results: The drying regimen neither increased survival nor decreased infection load, and treatment may have hastened mortality. All infected frogs died between 21 and 80 days after exposure, except one untreated frog.

Conclusions: Our results suggest that a drying regimen is not a viable treatment for chytridiomycosis. Infection may decrease the host's ability to cope with water stress.

Implications: The failed drying treatment in *P. corroboree* suggests that drying is not an appropriate treatment for chytridiomycosis and treatment efforts should be focussed on chemotherapies for cold-adapted species. *P. corroboree* is a critically endangered species for which chytridiomycosis is the primary cause of decline, yet few experiments have been carried out on this species with regards to chytridiomycosis. All experimental information about this species and disease will benefit the management and protection of the species.

TOC abstract

The disease chytridiomycosis is causing massive declines globally, and the current treatment methods can be harmful or impractical for some species. We trialled the

treatment method of drying, but found that neither mortality nor infection was decreased, and drying may have hastened mortality. Infection may decrease an animal's ability to cope with water stress, which may cause further declines in the face of global climate change.

Additional keywords

dry treatment, non-chemotherapeutic treatment, *Pseudophryne corroboree.*

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Introduction

Amphibians are currently experiencing the greatest biodiversity decline of all vertebrate taxa (Stuart et al. 2004). Chytridiomycosis, a skin disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), is one of the major causes of this dramatic decline (Skerratt et al. 2007). Bd is the worst pathogen on record for causing biodiversity loss; it is known to infect over 600 species of amphibian, and has caused declines in over 300 species, of which ~200 species have become critically endangered or extinct (Wake and Vredenburg 2008). *Bd* is not host specific and it is unclear what host factors cause susceptibility. Chytridiomycosis causes death by disrupting ion channels in the skin, resulting in electrolyte loss and heart failure (Voyles et al. 2007, 2009). Because many species are declining at an alarmingly rapid rate, captive assurance colonies and breeding programs are important conservation measures, and may be the only lifeline for some species (Zippel et al. 2011). Establishing and maintaining disease-free colonies represents a challenge to amphibian management facilities.

The current chemotherapeutic methods for treating *Bd* infections, although effective, can be harmful and no single treatment method has been successful across species and age classes of amphibians (Berger et al. 2010; Baitchman and Pessier 2013). Adverse effects of one of the more widely used chemotherapeutic treatments, itraconazole, can be dose and life-stage dependent (Jones et al. 2012; Brannelly et al. 2012). Chloramphenicol, an antibacterial, is another possible chemotherapy for *Bd* (Bishop et al. 2009; Young et al. 2012; Holden et al. 2014), but may be impractical for terrestrial animals because it requires constant submersion for 2–4 weeks. Pharmacology in amphibians has been poorly studied (Woodward et al. 2014), and even for treatments that appear safe, none have undergone extensive testing to examine long-term effects on reproduction or immunity (Berger et al. 2010).

As *Bd* is a sensitive organism that occurs in superficial skin layers, non-chemotherapeutic treatments have potential and would avoid drug side-effects. The only non-chemotherapeutic treatment of *Bd* infection shown to be effective in clinical trials is heat treatment, such as exposing frogs to at least 30°C for 10+ days. It appears to have few side effects and has been successful for many species and life stages (Woodhams et al. 2003, 2012; Chatfield and Richards-Zawacki 2011). Many cold-adapted species,

however, cannot survive 30°C for an extended time. Therefore, an alternative non-chemotherapeutic method is needed.

Drying may be an alternative treatment option. *Bd* is highly sensitive to desiccation; in culture it is killed within 1 h of drying (Johnson et al. 2003). In a laboratory experiment an endangered toad, *Anaxyrus boreas*, had higher survival and lower *Bd* zoospore loads in drier and warmer terraria (Murphy et al. 2011). This study mimics what has been found in field studies around the world; that *Bd* is less pathogenic in drier habitats (Kriger and Hero 2007; Puschendorf et al. 2011; Terrell et al. 2014). Additionally, the same species in different environments has a different response to infection where the impact and distribution of *Bd* is limited by low rainfall (Kriger and Hero 2009; Puschendorf et al. 2011; Terrell et al. 2014). Based on the laboratory and field evidence, a low humidity treatment trial can be justified.

The southern corroboree frog, *Pseudophryne corroboree*, is a critically endangered alpine species endemic to Kosciuszko National Park in New South Wales, Australia (Hunter 2000). The species is highly susceptible to *Bd*, and due to the introduction of the pathogen in the 1980s (Hunter et al. 2010a) the species is now functionally extinct in the wild. Large-scale captive-assurance colonies are being maintained in numerous locations in New South Wales and Victoria (Hunter et al. 2010b). Species of *Pseudophryne* are terrestrial and have a high tolerance for low-humidity environments. For example, wild frogs have been found in dry grass tussocks (D. Hunter, pers. comm.).

Anecdotal evidence from captive managers suggests that *Pseudophryne* species can cope with dehydration and recover easily. When automated misting systems have malfunctioned, animals were left without water for a few days, yet *Pseudophryne* species seem to be well adapted to water stress. Some examples from captive colonies are (1) four northern corroboree frogs, *Pseudophryne pengilleyi*, were found huddled together in a water-conserving position after lacking water for five days, with the moss remaining dry for the last three days (M. McFadden, pers. comm.); (2) one P. corroboree was accidentally denied water for three days on a paper towel substrate and, while initially slow in movement, recovered quickly after being provided water and survived (L. Brannelly, pers. obs.); and (3) individuals from three species of *Pseudophryne* have been so severely desiccated after more than five days without water that they appeared

shriveled and lacked movement, but were successfully rehydrated (G. Marrantelli, pers. obs.).

Because data available for successful treatment options, both chemotherapeutic and non-chemotherapeutic, are limited for the critically endangered heat-sensitive *P. corroboree*, we investigated drying as a non-chemotherapeutic treatment for *Bd* infections. We tested the hypothesis that restriction of water would clear infection by reducing the viability of the zoospores to successfully reinfect the epidermis. This study was part of a larger experiment investigating susceptibility to *Bd* in this species. We investigated the effectiveness of two low-humidity treatment regimens. The first restricted access to water by offering occasional water to the animals. The second drying regimen provided water by occasional rinsing. As water is believed to stimulate zoospore release from sporangia by dissolving the plug on the discharge tube (Berger et al. 2005), we trialled this second drying regimen in the hope that rinsing would stimulate the release and enable the removal of zoospores (Hyatt et al. 2007).

Methods

Animal husbandry

Fifty southern corroboree frogs that were excess to breeding programs were delivered to James Cook University from the Amphibian Research Centre. They had been captive raised, ranged in age from five to eight years old, ranged in snout–vent length from 26.3 to 30.7 mm and in mass from 1.3 to 3.3 g. Animals were housed individually in 300 × 195 × 205 mm terraria with a damp and crumpled paper towel substrate, at a room temperature of 18–20°C. They were fed *ad libitum* three times weekly with 5–10-mm crickets (*Acheta domestica*). Animals were misted twice daily for 60 s with Reverse Osmosis (RO) water. Temperature and humidity were monitored daily. Terraria were cleaned fortnightly by replacing the paper towel substrate.

Inoculation

Animals were allowed to acclimate to their new environment for no less than seven days. The animals were inoculated with a New South Wales strain of *B. dendrobatidis* (AbercrombieR-L.booroologensis-2009-LB1 passage no. 11). *Bd* was harvested from agar and tryptone, gelatin hydrolysate, lactose (TGhL) Petri plates after incubation at 23°C for five days. Plates were flooded with 3 mL of artificial spring water

and allowed to sit for 10 min to allow zoospores to be released from zoosporangia. Inoculum was poured off the plates and zoospores were counted using a haemocytometer. Animals were inoculated with 1×10^6 zoospores by applying 3 mL of inoculum onto the venter. Animals were placed in individual 40-mL containers for 6 h, and then transferred back into their terraria. *Bd*-negative control animals were mock-inoculated using *Bd*-negative Petri plates ($n = 10$).

Testing for Bd

We tested for *Bd* infection by using qPCR analysis of skin swabs (Boyle et al. 2004), which is standard practice for *Bd* diagnosis. The technique has been well validated and has high accuracy for quantifying heavy infections (Hyatt et al. 2007; Skerratt et al. 2011). The swabbing protocol was standardised by performing 45 strokes with a sterile rayon-tipped swab (MW-113, Medical Wire & Equipment) per animal, five on the middle of the venter, five on each side of the venter, five on each thigh, and five on each limb. The swab was gently rotated during and between strokes to ensure the greatest amount of DNA was gathered on the swab. Genomic DNA was extracted from the swabs by bead beating each sample for 2 min to disrupt the fungal cell wall and using the Prepman Ultra Kit, and analysed using quantitative PCR following Boyle et al. (2004), with a positive and negative control, and a series of dilution standards in singlicate. Zoospore equivalents (ZE) of the swab were capped at 16667 ZE based on the maximum standard run. Internal positive controls were run only if the reaction well was *Bd* negative.

Treatment for sensitive species

After inoculation, animals were swabbed, weighed and measured once weekly and monitored for clinical signs of chytridiomycosis. Treatments commenced three weeks after inoculation. Frogs were randomly assigned to one of four groups using a random number generator in Microsoft Excel. Two groups were used to test treatment protocols, while two groups were used as controls. The first treatment protocol was a drying regimen and the second was a combination of drying and rinsing. Treatment lasted for 10 days with a sample size of 10 individuals in each treatment group. An untreated, infected control group ($n = 20$), and an uninfected control group ($n = 10$) were also used. Control animals were randomly selected from a larger experiment investigating susceptibility to *Bd* in this species; therefore, an uninfected treated group was not used.

The drying regimen required that the animals remain in dry terraria (smaller: 200 × 150 × 100 mm), being provided water (0.5 mL applied to the centre of their dorsum and allowed to drip to their venter) once daily. The rinsing and drying regimen required that the animals remain in dry terraria, but instead of dripping water on the animals once daily, they were placed in a bath of water (200 mL to ensure submersion) for 5 min, removed from their bath and rinsed with clean water for 5 s and returned to their terraria. Animals were monitored twice daily for clinical signs of dehydration (e.g. sticky skin, immobility) and morbidity. If dehydration or morbidity occurred, animals were given their daily water dose in an effort to revive them. If movement did not return, the animals were killed with an overdose of MS-222.

Testing for *Bd* occurred at Week 1, 2, 3 and 4 after treatment was completed to assess effectiveness of the treatment, and after euthanasia on the date of death. All surviving animals were euthanised with an overdose of MS-222 at the completion of the experiment, four weeks after the end of treatment.

Data analysis

Survival data were analysed using Cox Regression analysis by comparing the different treatments (drying, dry and rinsing and *Bd*+ controls). Infection loads were non-normal, so the two-tailed non-parametric Mann–Whitney test was used to compare infection loads from animals that died during the treatment time with those that died after the treatment time. This test was also used to compare infection loads from the *Bd*+ control animals that died during the treatment time with those from the treatment animals that died during the treatment time. A two-tailed Kruskal–Wallis test was used when comparing the infection load between different treatment groups (drying, dry and rinsing and *Bd*-positive controls). All statistical analyses were performed in SPSS.

Animal ethics

Ethical approval was granted by James Cook University for this study under application A1875 entitled ‘Innate and adaptive immune mechanisms against amphibians chytrid fungus and non-chemotherapeutic treatment methods’.

Results

Mortality rate in the Bd-exposed groups was 97.5% overall. There was no difference in mortality or time until death between either drying treatment (dry and rinse, and dry alone) and the Bd+ control animals (Cox regression: $\chi^2_1 = 1.059$, exp(b) = 1.212, P = 0.303) (Fig. 1). There was no difference in zoospore equivalents between *Bd*+ treatment groups and the *Bd*+ control group at death (Kruskal–Wallis: $\chi^2_2 = 3.429$, P = 0.18) (Fig. 2). However, the zoospore loads of the animals that died during the treatment (7848 IQR 15579 ZE, n = 8; range = 16 to >16667 ZE) were significantly lower than those of the animals that died after treatment (>16667 ZE, n = 12) (Mann–Whitney: $Z_{8,12} = -3.043$, P = 0.002). The *Bd*+ control animals that died during the treatment period tended to have a higher zoospore load (16667 IQR 3375 ZE, n = 6) than the treated animals that died during the treatment period; however, this trend was not significant (Mann–Whitney: $Z_{8,6} = -1.787$, P = 0.074) (Fig. 3). In all, 40% of treatment animals died during the treatment period. The first animal died on Day 2 after treatment began and three weeks after exposure to *Bd*. All of the treatment animals that survived the treatment period died of chytridiomycosis before the experiment was terminated. All but one of the *Bd*+ control animals died of chytridiomycosis before the termination of the experiment (Fig. 1). The animals that died during the treatment period were desiccated and stiff, and they did not appear to exhibit the signs of chytridiomycosis, such as skin shedding in small pieces, or cracked and bleeding hands and feet. The *Bd*+ control animals and the treatment animals that died after the treatment period all exhibited clinical signs of chytridiomycosis. The 10 unexposed controls remained healthy and *Bd* negative until the experiment was terminated.

Discussion

Our test of drying as a non-chemotherapeutic treatment for *Bd* infection was unsuccessful for *P. corroboree* and neither mortality rate (Fig. 1) nor infection burden was reduced (Fig. 2). Deaths during the drying treatment may have been due to chytridiomycosis, or a combination of desiccation and chytridiomycosis, given that the *Bd* zoospore loads were significantly lower than in the animals that died after the treatment period. There was a non-significant trend for higher zoospore loads for the six

Bd+ control animals that died during the same period, and the eight treatment animals did not exhibit signs of chytridiomycosis (Fig. 3). These preliminary data suggest that, when dry, frogs die at a lower threshold of infectious burden, or are more prone to desiccation when infected with *Bd*.

Bd infection causes death by causing an imbalance in cutaneous electrolyte transport, resulting in depletion of Na+, K+ and Cl- leading to cardiac arrest (Voyles et al. 2007; Campbell et al. 2012). In addition to electrolyte imbalances, animals become dehydrated, and are unable to rehydrate as efficiently as non-infected animals (Carver et al. 2010; Wardziak et al. 2013). *P. corroboree* is a species known in both captive and natural environments to cope with much drier conditions for extended periods and to have the ability, when dehydrated, to recover completely when rehydrated (D. Hunter and M. McFadden, pers. comm.; G. Marrantelli and L. Brannelly, pers. obs.; see Introduction). As this treatment experiment provided water daily and animals began to die just 36 h after being placed in a low-humidity environment, our results suggest that *Bd* infection reduces the animals' ability to rehydrate.

A *Bd*– dry treatment control group was not tested in this study. In this experiment we sought to determine the efficacy of drying as a treatment alternative, which would be determined either by decreased zoospore burden or increased survival of the treated animals; therefore, a *Bd*– treatment group was not essential to determine whether the treatment was successful. Additionally, *P. corroboree* is a critically endangered species and sample size is a limiting factor for all experiments involving endangered species. We relied on previous knowledge of the species' ability to cope with extreme water stress as a justification for choosing *P. corroboree* as the study species.

Climate change is a global phenomenon, and for amphibians that are currently surviving in *Bd*-infected habitats, reduced tolerance to drought conditions via chytridiomycosis could decrease survival during climatic changes. *P. corroboree* is a critically endangered species, and if there is a synergistic interaction between disease and periods of drought to increase mortality, the fate of the species could be further endangered.

The treatment regimen trial lasted for only 10 days. The treatment was terminated on the tenth day because 40% of animals died during the treatment period. We did not observe any decrease in zoospore load or time until death between the animals that survived treatment and the *Bd*+ control animals that did not undergo treatment,

suggesting that the methods used in this treatment regimen did not negatively affect the pathogen. In vitro, *Bd* desiccates within 1 h of water removal (Johnson et al. 2003), but because *Bd* is an intracellular pathogen, it is possible that the water provided to the animals cutaneously was enough to sustain infection. Treatment of a drying regimen may be a slow process, as it aims to minimise zoospore mobility and viability rather than kill the intracellular sporangia. If treatment time were extended, treatment would need to be less stressful for the host. A possible alternative treatment protocol is total cutaneous water deprivation with oral or parenteral rehydration as this might enable an extended but less extreme drying treatment to clear infection.

In this treatment trial the animals were maintained at 18–20°C, which is at the higher end of this species' preferred temperature range and within the optimal range for *Bd*. It is possible that a drying treatment would be successful if retested at a lower temperature, perhaps as low as 4°C for a longer period, which would slow the growth of *Bd* (Piotrowski et al. 2004), but be favourable to corroboree frogs. However, reduced evaporative water loss at lower temperatures could actually improve the conditions for the pathogen. Additionally, attempting treatments during lighter subclinical infections might decrease the synergistic effects of infection and water stress and allow for recovery.

This study demonstrated that a treatment trial using a drying regimen for treating chytridiomycosis was unsuccessful for *P. corroboree. P. corroboree* is a critically endangered species, with very few individuals remaining in the wild, and chytridiomycosis is the primary cause of decline for this species. Safe and effective treatment of this species is critical for the maintenance of these individuals in captivity. There are no published accounts of successful treatment for this species to date, and future research into treatment methods for this species should be focussed on chemotherapeutic antifungals that have been successful in other species, before reassessing drying as a method of treatment.

Acknowledgements

We thank R. Webb and S. Bell for help testing the animals for *Bd* infection, D. Hunter and M. McFadden for their contribution to the ecological and husbandry knowledge on *P. corroboree* and other *Pseudophryne* species, R. Speare for commenting on the

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Figure legend

Figure 1. Survival of animals after treatment began.

Day 0 indicates the first day of the low-humidity treatment. The light grey line represents animals that underwent the drying and rinsing treatment, the grey line represents animals that underwent the dry treatment and the black line represents animals in the *Bd*+ control group that did not undergo a treatment. The dotted black line represents the *Bd*– animals. The vertical arrow indicates the end of the treatment period (Day 10).

Figure 2. Median zoospore equivalents for each treatment group.

The light grey line represents animals that underwent the drying and rinsing treatment, the grey line represents the animals that underwent the dry treatment and the black line represents animals in the *Bd*+ control group. Week 0 is the start of the treatment period, which lasted 10 days. The vertical arrow indicates the end of the treatment period. Week –2 is one week after inoculation. The error bars indicate interquartile range.

Figure 3. Median zoospore load at day of death.

Bd+ control animals (black) that died during the treatment period when the treatment groups were undergoing treatment, animals in the treatment regimen that died during the treatment period (dark grey), and animals that survived the treatment regimen but died of chytridiomycosis after treatment period had been completed (light grey). The error bars indicate interquartile range

Figures

Figure 1

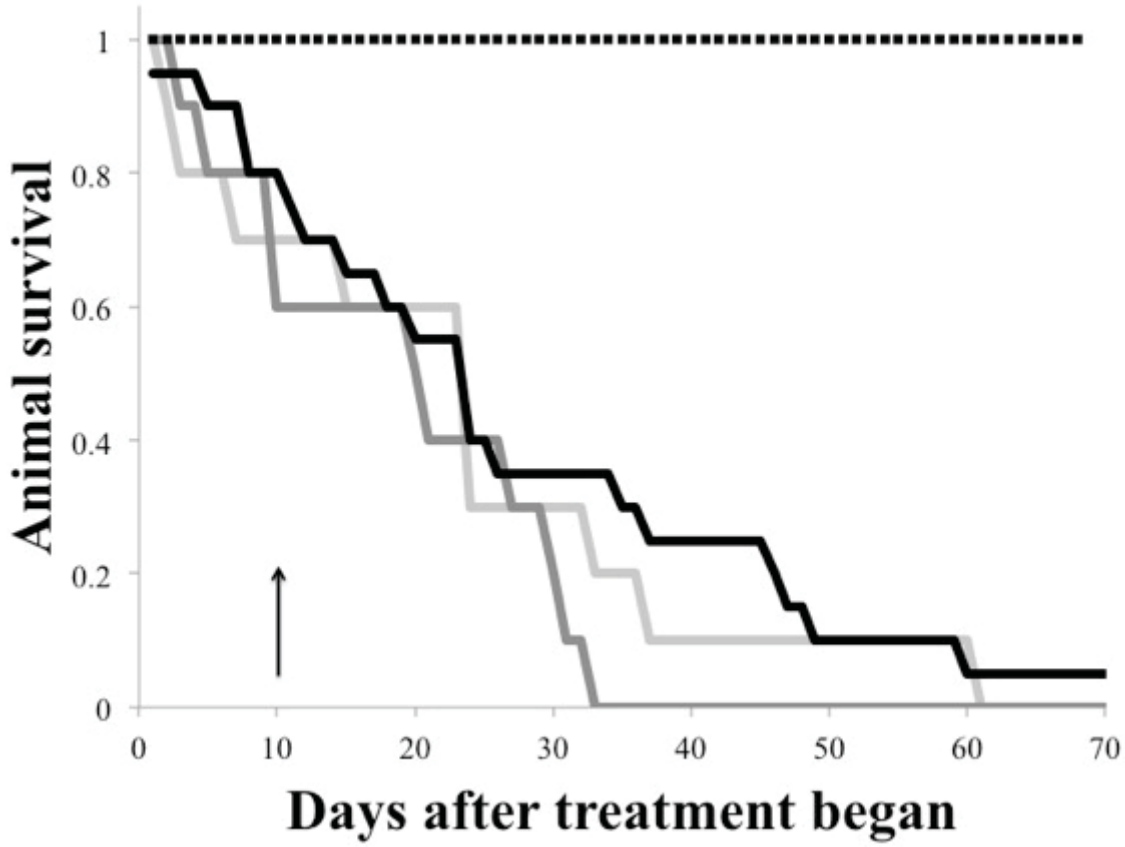
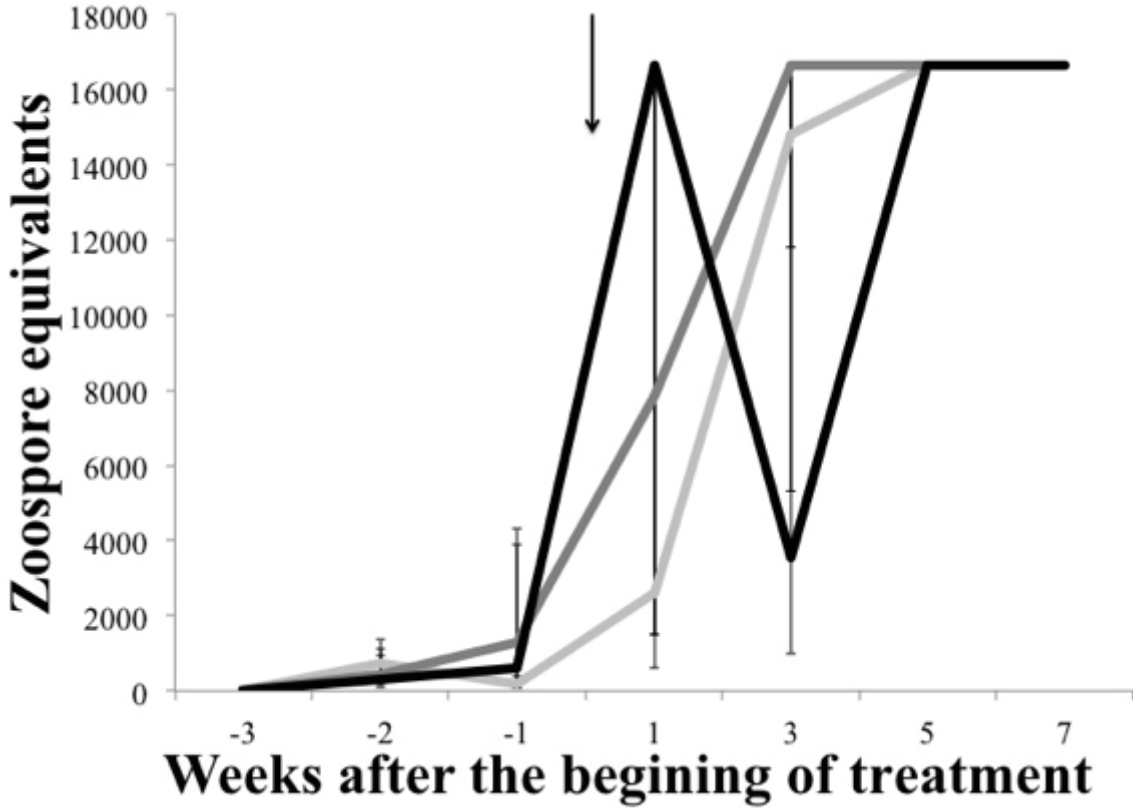


Figure 2


Zoospore equivalents
18000
16000
14000
12000
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8000
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4000
2000
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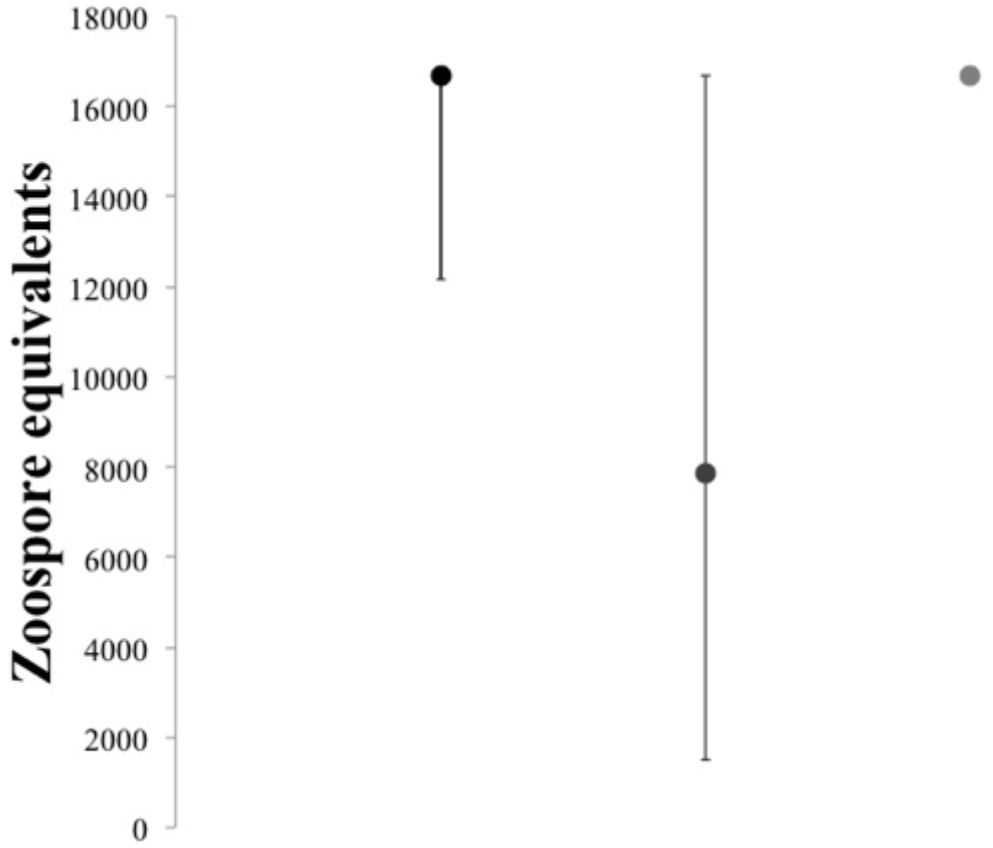
-3
-2
-1
1
3
5
7
Weeks after the begining of treatment

Figure 3


Zoospore equivalents
18000
16000
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12000
10000
8000
6000
4000
2000
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Paper 2

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Treatment trial of clinically ill amphibians with chytridiomycosis with two triazole antifungals and electrolyte therapy

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Abstract

Chytridiomycosis caused by the pathogen *Batrachochytrium dendrobatidis* is an important cause of amphibian declines globally, and is the worst pathogen on record for causing biodiversity loss. The critically endangered southern corroboree frog, *Pseudophryne corroboree*, is functionally extinct in the wild and is surviving in captive assurance colonies. These captive colonies must remain disease free, and there is no known treatment for corroboree frogs with terminal chytridiomycosis. In this study we tested two triazole antifungals (itraconazole and voriconazole) coupled with aggressive electrolyte therapy on moribund corroboree frogs with severe chytridiomycosis.

Six moribund frogs were given 20mL baths of 0.5μg/mL itraconazole for 5 min/day for five days coupled with electrolyte injections every eight hours for three days followed by every 12 hours for three days. Six other moribund frogs were given 1mL drops of 1.25μg/mL voriconazole daily for seven days coupled with the aggressive electrolyte therapy. While only one animal survived this treatment regime, time until death was extended for all animals by at least three days and infection load decreased by an average of 89.3%. Our results suggest there is potential for recovery of terminally ill *P. corroboree*, and we suggest further trials include antibiotics as well as exploring variations on the above treatment regime with other antifungals.

Keywords

Batrachochytrium dendrobatidis; Chytridiomycosis; Itraconazole; *Pseudophryne corroboree*; Treatment; Voriconazole

Introduction

Amphibians are in a desperate state of decline with over 30% of the world's species threatened: the highest threat of any vertebrate taxon (Stuart et al. 2004). Chytridiomycosis caused by a fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) is a primary cause of decline in over 200 species (Skerratt et al. 2007). With many species critically endangered, captive assurance colonies may be the only lifeline for some species.

The southern corroboree frog, *Pseudophryne corroboree*, is a critically endangered species endemic to Kosciuszko National Park in New South Wales, Australia. The species is highly susceptible to chytridiomycosis (Hunter et al. 2009; Brannelly et al. 2015) and is now functionally extinct in the wild, with only a handful of individuals remaining. The species is currently being sustained by captive assurance colonies in New South Wales and Victoria, Australia, with the hope of reintroduction (Hunter et al. 2010; Brannelly et al. 2015). The ability to maintain chytridiomycosis-free colonies and halt outbreaks is a high priority (Berger et al. 2010).

Bd can enter a captive colony, spread, and incubate unnoticed for weeks until clinical signs occur. Frogs with signs of disease deteriorate rapidly and become moribund within a few days. Current antifungal chemotherapies are able to treat subclinical chytridiomycosis, including frogs with heavy infections, but not animals with severe clinical disease (Pessier and Mendelson 2010; Berger et al. 2010; Baitchman and Pessier 2013; Jones et al. 2012). In captive assurance colonies for endangered species, saving every individual is important.

Chytridiomycosis causes death in amphibians by disturbing the electrolyte transport pathways and leading to cardiac arrest. Voyles et al. (2009) demonstrated that electrolyte therapy for moribund animals could prolong life by greater than 20 hours. The authors suggest that although electrolytes would not reverse the effects of chytridiomycosis, they may work in conjunction with antifungals to cure terminal animals. To test this hypothesis, Young et al. (2012) used electrolyte therapy in addition to chloramphenicol on three terminally ill *Litoria caerulea* and successfully cured their infection. This study has not been duplicated in threatened species or with other antimicrobials.

Effective treatments have not been extensively studied in *P. corroboree*. The antibacterial chloramphenicol appears to be an effective and safe treatment for *Bd* infection in *Litoria* species (Young et al. 2012). Chloramphenicol may be effective and appears to have few side effects for amphibians, yet its use in veterinary applications has been restricted in some countries because of the risk of chloramphenicol resistance in pathogens, and because human exposure has been associated with aplastic anemia (Page 1991). In addition, no clinical trials have been conducted to determine dosage and treatment, and treatment was unsuccessful in *Rana pipiens* at 200ug/ml of constant exposure for 56 days (Holden et al. 2014). The triazole antifungal itraconazole is popular for treating *Bd* infection in amphibians, with the recommended treatment regimen of 5min bath/day of 10μg/mL itraconazole for 11 days (Pessier and Mendelson 2010). Itraconazole has been shown to be effective at curing subclinical adult *P. corroboree*, although side effects of treatment in this species are unknown (5min bath/day of 5μg/mL for 10 days. Cashins pers. comm.; Marantelli pers. comm.). Aqueous itraconazole treatment, although effective at curing *Bd* infection, can cause death in certain species (particularly Ranids) and age groups (such as tadpoles) of amphibians (Pessier and Mendelson 2010). Direct toxicity to itraconazole has never been identified in amphibians, but the pH of the treatment solution has been suggested as a contributing factor (Georoff et al. 2013). Using itraconazole at lower doses and treatment times has reduced negative side effects of the therapy, while still remaining effective (Brannelly et al. 2012). Voriconazole is an alternative triazole antifungal that has been suggested as a safe antifungal treatment of *Bd* infection (Martel et al. 2011). Voriconazole is more water soluble than itraconazole, making delivery to amphibians easier, and is even safe for tadpoles (Martel et al. 2011). While these two triazole antifungals appear to be effective against *Bd* infection, we do not know the full extent of drug toxicity or long-term impacts of the treatment in amphibians.

We tested itraconazole and voriconazole in conjunction with electrolyte therapy on terminally ill *P. corroboree* with the hypothesis that this multifaceted treatment approach would cure amphibians with severe chytridiomycosis.

Methods

Animal husbandry

Adult southern corroboree frogs (*Pseudophryne corroboree*) were delivered to James Cook University from the Amphibian Research Centre. These frogs were excess to a captive breeding program, and this experiment was part of a larger study assessing innate resistance in this species. Animals were housed individually in 300 x 195 x 205mm terraria with damp paper towel substrate (Earthcare®, ABC Tissue), at a room temperature of 16–19°C. Animals were fed small (5–10mm) crickets (*Acheta domestica*) *ad libitum* three times weekly. Animals were misted twice daily with reverse osmosis water for sixty seconds. Terraria were cleaned fortnightly by replacing the paper towel substrate. Once a week animals were swabbed for *Bd* presence (outlined below), weighed and measured snout to venter length (SVL).

Inoculation

Animals were acclimatised to their environment for one week after their delivery to James Cook University before the study commenced. Animals (n = 23; 12 treated, 11 untreated) were inoculated with a New South Wales strain of *Bd* (AbercrombieR-L.booroolongensis-2009-LB1) at passage 11. *Bd* was harvested from tryptone, gelatin hydrolysate, lactose (TGhL) agar plates after incubation at 23°C for 5 days. Plates were flooded once with 3mL of artificial spring water before harvesting zoospores and counting with a hemocytometer. Animals were placed in 40mL clear plastic containers (Genfac Plastics Pty Ltd) and inoculated with $1x10^6$ zoospores by applying 3mL of inoculum onto the venter. After 6 hours they were transferred back into their terraria. Uninfected control animals (n = 6) were inoculated with solution collected off sterile nutrient agar.

Treatment

Animals were checked daily for clinical signs of chytridiomycosis and morbidity using three steps. The first step was checking for movement when the terrarium was tilted. If the animal moved (head bob, adjustment of limb), they were considered alert. The second step involved touching the back of the animal and checking for attempts to escape. Thirdly, the righting reflex was assessed by positioning the frog on its back. If the

animal failed all three alertness steps it was considered moribund and treatment commenced (Brannelly et al. 2015).

Moribund animals entered treatment between day 47 and 54 post inoculation. Animals were randomly assigned a treatment group, six underwent itraconazole and electrolyte treatment, six underwent voriconazole and electrolyte treatment, and 11 received no treatment. The six *Bd*-negative control animals underwent no treatment. No *Bd*-negative azole plus electrolyte therapy treatment group was incorporated in this study. During azole and electrolyte treatment animals were removed from their large terraria and placed in a smaller terraria (200 x 150 x 100 mm) with a damp paper towel substrate. Terraria were changed once daily during the treatment period. Upon treatment completion, animals were returned to the larger terraria.

Upon morbidity, animals that were assigned to either treatment group were swabbed, weighed and then injected subcutaneously with Hartmann's solution at 50mL/kg with a 26g needle (averaging 0.7mL per frog, mean mass = 1.48g +/- 0.34) into the left side of the venter (Young et al. 2010). Where possible, the injection site was the same as the day before to reduce wounds. Electrolyte therapy was administered every eight hours for the first three days, and every 12 hours for the next three days. Six moribund animals were treated with itraconazole, and six were treated with voriconazole. Itraconazole (Sporanox® solution, Janssen-Cilag Pty Ltd) was diluted to 0.5µg/mL in Amphibian Ringers solution (Garner et al. 2009; Pessier and Mendelson 2010) and applied as a 20mL bath for five minutes a day for six days. Baths occurred 30 minutes after the electrolyte injection. Voriconazole (VFEND® solution, Pfizer Inc.) was diluted to 1.25µg/mL in reverse osmosis water (Martel et al. 2011) and given daily for 7 days as 1mL applied to their whole dorsum where it dripped around to their venter to form a puddle, which they sat in for 5min. Eleven animals were left untreated and served as *Bd*+ no-treatment controls. The uninfected controls did not undergo treatment. After daily antifungal treatments, animals were returned to disinfected terraria (10 x 6 x 6cm). On the last day of treatment, surviving animals were returned to their larger terraria. Each week for four weeks after treatment finished animals were swabbed, weighed and measured.

Upon morbidity all treated and positive control animals were euthanized with an overdose of MS222 in compliance with animal ethics: no animal died naturally. On date of death, animals were weighed, swabbed and fixed in 4% phosphate buffered formaldehyde for histological examination. At the end of the experiment, four weeks

after treatment finished, all negative control animals were euthanized following the same method and preserved. One animal survived treatment and was not euthanized.

Histological examination

Skin tissue from all treatment animals was examined histologically from the venter, thigh and the plantar surface of the left hind foot. Tissues were fixed in 4% phosphate buffered formaldehyde, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin according to standard histology protocols. A further two animals from each of the four groups (*Bd*+ non-treatment controls, *Bd*- non-treatment controls, itraconazole treated, voriconazole treated) were histologically examined in full. The tissues examined were liver, kidney, spleen, reproductive organs, heart, lungs, digestive tract, brain and spinal chord and processed the same as above. Histopathology examination was carried out with the help of trained veterinary pathologists.

Testing for Bd

Frogs were tested for *Bd* infection by using skin swabs and a qPCR assay (Boyle et al. 2004). The swabbing protocol was standardized by performing 45 strokes with a sterile rayon-tipped swab (MW-113, Medical Wire & Equipment) per animal, five on the venter, five on each thigh, each side and each limb. The swab was gently rotated during and between strokes to ensure the greatest amount of DNA was gathered on the swab. DNA was extracted from the swabs using a bead beater for two minutes per sample to open the fungal cell wall and Prepman Ultra (Applied Biosystems®, Life Technologies Pty Ltd) and diluted 6:100 in PCR grade water. The samples were then analysed using quantitative real time PCR (qPCR) following Boyle et al. (2004). Samples were run in singlicate with a positive and negative control, and a series of dilution standards. Zoospore equivalents (ZE) were calculated as the number of *Bd* zoospores present on the swab, and used to quantify infection intensity.

Bd *as a biohazard*

All animals were handled with a new nitrile glove, which was changed between each animal or substrate. Countertops were disinfected with 4% bleach daily, and laboratory floors were disinfected with 4% bleach three times per week. Between uses,

terraria were soaked for at least 30sec in 4% bleach, rinsed twice with running water, and then dried for over 24hr.

Statistical analysis

Infection loads were compared between the start and end of treatment, and between itraconazole and voriconazole treated animals at end of treatment using paired and independent t-tests respectively on logarithmic base 10 transformed ZE values. Effect size was determines using Cohen's d statistic. Survival was compared between treatment groups, and with *Bd*+ non-treatment controls using Kaplan-Meier Survival tests. All analyses were completed in SPSS (v21).

Results

Treatment survival and clinical diagnosis

Treatment extended survival by at least three days compared to *Bd*+ control animals (Kaplan-Meier: $\chi^2_{1,22} = 22.00$, $p < 0.01$) (Fig.1). One animal (treated with itraconazole) was successfully cleared of *Bd* infection and fully recovered from severe chytridiomycosis. There was no difference in survival between the itraconazole and the voriconazole treatment groups (Kaplan-Meier: $\chi^2_{1,11} = 1.439$, $p = 0.23$; Fig. 1).

Once frogs showed clinical signs they deteriorated rapidly: treatment commenced when frogs appeared moribund with three of 12 unable to move. All animals recovered their righting reflex, mobility and alertness less than 8hr after the first electrolyte injection and triazole treatment, and normal feeding behaviour returned between one and three days of the first treatment in all animals. In the voriconazole treatment, 100% of the animals died during or directly after treatment finished, and in the itraconazole treatment, four of the six animals survived the treatment, but three of those animals died by day ten.

All 12 treated animals (both itraconazole and voriconazole treated) developed ulcerative, haemorrhagic lesions on the feet, nine also had lesions on the hands, and three on the pelvic patch (Table 1). The one survivor had lesions and ulcers on hands, feet and pelvic patch, and took longer than 8 weeks to heal completely from the ulcerations. All animals experienced irregular skin sloughing (1 – 5 mm^2 sections flaked off during handling) throughout the treatment period. Lethargy returned between 18hr and eight days after treatment began in all animals, often associated with the formation of skin

ulcerations. For the voriconazole treatment, animals became lethargic at an average of two days after treatment commenced, and for the itraconazole treatment, an average of three days.

Infection status

Infection loads of all animals increased throughout infection (Fig. 2a) and when the animals entered treatment, their *Bd* loads were very high (303,391 ZE +/- 221,516) and did not differ between the two treatment groups (Independent T-Test: $t_{10} = -1.082$, $p = 0.305$). Treatment with either triazole significantly decreased the infection burden of the animals (Paired T-Test: $t_8 = 3.469$, $p < 0.01$). Treatment reduced infection by an average of 99.8% in itraconazole treated animals, and 76.3% in voriconazole treated animals. Upon date of death, or four weeks after treatment (for the one animal that survived) infection load for itraconazole treated animals (523 ZE +/-1,263) had 99.5% fewer zoospores after treatment than the voriconazole treated animals (106,646 ZE +/- 178,768) (Fig. 2a) (Independent T-Test: $t_{10} = -5.857$, $p < 0.01$, $d = 3.38$).

Histopathological findings

The two *Bd* infected controls had typical heavy *Bd* infections, whereas there were few sporangia observed in four of six voriconazole treated animals through histology, and one of the itraconazole treated animals (Table 1) (3). Infection in controls was associated with typical lesions such as hyperkeratosis, erosions, and irregular epidermal layers on feet, thighs and ventral skin (Fig. 3b). Skin lesions in both voriconazole and itraconazole treated frogs were more severe, with eroded, necrotic, thin or disordered epidermis, and ulceration (Fig. 3c). Histological evidence of suprabasal acantholysis was observed in epidermal cells attached to the basement membrane with a tombstone appearance (Fig. 3d). There was some evidence of epidermal regeneration with flattened epidermal cells expanding to cover the dermis (3d). Melanin is typically found in the dermis in amphibian skin (Fig. 3a,b), but in 9 / 12 treated animal, melanin was also found throughout the epidermis (Fig. 3e). Bacterial overgrowths occurred in hyperkeratotic skin layers and ulcerations in all itraconazole and voriconazole treated animals (Table 1), but bacteria were not seen in internal organs of those where full histopathology was done. No abnormalities were seen in the skin of the uninfected controls examined, and a 2-cell layer stratum corneum was noted (Fig. 3a).

Examination of internal organs in two animals per group did not reveal any severe or consistent lesions that were likely to have caused death. There were no noticeable differences observed between the treated animals and the *Bd*-positive untreated animals, and were consistent with typical acute terminal changes in these animals.

Discussion

Treatment efficacy

While electrolyte injections and triazole chemotherapy treatment increased time until death, few animals survived long enough to complete treatment. Only 33% of itraconazole treated animals survived six days of treatment, and 17% of voriconazole animals survived seven days of treatment. Only one animal, an itraconazole treated animal, survived past the end of treatment and was able to clear *Bd* infection completely.

The most promising result was that moribund animals, with no righting reflex and little to no response to external stimulation regained full mobility and responsiveness within a few hours of the first electrolyte injection and treatment. Normal behaviour was regained within the first day of treatment. Survival was extended by at least three days after treatment commenced. Electrolyte supplementation alone can prolong survival of infected frogs a few days (Voyles et al., 2009), which may be enough time for an effective treatment to enable recovery.

We chose electrolyte supplementation via subcutaneous injection to ensure systemic delivery, as described by Young et al. (2012). Electrolyte baths would be less stressful and have been suggested as an alternative to injections to reduce stress in sick animals (Pessier and Mendelson 2010; Young et al. 2012; Baitchman and Pessier 2013). However, in animals clinically ill with chytridiomycosis, absorption through the skin may be compromised (Carver et al. 2005; Brannelly et al. 2015) and therefore delivery is uncertain and needs testing. Oral electrolyte dosing appears effective (Voyles et al. 2009) but is also stressful.

In both triazole treatment trials, infection burden was significantly reduced, as determined by qPCR and histology. Quantitative real time PCR is a sensitive and specific test for *Bd* detection, but cannot determine viability of zoospores. Histology is less sensitive but allows for visualization of sporangia and zoospores within the skin (Skerratt et al. 2011). Using both methods of detection after treatment provides a greater

understanding of the effectiveness of treatment. There were few itraconazole treated frogs (in one of six animals) with *Bd* sporangia detectable by histology, and qPCR revealed an average 99.8% decrease in infection load after treatment. Voriconazole was less effective and reduced infection by only 76.3% and sporangia were visible on histology in the skin in four of six animals. The qPCR and histology results were surprising given the short treatment in some animals, as short as three days, and some animals died before treatment completed. Voriconazole had never been trialled on *P. corroboree* before, and the treatment dose and time followed the one published study using this treatment (Martel et al. 2011). The itraconazole dosage and treatment time was 1/20 the standard dose, and half the treatment time. It was an even lower dose than the lowest successful dose reported by Brannelly et al. (2012), and a shorter treatment time than was successfully used in tadpoles (Garner et al. 2009). The treatment was successful at reducing the intensity of infection but recovery did not occur, likely due to the remaining severe lesions on the skin.

Histopathology

There was evidence of epidermal regeneration and in five treated frogs some parts of the skin appeared fully intact with no infection. Treated frogs also had areas of thin epidermis that appeared to be regenerating with flattened, attenuated epidermal cells (Fig. 3c,d). Curiously, we found melanin scattered through some parts of the epidermis in ten of the 12 treated animals (Fig. 3e), but not in either control group. While we do not know the significance of melanin being incorporated into to epithelium, this likely occurred after ulceration damaged the basement membrane and melanocytes. It is possible that during regeneration of the epithelium the loose melanin was incorporated into the epidermis (Fig. 3e).

Grossly visible skin damage with ulceration and haemorrhage occurred in all animals that died during treatment. Histopathology revealed treated animals had more skin ulceration, necrosis and foci of bacteria in skin than infected controls. Although septicaemia was not observed, superficial bacterial overgrowths may have contributed to the deaths. Frogs with clinical chytridiomycosis tend to have minor skin damage (hyperplasia, hyperkeratosis, erosions) or more severe lesions such as ulcerations especially on the hands and feet or other points of pressure (Berger et al. 2005). The treatments or the extended survival and increased mobility observed in this study

appeared to exacerbate epidermal lesions. While the specific cause of these lesions are unknown, they occurred on the plantar surface of the fore and hind limbs, and in extreme cases the pelvic patch, which suggests deterioration of already damaged skin from heavy infection at points of surface pressure, rather than a side effect of the azole treatment

Implications and future directions

Combining an antibacterial treatment along with an antifungal, especially at the site of lesions on the fore and hind limbs, may improve survival in moribund frogs. The previous study that successfully treated moribund frogs involved a larger species and used chloramphenicol, which has antibacterial and antifungal properties, and severe skin lesions did not occur (Young et al. 2012). Chloramphenicol as a treatment in veterinary practice has been restricted in some countries. The dose and treatment time has not been optimized, but is most commonly used for chytridiomycosis as a constant submersion for several weeks (Young et al. 2012). Therefore, chloramphenicol bath may not be an appropriate treatment for terrestrial species such as *Pseudophryne corroboree*, and an alternative antibiotic, or chloramphenicol ointment (Bishop et al. 2009), should be trialled.

Although the two triazole antifungals along with the electrolyte therapy trialled in this study did not cure all frogs, infection was significantly reduced, and the survival time of the animals was significantly extended. The extended survival leaves hope for future treatment of moribund southern corroboree frogs using an alternative but more effective treatment, combined with an antibiotic, because all animals developed lesions and bacterial overgrowth on the skin. As one of 12 (8%) of the animals in the treatment trial was cured of infection and recovered, this indicates it is possible to save terminally ill corroboree frogs, and further research is needed to develop a more successful treatment protocol.

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Conflict of interest

The authors declare they have no conflicts of interest in the research undertaken for this study.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of James Cook University Animal Ethics Council, and the study was conducted under ethics application A1875 entitled “innate and adaptive immune mechanisms against amphibian chytrid fungus and non-chemotherapeutic treatment methods.”

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Tables

Table 1. Description of each treated individual, including snout to vent lengths, mass in g before infection, at morbidity and start of treatment, and then at death or completion of treatment.

Infection intensity (in zoospore equivalents, ZE) at morbidity and then at end of treatment is also displayed. Position of visible ulcers at the start and end of treatment is noted. Finally, skin pathology for each animal was recorded in the thigh, ventral and skin of the planar surface of the back left foot. Observed pathology in the skin tissue is recorded.

Itraconazole Treatment						
ID	I1	I2	I3	I4	I5	
Days survived after treatment began	3	5	6	7	10	Recover
SVL (mm)	29.3	24.6	27.4	26.7	25.0mm	26.3m
$Mass_0$ (g)	2.3	1.5	2.1	2.1	1.3	2
$Mass_{Treatment}$	1.9	1.4	1.0	1.8	1.5	1
$Mass_{death}$	1.3	0.9	0.7	1.2	1.5	
$ZE_{morbidity}$	266,766	136,845	265,991	397,066	151,095	91,41
$ZE_{end\_treatment}$	3,101	33	2	0	0	
Ulcers before treatment	none	none	one hind foot	one hind foot	none	no
Day of first ulcer	3	4	0	0	2	
Ulcers after treatment	fore and hind feet	hind feet	fore and hind feet	fore and hind feet	pelvic patch, fore and hind feet	pelv patch, fore and hi fe
Skin pathology:						
Parts of skin look intact (recovered)	x	x		x		
Melanin present in epidermis	x	x	X	x	x	

Hyperkeratosis	x	x	X	x	x	
Thin epidermis, erosion	x					
Ulceration	x	x	X	x	x	
Severe ulceration (muscle is exposed)	x	x			x	
Red blood cells exposed on surface	x	x	X		x	
Bacterial infection	x	x	X	x	x	
Bd Sporangia	x					

Voriconazole Treatment

ID	V1	V2	V3	V4	V5	V
Days survived after treatment began	3	5	6	6	8	
SVL (mm)	22.6	24.4	27.7	27.8	28.3	30
$Mass_0$ (g)	1.6	1.6	2.0	1.7	1.5	2
$Mass_{Treatment}$	1.3	1.1	2.0	1.3	1.3	1
$Mass_{death}$	1.0	1.0	1.1	0.9	0.9	1
$ZE_{morbidity}$	498,250	198,443	770,959	206,960	90,532	467,8(
$ZE_{end\_treatment}$	5,987	77,516	29,100	13,432	46,042	315,5
Ulcers before treatment	hind foot	none	none	hind feet	none	no
Day of first ulcer	0	1	1	0	1	
Ulcers after treatment	fore and hind feet	fore and hind feet	fore and hind feet	pelvic patch, fore and hind feet	hind feet	one hi fo

Skin pathology:

Parts of skin look intact (recovered)			X		x	
Melanin present in epidermis	x			x	x	
Hyperkeratosis	x	x		x		
Thin epidermis, erosion	x		X		x	x
	x	x	x	x	x	x

Ulceration						
Severe ulceration (muscle is exposed)				x		
Red blood cells exposed on surface		x				x
Bacterial infection	x	x	X	x	x	x
Bd Sporangia		x		x	x	x

Figure legend

Figure 1. Survival of critically ill animals over time.

The solid black line: untreated animals with severe chytridiomycosis starting on the day they became morbid (n = 12). The light grey line: animals treated with electrolyte therapy and voriconazole. Treatment of voriconazole lasted seven days (n = 6). The dark grey line: animals treated with electrolyte therapy and itraconazole. Treatment with itraconazole lasted six days (n = 6).

Figure 2. Average infection load for treatment groups.

a) Infection over time of all animals throughout the experiment. Inoculation occurred at week 0, and infection intensity was measured every other week until morbidity, which is illustrated as week 9. Groups are combined because there was no difference in infection loads among the itraconazole treated, voriconazole treated and *Bd*+ control animals. b) Zoospore equivalents at the start of treatment and upon date of death or 4 weeks after treatment completed. The light grey line: voriconazole treatment. The dark grey line: itraconazole treatment. Infection intensity was calculated by the log, base 10 of zoospore equivalents. Error bars indicate standard error.

Figure 3. Ventral skin from *Pseudophryne corroboree*.

a) Uninfected healthy frog skin. Notice the two layers of the stratum corneum. b) *Bd* infected, untreated skin. Hyperkeratosis, erosions, irregular and degenerate epidermal layers, and *Bd* sporangia. c) Itraconazole treated skin. No *Bd* sporangia, epidermis is eroded, with a thin basal layer remaining. d) Itraconazole treated skin. Some epidermal cells are flattened and appear to be spreading to cover the dermis (A), and some epidermal cells have a tombstone appearance (B). e) Itraconazole treated skin. Epidermis appears intact, and melanin granules are scattered through cell layers. Melanin may have been incorporated into the epidermis during regeneration. Magnification 200x, scale bar is 0.05mm.

Figures

Figure 1

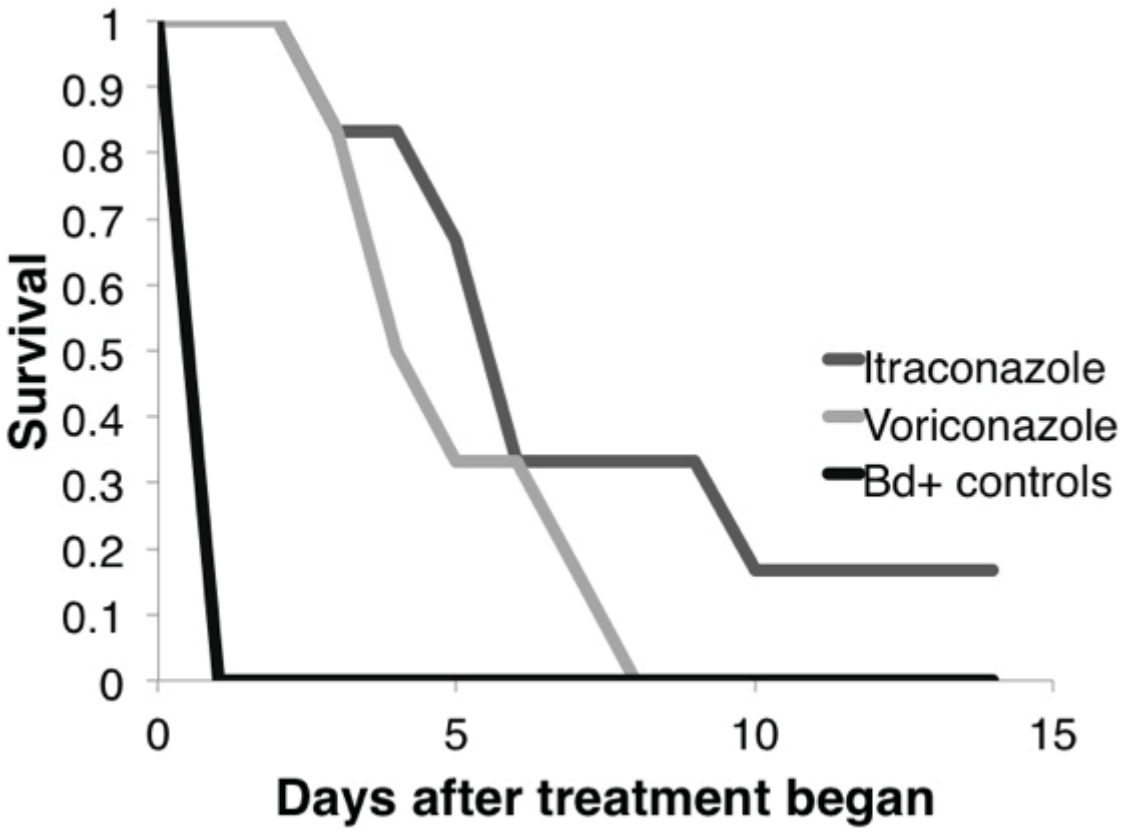
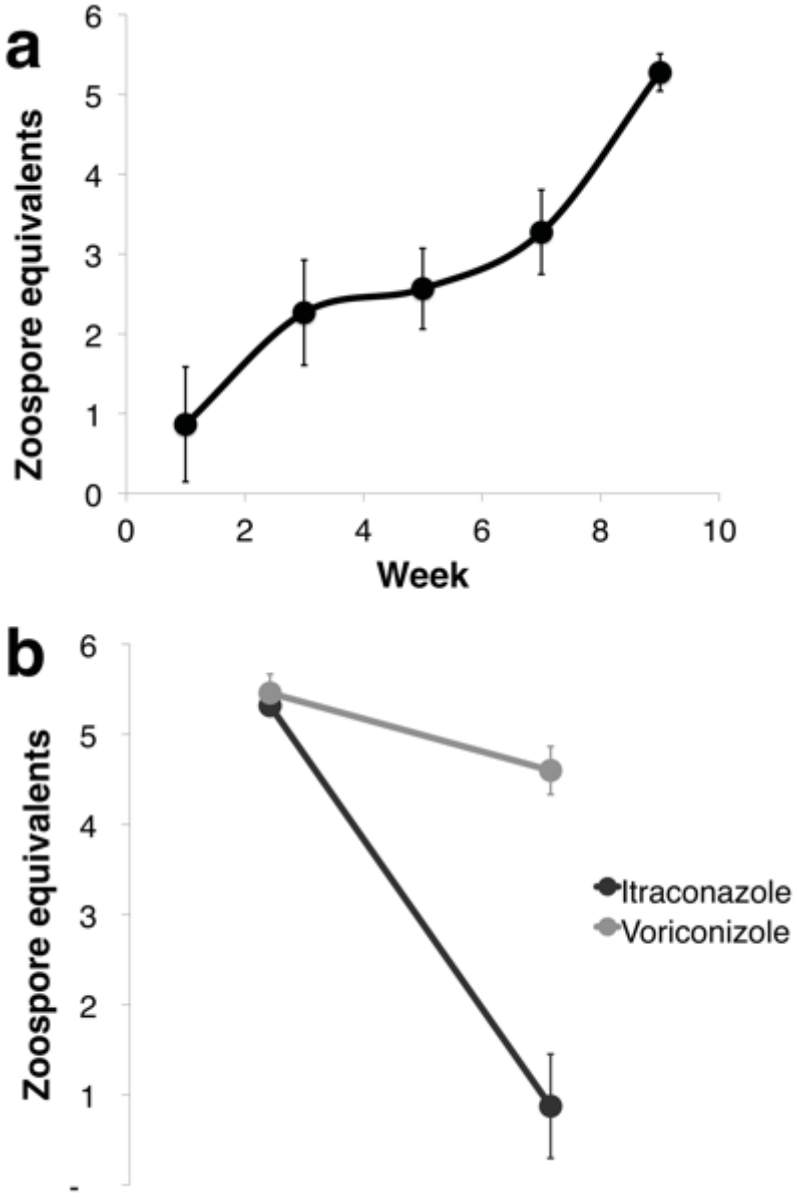


Figure 2


a
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0
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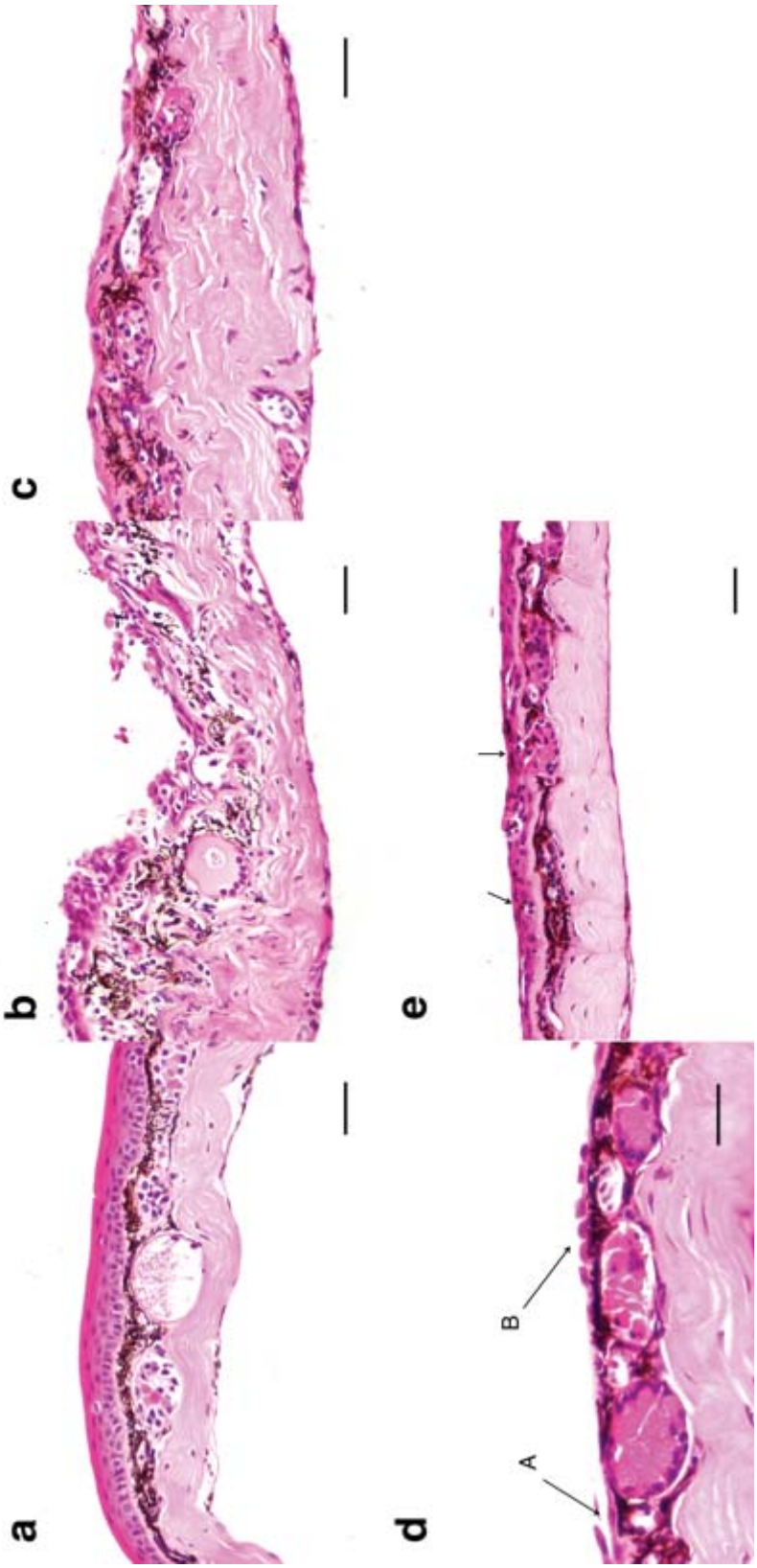
Itraconazole
Voriconizole

Figure 3



CHAPTER 8

Conclusions and management implications

The overarching theme of my thesis research was to explore the impact of chytridiomycosis on understudied and declining species, particularly focused on the alpine regions of temperate Australia. Since the discovery of the cause of chytridiomycosis, *Batrachochytrium dendrobatidis*, *Bd*, in 1998, researchers globally have been investigating the impacts, outcomes and immune mechanisms of this disease on amphibians. However, much of the research focus has been on commercially available species, like *Xenopus laevis, X. tropicalis, Rana pipiens, Rana catesbeiana* and other species that are often tolerant of infection and have not experienced declines in the wild due to *Bd* infection. Susceptible and declining species, like *Litoria verreauxii alpina* and *Pseudophryne corroboree* remain understudied, yet require adequate research to help inform management of these highly endangered/functionally extinct species. In this chapter I highlight my major discoveries in the context of their relevance to applied outcomes.

Understanding disease ecology

Understanding the context-specific disease ecology of a species is crucial for devising effective and efficient conservation management to mitigate chytridiomycosis. In chapter two of this thesis (Brannelly et al. 2015b), I explored the factors that influence disease within the breeding season of a critically endangered species, and found that despite coexistence with *Bd* for more than 20 years, infection prevalence is near 100% by the end of the breeding season, intensity of infection is high and there is little recovery. These results, along with other studies conducted by my collaborators (Scheele 2015; Scheele et al. 2015), suggest that *Bd*-free *L. v. alpina* are arriving at ponds for the start breeding season, gaining an infection throughout the breeding season and then succumbing to disease post-breeding. Disease has caused this species to shift in its life history; individuals now survive only one breeding event. Such a shift prioritises recruitment for population persistence (Muths et al. 2011; Phillott et al. 2013; Scheele et al. 2015), and as such, management efforts should focus on habitat management.

Habitat management should include increasing water body size to reduce the impacts and effects of drought, which minimise tadpole death due to desiccation and ensure progeny survival. Infection spread is density dependant in *L. v. alpina* (Brannelly et al. 2015b); therefore, habitat management should also focus on decreasing population

density. I suggest that managed sites should focus on increasing water body size, while maintaining breeding habitat area. The increased water volume will lessen the density of the pathogen within the water body. In addition, managed sites should also aim to increase water flow in and out of the water bodies. Increased flow will buffer the effects of drought by ensuring water presence throughout the breeding season and tadpole development, while also reducing pathogen occurrence. Because recruitment is important for population persistence, habitat management is a priority for the preservation of this species.

The impact of disease on reproduction efforts

Because an important mechanism of population persistence is high recruitment and reproduction after high adult mortality at the end of each breeding season, I explored the influence of disease on reproductive output in two species. The effect of disease on reproduction in amphibians is rarely studied: only two published studies to date have investigated the effects of chytridiomycosis on reproduction and reproductive effort, one of which was undertaken as part of my Masters of Science research at Tulane University (Chatfield et al. 2013). I lead the reproductive aspect of that study and found that testis size increased in animals that were infected with *Bd* (Chatfield et al. 2013). The second study determined that infected animals in good body condition were more likely to be found calling than uninfected males or infected males in poor body condition (Roznik et al. 2015). Both of these previous studies investigated only one aspect of reproductive effort and only investigated males. In Chapter 3, I investigated the effects of disease on gametogenesis in males and females, and explored the effects in two distinct species. The results of this study support the previous findings and suggest that disease is causing animals to increase reproductive effort: a process called terminal investment.

Terminal investment in the *Bd*-amphibian system is an important finding for population and species management because it can direct population evolution. In a species that is highly susceptible to *Bd* infection and disease, if animals are prioritising reproductive effort over self-maintenance and survival, they will produce susceptible offspring. If those susceptible and infected animals produce more offspring than uninfected animals, this will reduce any evolution towards disease resistance. This

pattern may explain why there is little evidence of an effective immune response in *L. v. alpina* populations with endemic infection (Bataille et al. 2015; Grogan 2015).

While populations of *L. v. alpina* appear to be stable in the habitats in which they currently persist, relying solely on recruitment for population persistence is a risky strategy. Climate change is impacting the Australian Alps more dramatically than many other regions of the world (Hughes 2003; Bicknell & McManus 2006), and drought, fire or other extreme climatic event could affect recruitment. If extreme climatic events were to impact the breeding habitats over multiple years, the population could be decimated. While high recruitment and reproductive effort is important in the short term for bolstering population size, the most sustainable population persistence mechanism is decreased disease susceptibility. Management efforts should focus on decreasing population density in this species because with decreased population density, transmission and mortality rates may fall. Frogs with higher levels of resistance may be enabled to survive multiple years, hence allowing resistance to evolve naturally.

The importance of trialling management techniques

While it is important to understand disease impacts, disease dynamics, and mechanisms of population persistence in declining and endangered species, urgent interventions need to be initiated based on current knowledge. While resources should be invested in this research, management trials should occur concurrently. The second paper of Chapter 2 (Brannelly et al. 2015c) trialled an experimentally designed reintroduction program understanding the disease ecology and infection dynamic of the critically endangered *L. v. alpina*. I assessed the affect of source population and site of release by releasing animals from three populations with different population exposure histories, into four sites. I was able to recapture released animals months after release, and found that released animals were similar in both survival and disease susceptibility to extant animals at the different sites. The results of this reintroduction trial are encouraging, and suggest that reintroduction might be a valid and valuable management technique for this species.

Population of origin appeared to influence release success. I found that frogs sourced from one population of long disease exposure (>20 years) had higher survivorship than released animals of a second population of long disease exposure and

animals from a *Bd*-naïve population. This result suggests that disease exposure of the population might influence release success. Because *Bd* is the key threatening process causing the declines, we assume that some disease resistance has evolved, and might explain the difference in survival between the released populations. A laboratory infection trial with *L. v. alpina* investigating susceptibility between populations revealed that populations differed slightly in susceptibility (Grogan 2015); some populations had higher survivorship than others and survivorship was related to exposure history. My reintroduction trial and the lab susceptibility trial seem to draw the same conclusion; that population exposure history influences survivorship when exposed to disease. However, it is interesting to note that the long-exposed populations with higher survivorship were different populations in both the laboratory trial and the field trial, even though the same populations were trialled in both studies. The population that did not survive to recapture as well in the release was the population with the lowest susceptibility to disease in the lab (Brannelly et al. 2015c; Grogan 2015).

The implications of these disparate findings are important to consider when investigating the effects of disease and potential management opportunities. While it seems obvious that reduced susceptibility to disease might confer improved survivorship, there are a multitude of other factors that contribute to survival of an individual. Reduced susceptibility may come at a survivorship cost when released into the wild for *L. v. alpina*, and other factors may be more important, such as predator avoidance behaviour (Brannelly et al. 2015c). While selection for disease resistance has been successful in livestock (Bishop et al. 2002; Bishop & Morris 2007), it has not yet been attempted for any wildlife (Woodhams et al. 2011; Scheele et al. 2014). Improving fitness of free-ranging animals is obviously a complicated objective, especially as environmental conditions are much more changeable than for captive animals where individuals with uniform phenotypes can all survive. Many factors need to be considered in breeding plans for endangered wildlife, and my research has been a first step.

A similar approach can be undertaken using results from Chapter 6 of this thesis: an investigation of difference in susceptibility between populations of *Pseudophryne corroboree*. The results of that trial suggest that susceptibility to disease varies across populations, and one population was substantially more susceptible to *Bd* infection and disease than the others, and one population had higher survivorship. While these results suggest that breeding efforts be focused on the less susceptible population, susceptibility

to disease in the lab may not be the most important factor in survivorship of individuals. A preliminary reintroduction trial is a proactive step for both management and research objectives. *Pseudophryne corroboree* is a longer lived species with lower fecundity than *L. v. alpina* (see Chapter 1); therefore, there may have been more previous opportunity for evolution of disease resistance due to more generations since the introduction of *Bd* into the system. Alternatively, attempting to increase recruitment in this species may be an efficient approach.

While it is important to investigate many factors when deciding appropriate management options, and it is also important to trial management options so that past failures can inform future directions.

Investigating underexplored pathogenesis of *Bd* infection

Although *Bd*, the pathogenic agent of chytridiomycosis, was discovered and identified nearly 20 years ago, it remains unclear what host qualities allow for *Bd* infection to develop into the disease chytridiomycosis. The pathogen appears to cause immunosuppression in the host, or may evade detection while it reproduces and spreads. Epidermal damage leads to disrupted homeostasis as amphibian skin is important for ion exchange, and water and oxygen uptake and regulation. Understanding pathogenesis and how it varies between species is an important step for mitigating disease impacts, and also developing effective treatment options. Chapters 4 and 5 of this thesis investigated pathogenesis of disease on susceptible and endangered species, where I investigated two disease impacts: hematopoietic tissue depletion within different organs in order to investigate immunosuppression, and epidermal apoptosis in order to assess tissue damage by the pathogen. I found that hematopoietic tissue types and species vary in depletion patterns, suggesting that immunosuppression by the disease differs between hosts. I found that apoptosis in the epidermis is suppressed early in infection and then increases towards the development of chytridiomycosis. Both of these pathologies are understudied in the literature, they might represent important mechanisms to help determine species and individual differences in susceptibility to disease. In addition, mechanisms of pathogenesis are important for developing treatment options. Safe and effective treatments are important for captive managers because maintaining healthy and disease

free captive colonies are imperative to the conservation effort. Currently, no treatment works across species and age classes, and sensitive species require the development of new and effective treatment options (Brannelly et al. 2012, 2015a, 2015d; Brannelly 2014). Treatments that address the specific pathogenesis of infection might be more successful than the current treatment options available.

Conclusions

Amphibians are dying globally at an alarming rate, and alpine species in seemingly pristine habitats have been hit particularly hard by the fungal pathogen *Bd.* I focused on endangered and functionally extinct amphibian species endemic to the alpine region New South Wales and Victoria for my PhD research because knowledge is needed to optimise current management efforts focused on protecting these species. My research was devised in conjunction with amphibian managers and has contributed key knowledge on individual and population level effects of disease on these species. As species differ greatly in disease ecology and pathogenesis, research needs to focus on the target species. With the support of conservation agencies and zoos, I have been able to use individuals of endangered species that are excess to reintroduction programs. By focusing on understudied susceptible and declining species, we can begin to understand the direct impacts of disease on the ecology, susceptibility, mechanism of population persistence and pathogenesis, which will be directly used by conservationists, captive breeders and land managers. The collaboration between research and management is imperative to the protection of declining species. As frogs have gone extinct in pristine areas, protection of habitat is not sufficient to counteract an invasive disease – proactive and targeted interventions are required to save Australia's most endangered frogs.

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