Contrasting population responses of ecologically-similar sympatric species to multiple threatening processes

By

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Bachelor of Science
Masters of Reproductive Science

This thesis is submitted in total fulfilment of the requirements for the degree of Doctor of Philosophy

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Produced on archival quality paper

23 December 2015
Abstract

Understanding drivers of population change is crucial when species are declining. However, uncertainty about species declines and causal factors are pervasive problems hampering conservation efforts. This is because populations can naturally fluctuate and pre-decline population data are often insufficient to diagnose causes of decline which may be driven by multiple threats, environmental processes and possible interactions. Globally, amphibians are declining at a rate exceeding other assessed vertebrate taxa. Chytrid fungus (*Batrachochytrium dendrobatidis*) is a key threat that has caused rapid amphibian declines. However, species and population responses to chytrid can vary and may be influenced by interactions with sympatric species, climatic conditions or other threats. Understanding why species respond differently to chytrid is crucial as few options currently exist to manage the threat.

I examined a case for the critically endangered Spotted Tree Frog (*Litoria spenceri*) and the non-threatened Lesueur’s Frog (*Litoria lesueurii*). *Litoria spenceri* has been recorded at 50 sites at elevations of 300 - 1110m asl in south-eastern Australia. *Litoria lesueurii* co-occurs at some sites but has a much broader distribution. Both are stream-breeding species that respond differently to introduced trout (*Salmo trutta* and *Oncorhynchus mykiss*) and were suspected to respond differently to chytrid.

I constructed a two-species dynamic occupancy model to examine the historic and future changes in site occupancy of both frog species, and evaluated factors that influence their probabilities of local extinction and colonization. Models combined fragmented historic data and more intensive post-decline data collected over a 55-year period (1958-2012) at 49 historic *L. spenceri* sites. Trout influence could not be examined as they occurred at all but one site. My analysis revealed that *L. spenceri* has declined from approximately 50% of known historic sites. This decline could be influenced by either (or both) the presence of chytrid or the presence of *L. lesueurii*, but was most severe when both chytrid and *L. lesueurii* were present at sites. In contrast, *L. lesueurii* tended to become more prevalent over time and changes were uncorrelated with the occurrence of chytrid or *L. spenceri*. Without intervention, the model extrapolated that *L. spenceri* will continue to decline, and
may be extinct by as early as 2035. I hypothesize that *L. spenceri* decline is most severe at sites when the presence of pathogen-host-reservoir species (or total frog density) facilitates chytrid persistence and maintains chytrid transmission.

Several PCR tests exist to detect chytrid but their results may be imperfect. Prior to evaluating the impact of chytrid on wild frog populations, I assessed the diagnostic sensitivity and specificity of a Qiagen quantitative PCR method to detect chytrid using spiked samples. The Qiagen quantitative PCR was selected as preliminary results indicated equal or superior sensitivity to other published methods. The Qiagen diagnostic sensitivity was high (mean generally > 0.937) but varied in response to the concentration of zoospores in the sample. Diagnostic sensitivity could be improved by classifying equivocal results as positive, with minimal impact on diagnostic specificity. Mean diagnostic specificity was 0.961 (95% CI: 0.897-0.995). The results highlight a risk of misclassifying samples due to imperfect diagnostic sensitivity and specificity which should be considered by practitioners when evaluating the impact of disease on wildlife.

To clarify the response of *L. spenceri* and *L. Lesueurii* to chytrid I assessed the infection state of all frogs each time they were encountered during a four – six year mark-recapture study at 4 sites. I quantified individual’s probabilities of infection, recovery from infection and survival in each infection state using multi-infection-state models. Nine candidate models were compared to account for four factors that could bias parameter estimates: 1) potential misclassification of disease state, 2) disease state affecting detection, 3) impacts of marking on survival or capture probabilities, and 4) the assumed ability of individuals to recover from an infection. The model with the most support (lowest DIC value) for both species assumed some misclassification of an individual’s disease state, equal detection of infected and uninfected frogs, no effect of marking on return rates, and that frogs could recover from an infection. Marking did not reduce the probability of capture or survival of *L. lesueurii* and the impact upon *L. spenceri* was uncertain. Chytrid infection reduced the survival of both species. *Litoria lesueurii* had the higher probability of becoming infected and the lower probability of survival when infected. Both species had similar probabilities of recovery from infection.
Discrete-time deterministic multi-state population models were constructed using multi-state mark-recapture and published parameter estimates to assess the combined influence of chytrid and introduced trout upon each frog species. High, mid and low elevation site models were constructed for each species to reflect demographic differences that are known to be correlated with climate and elevation. Age to maturation, clutch size and egg-year1 survival influenced interspecific and intraspecific population-level responses of the two frogs to threats. Crucially, *L. spenceri* populations were non-viable at high and mid elevations but had some capacity to persist at low elevations when impacted by both threats (in the absence of stochastic processes and pathogen reservoir species). In contrast, *L. lesueurii* populations had a greater capacity to persist at both low and mid elevation sites.

This thesis highlights that population dynamics and the causes of decline must be carefully evaluated to understand species’ contrasting responses to multiple threats and to identify effective conservation management solutions. Chytrid can severely impact both threatened and non-threatened frogs at an individual-level. However, species population-level responses to chytrid are influenced by their ability to compensate for pathogen-induced mortality through recruitment. Interspecific and intraspecific differences in amphibian populations’ risk of extinction can occur when species recruitment is influenced by site-specific environmental processes and other threats. Furthermore site-specific extinction risk may also be exacerbated by the presence of native sympatric species that act as pathogen reservoir hosts or competitors. In this case, mitigation of threats to *L. spenceri* might be achieved at some sites by enhancing recruitment through trout management. However, trout mitigation may increase chytrid infection risk, which should be monitored and potentially concurrently managed. Prudent management action is required for species like *L. spenceri* that are facing multiple threats to avoid undesirable conservation outcomes and to prevent extinction.
Statement of Authorship

This is to certify that

i) the thesis comprises only original work towards the degree of Doctor of Philosophy except where indicated in the preface

ii) due acknowledgement has been made in the text to all other material used

iii) the thesis is fewer than the maximum (100,000) word limit in length, exclusive of tables, maps, bibliographies and appendices as approved by the Research Higher Degrees Committee.

Matt West

23 December 2015
Statement of Ethics and Research Approval

This research was conducted under animal ethics approval (ZV09016, ZV11022) and with authorized Wildlife Act 1975 and National Parks Act 1975 Research Permits (Permit. No’s: 10004700, 10006138, 1007397).
Acknowledgments

First and foremost, I thank my family for allowing me the time to undertake this PhD. By thanking you first I want you to know how important you all are to me. Thank you Michelle, Toby and Sierra I am grateful that you coped with my absence and welcomed me home after frequent field trips, conferences, workshops, and long nights testing samples, running analyses and ultimately writing this thesis. Thanks Mum and Dad, Pete and Ian for all your help particularly looking after Toby and Sierra whilst I have been studying. I am looking forward to spending more time with you all now that this thesis is complete.

To my supervisors Graeme Gillespie and Michael McCarthy: I am in your debt. Your ecological experience and knowledge is truly inspiring and this thesis would have been nearly impossible without your help and ecological insight. This thesis builds on a substantial research base, largely a legacy of Graeme’s efforts over the last 25 years. Graeme – I appreciate all the work that you and others have put in to maintain this long-term monitoring program, I am particularly grateful for this opportunity and hope this research is a worthy contribution. Modelling was not one of my strengths prior to commencing this thesis, I wish I had reattained more of the mathematical and statistical training that I learnt as an undergraduate student. After many years working in an industry that did not require these skills (as a Zookeeper) I let that knowledge slip out of my head. But fortunately for me, Mick’s expertise in data analysis is outstanding. Mick - I am very grateful for all of the support that you have given me, I will actively try to maintain and expand this skill as I now realise how useful and important it is. This has honestly been a terrific opportunity, thank you. I hope we will all continue to stay in touch and work with each other into the future.

This thesis would have been impossible without the very generous financial support of Zoos Victoria, the Holsworth Wildlife Research Endowment, an Alfred Nicholas Fellowship, the University of Melbourne, the Australian Research Council (ARC) Centre of Excellence for Environmental Decisions (CEED) and in-kind support from cesar Pty Ltd.

The most enjoyable component of this research was the time that I have spent in the field. This is largely due to the company and assistance of Graeme Gillespie, Kwai Chang-Kum, Michael Williams, Michael Shelton, Michael Saunders, Daniel Wilcox, Damien Goodall,
Natalie Radojcic, Glen Johnson, Steve Smith, Rowhan Marshall, Felicity Smith, Nigel Watts, David De Angelis, Craig Reid, Raelene Donelly, Deon Gilbert, Glen Wiesner, Chris Jaffer, Michael Swan, Paula Watson, Yvette Pittao, Casey Visintin, Micha Plein, Grant Harris, Jim Thomas and many others. Despite the climatic challenges, lack of sleep and the busy field schedule we have seen some awesome country, consumed delicious camp meals, had plenty of memorable adventures and heaps of fun. I am particularly grateful for your generous support and friendship.

I have had the privilege of working with a wide range of people during this project. Thank you to Glen Johnson, Steve Smith, Dave Hunter, Charles Todd, Nick Clemann, Michael Scroggie, Gerry Marantelli, Andrew Weeks, Anthony van Rooyen, Lee Skerratt, Lee Berger, Rebecca Webb, Simon Crawford, Barbra Howlett, Alex Idnurm, Peter Vesk, Tracey Regan, Jane Elith, Jose Lahoz-Monfort, Stefano Canessa, Kirsten Parris, John Baumgartner, Chris Banks, Michael Magrath, Murray Littlejohn, Greg Hollis, Scott Cunningham, Peter Liepkalns, and Geoff Heard for your advice and support. Another great part of this project has been getting to know you all. Thank you to Geoff Heard, Charles Todd, Dave Hunter, Casey Visintin, Gerry Ryan, Micha Plein, Andrew Weeks, Anthony Van Rooyen, Michelle Cleary and Ros West for all providing feedback on my drafts, it has been a huge help.

During this project I had several amazing opportunities to learn skills crucial to this thesis. Early into the project, I attended a chytrid conference at James Cook University where I spent some time with Lee Berger learning chytrid collection and culturing techniques, and also techniques to detect other potential amphibian diseases. This was a fantastic opportunity and a great introduction to the work I faced. Thank you Lee and to everyone involved in the conference. I managed to get involved in a number of other interesting related projects during this period and particularly thank Tracey Regan, David Keith, Kirsten Parris, Stefano Canessa and Sarah Converse for inviting me to participate. CEED and the University of Melbourne supported me to attend a workshop on matrix population modelling at the Centre d’Ecologie Fontionelle et Evolutive (CEFE), in Montpellier, France. The workshop was run by Jean-Dominique Lebreton, Olivier Gimenez and Dave Koons with a guest appearance from Madan Oli. It was an absolute privilege to spend time in Montpellier and learn from you all. What a terrific course! Thank you everyone, I hope to visit and work with you again.
I undertook this thesis as a member of a large and diverse research team, the Quantitative Applied Ecology Group at the University of Melbourne. It has been a terrific group to work with. I have enjoyed the comraderie and have learnt a lot just by association. I also realise how much I still have to learn. Thankfully I now know where to find you all. Thank you everyone.

To everyone involved, I look forward to thanking you all in person. Please keep in touch.
Dedication

Toby and Sierra – conserving wildlife was important to me before you were born and it is even more important to me now that I have you. I hope I can inspire you to value nature and to be as fascinated by it as I am.
A male Spotted Tree Frog *Litoria spenceri* at Taponga River Victoria Australia. Photo taken by Michael Williams © It’s a Wildlife.
Table of contents

Abstract .......................................................................................................................... iii

Statement of Authorship ................................................................................................ vii

Statement of Ethics and Research Approval ............................................................... viii

Acknowledgments ...................................................................................................... ix

Dedication .................................................................................................................... xii

List of Figures ............................................................................................................... xix

List of Tables .............................................................................................................. xxi

Chapter 1. General Introduction ................................................................................. 1

1.1 Contemporary challenges for amphibian conservation................................. 2

1.1.1 Amphibian population declines ................................................................. 2

1.1.2 Key threatening processes and their interactive effects ....................... 4

1.1.3 Chytrid: a pervasive problem for amphibian conservation ............... 6

1.2 Case Study: Sympatric frog species with suspected differing responses to a fungal pathogen ................................................................. 14

1.3 Thesis Aims and Structure ............................................................................ 20

Chapter 2. Evaluating long-term patterns of population change and multiple factors linked to decline in sympatric frog species, using 55 years of data ................................................. 24

2.1 Introduction ..................................................................................................... 24

2.2 Methods ......................................................................................................... 27

2.2.1 Frog Surveys ............................................................................................ 27

2.2.2 Chytrid records ....................................................................................... 27
2.2.3 Multi-species dynamic occupancy model ............................................ 28
2.2.4 Predicted future change in species site occupancy ......................... 32

2.3 Results ............................................................................................................... 32
  2.3.1 Historic change in site occupancy ....................................................... 32
  2.3.2 Detection probability ............................................................................ 33
  2.3.3 Annual infection probability ................................................................. 33
  2.3.4 Factors influencing annual extinction probability ................................. 33
  2.3.5 Annual colonization probability ............................................................ 38
  2.3.6 Future change in occupancy ............................................................... 38

2.4 Discussion ........................................................................................................... 40

Chapter 3. Imperfect disease classification: can PCR tests accurately detect wildlife pathogens? ................................................................. 46

3.1 Introduction .......................................................................................................... 46

3.2 Methods ............................................................................................................... 49
  3.2.1 Production and quantification of zoospores ........................................ 49
  3.2.2 DNA extraction .................................................................................... 49
  3.2.3 PCR methods ...................................................................................... 50
  3.2.4 Preliminary assessments of diagnostic sensitivity and specificity ...... 51
    3.2.4.1 Preliminary Experiment 1: Comparing diagnostic sensitivity and specificity of four PCR methods: spiked samples, no other contaminants. .............................................................................. 51
    3.2.4.2 Preliminary Experiment B: Comparing diagnostic sensitivity of four PCR methods: spiked samples, with natural contaminants present. ................................................................. 52
  3.2.5 Qiagen PCR diagnostic sensitivity and specificity .................................... 52
3.3 Results .................................................................................................................. 53

3.3.1 Qiagen PCR diagnostic sensitivity and specificity ......................... 54

3.4 Discussion ............................................................................................................. 57

Chapter 4. Quantifying the demographic impact of disease upon sympatric threatened and non-threatened frogs ......................................................... 62

4.1 Introduction .......................................................................................................... 62

4.2 Methods ............................................................................................................... 65

4.2.1 Study Species ................................................................................................. 65

4.2.2 Mark-recapture study design ................................................................. 66

4.2.3 PCR Analysis .............................................................................................. 66

4.2.4 Multistate Mark-recapture Analysis ....................................................... 67

4.2.5 Model evaluation ......................................................................................... 71

4.2.6 Model selection ........................................................................................... 71

4.3 Results ............................................................................................................... 72

4.3.1 Sample data and observations ............................................................... 72

4.3.2 Multistate Mark-recapture Model Analysis ........................................... 75

4.3.2.1 Model selection ................................................................................... 75

4.3.2.2 Effect of marking frogs on capture and survival probabilities .......... 75

4.3.2.3 Monthly probability of acquiring an infection ..................................... 76

4.3.2.4 Monthly probability of recovery from an infection ......................... 77

4.3.2.5 Impact of chytrid on probability of detection .................................... 78

4.3.2.6 Probability of apparent annual survival ............................................. 79
4.4 Discussion ........................................................................................................... 80

Chapter 5. Population-level responses of two frogs to multiple threats: chytrid fungus and introduced trout .......................................................... 86

5.1 Introduction ........................................................................................................ 86

5.2 Methods ............................................................................................................... 89

5.2.1 Case Study ................................................................................................... 89

5.2.2 Population Models ....................................................................................... 94

5.3 Results ............................................................................................................... 99

5.4 Discussion ........................................................................................................ 105

Chapter 6. Synthesis and General Discussion .......................................................... 110

6.1 Species decline driven by multiple interacting threats ..................................... 110

6.2 Implications for Spotted Tree Frogs: future research priorities and recommendations .......................................................... 113

6.3 Broader implications for addressing species declines ..................................... 119

6.4 Conclusion ....................................................................................................... 121

Appendix 1 Other occupancy model results .......................................................... 123

Appendix 2 Results of the preliminary assessment to compare the diagnostic sensitivity and specificity of four PCR methods ........................................... 129

Appendix 3 Life graphs for each species at mid and high elevation sites .......... 134

References ............................................................................................................. 137
List of Figures

Figure 1.1 Major threats to amphibians, determined during a global assessment in 2008 (Stuart & Edicions 2008). Direct human impacts include persecution (such as harvesting), disturbance and accidental mortality………………… 5

Figure 1.2 Scanning electron micrograph images of Chytrid fungus Batrachochytrium dendrobatidis…………………………………………… 8

Figure 1.3 The Spotted Tree Frog Litoria spenceri and Lesueur’s Frog L. lesueurii.. 13

Figure 1.4 Comparative distributions of L. spenceri and L. lesueurii, both species are restricted to south-eastern Australia…………………………………..... 15

Figure 1.5 Examples of the stream habitats used by L. spenceri and L. lesueurii……. 16

Figure 2.1 Factors influencing the probability of extinction of L spenceri and L. lesueurii assuming chytrid was present from 1996………………………… 34

Figure 2.2 Factors influencing the probability of extinction of Litoria spenceri and L. lesueurii assuming chytrid was present at sites prior to 1996……………. 35

Figure 2.3 Estimated historic (1958-2012) and predicted future (2013-2057) change in proportion of sites occupied by L. spenceri and L. lesueurii……………. 39

Figure 3.1 Diagnostic sensitivity and specificity of the Qiagen qPCR method for detecting chytrid……………………………………………………………. 55

Figure 3.2 Estimated number of a) chytrid zoospores and b) copies of the ITS1 region per chytrid fungus zoospore in swab samples…………………... 56
Figure 4.1 Estimated monthly probabilities of uninfected *L. spenceri* and *L. lesueurii* becoming infected during the survey (Nov – March) and non-survey (April-Oct) periods…………………………………………………………. 76

Figure 4.2 Estimated monthly probabilities of recovery of infected *L. spenceri* and *L. lesueurii* during the survey (Nov – March) and non-survey (April-Oct) periods…………………………………………………………………………………………………………………………….. 77

Figure 4.3 Estimated probabilities of detection of infected (+ve) and uninfected (-ve) *L. spenceri* and *L. lesueurii*…………………………………………… 78

Figure 4.4 Estimated probabilities of apparent annual survival of infected (+ve) and uninfected (-ve) *L. spenceri* and *L. lesueurii*………………………………. 79

Figure 5.1 Multi-state life-cycle graphs and corresponding population matrices for *L. spenceri* and *L. lesueurii* at low elevation sites when chytrid is present……………………………………………………………………… 90

Figure 5.2 Sensitivity of the population growth rate to each of the vital rates of *L. spenceri* and *L. lesueurii* for multi-state age-structured models……………102

Figure 5.3 The potential change in deterministic population growth rates (lambda) of *L. spenceri* and *L. lesueurii* if vital rates vary from current mean parameter estimates…………………………………………………………………….103
List of Tables

Table 2.1 Combinations of factors evaluated by multi-species dynamic occupancy models to estimate the probability of extinction of Litoria spenceri and L. lesueurii. Bd.………………………………………………………………… 29

Table 2.2 Estimated mean current (2012) and future (2057) proportion of the 49 historic Litoria spenceri sites occupied by L. spenceri and L. lesueurii…………………………………………………………………….. 36

Table 2.3 Estimated probability of detection and colonization of Litoria spenceri and L. lesueurii in 2012………………………………………………………………………… 37

Table 4.1 Assumptions of the candidate multistate mark-recapture models to account for four potential sources of bias when estimating the demographic impact of disease on frogs……………………………………………… 68

Table 4.2 Chytrid PCR results of L. spenceri and L. lesueurii during successive sampling (capture) occasions over a 4 – 6 year mark-recapture study…….. 73

Table 4.3 Demographic parameter estimates for L. spenceri and L. lesueurii as determined by the four candidate Bayesian multi-state mark-recapture models……………………………………………………………………… 74

Table 5.1 Initial annual demographic parameters of L. spenceri and L. lesueurii at low, mid and high elevation sites…………………………………….. 91

Table 5.2 Estimated deterministic population growth rates of L. spenceri and L. lesueurii at low, mid and high elevation sites, under current conditions or varying hypothetical management scenarios…………………………… 101
General
Introduction
Chapter 1. General Introduction

1.1 Contemporary challenges for amphibian conservation

1.1.1 Amphibian population declines

Conservation biology depends on determining when species are declining and identifying factors that cause decline (e.g. Caughley 1994), but gathering evidence to inform management and conserve species is challenging. Globally, the number of threatened species continues to increase (Butchart et al. 2010; Hoffmann et al. 2010). Most species are only assessed after a suspected decline has occurred; if any pre-decline data exists, it is often incomplete (Tingley & Beissinger 2009; Lindenmayer et al. 2012). Consequently, the degree of population change is often uncertain. In addition, the causes of decline are frequently unknown, since multiple factors can interact and resources are rarely allocated to disentangle their effect on population change (Blaustein & Kiesecker 2002; Brook, Sodhi & Bradshaw 2008; Bolten et al. 2010; Doherty et al. 2015). Given these challenges, conservation management decisions often occur before systematically appraising the evidence (Sutherland et al. 2004). Failing to consider the evidence of decline or causes of decline and their interactions can lead to biased assessments of population viability, wasted or misdirected resource investment and ineffective or undesirable conservation outcomes (Sutherland et al. 2004; McKelvey, Aubry & Schwartz 2008; Sabo 2008; Evans, Possingham & Wilson 2011; Lindenmayer et al. 2012). Understanding population decline and its causes requires assessments that either synthesize historic (often fragmented) data to quantify population trends and identify threats post hoc, or directly evaluate the current impact of perceived threats upon populations using observations or experiments.

Globally, amphibians are declining at rates that both exceed those of other vertebrate groups (Hof et al. 2011) and background extinctions (McCallum 2007; Alroy 2015). Contemporary extinction rates cannot be explained by natural catastrophic events or continental drift which are hypothesised causes of previous mass extinctions (McCallum 2007; Wake & Vredenburg 2008). The International Union for the Conservation of Nature (IUCN) currently lists 38 extinct amphibian species and suggests that at least another 120 species
could be extinct as they have not been observed in recent years (IUCN 2008). Furthermore, 42% of the 6,260 internationally recognized amphibian species are considered to be in decline (IUCN 2008). Alarmingly, amphibian extinction rates are predicted to increase into the future (McCallum 2007; Hof et al. 2011; Alroy 2015).

Widespread amphibian declines were first noticed during the 1980’s (Barinaga 1990; Blaustein & Wake 1990; Wake 1991). However, researchers have not always agreed about whether population changes represent declines (Pechmann et al. 1991; Blaustein 1994; Pechmann & Wilbur 1994; Reed & Blaustein 1995), or about the timing (Houlahan et al. 2000; Alford, Dixon & Pechmann 2001) and causes of amphibian decline (McCallum 2005). This is because amphibian declines can be difficult to distinguish from natural population fluctuations (Pechmann et al. 1991; Pechmann & Wilbur 1994) and multiple interacting factors can influence population change (Blaustein & Kiesecker 2002). Amphibian populations may naturally experience boom bust cycles (Wells 2010), particularly as breeding success can vary widely with climatic conditions (such as rainfall) (Alford & Richards 1999). As a result, natural events like drought can explain some population fluctuations that could otherwise be perceived as a decline (Corn & Fogleman 1984; Pechmann et al. 1991).

Evaluation of amphibian population trends and the factors influencing population change have also been ‘hampered by a dearth of long-term census data’ (Pechmann et al. 1991). The intensity and purpose of historic amphibian surveys varies widely within and between regions and over time (Alford, Dixon & Pechmann 2001). A paucity of data undermines attempts to distinguish apparent population declines from natural fluctuations, given limited baseline data and low statistical power (Pechmann et al. 1991; Alford, Dixon & Pechmann 2001; McCallum 2005). In response, conservation biologists have called for 1) long-term monitoring programs to assess amphibian population trends (e.g. Pechmann et al. 1991; Blaustein, Wake & Sousa 1994), 2) detailed well replicated studies in natural settings to evaluate suspected causes of population decline and 3) laboratory or mesocosom (artificial field) trials to explicitly evaluate threats (Alford & Richards 1999).

Species population change can be assessed with either occupancy, count or mark-recapture techniques. Occupancy methods can inform population trends by evaluating changes in
species distributions or the number of sites occupied by species (MacKenzie et al. 2002; MacKenzie et al. 2003; MacKenzie 2006; Royle & Kery 2007). Counts can be used to estimate and understand changes in species abundance or population density within a defined patch (assessed using point surveys or distance sampling techniques) (Seber 1982; Schwarz & Seber 1999; Schmidt 2003). Mark-recapture assessments can provide detailed information about population demography by tracking the fate of individuals within discrete populations, enabling assessment of the factors regulating survival and recruitment rates and ultimately population growth rates (Cormack 1964; Jolly 1965; Seber 1965; Lebreton et al. 1992; Lebreton et al. 2009). All three methods can now account for imperfect detection which is crucial as an assumption that failed detections represent true absences will bias demographic estimates (Lebreton et al. 1992; Nichols et al. 2000; MacKenzie et al. 2002; MacKenzie et al. 2003; Royle & Nichols 2003; Schmidt 2003; Conn & Cooch 2009). The best monitoring strategy depends upon the reason for assessing populations. Studies examining long-term trends are particularly informative when the reason for monitoring is to determine if changes in species occurrence, abundance or demographic rates are caused by threats rather than natural processes (Lindenmayer et al. 2012).

1.1.2 Key threatening processes and their interactive effects

Habitat loss, pollution, invasive species and disease are amongst the most important threats to amphibians (Table 1.1) (IUCN 2008). Habitat loss alone is considered to threaten almost 4000 amphibian species (IUCN 2008). But many suspected population declines have been reported in protected or relatively undisturbed areas (Alford & Richards 1999) indicating that habitat protection in isolation will not prevent all amphibian declines and that other threatening processes are also important. Indeed, most threatened amphibians are considered to be adversely affected by multiple factors (Hof et al. 2011).
Interactive effects amongst multiple environmental factors can be complex and must be disentangled to halt amphibian declines (Blaustein & Kiesecker 2002; Blaustein et al. 2010; Blaustein et al. 2011; Hof et al. 2011; Wake 2012). For example, habitat degradation in North America may have permitted expansion of invasive bullfrogs (*Rana castebeiana*) that adversely affect native amphibian populations through competition and predation (Blaustein & Kiesecker 2002). Furthermore, invasive bullfrogs can alter microhabitats and consequently increase native amphibian species’ risk of predation by introduced fish (Kiesecker & Blaustein 1998). The risk to species is most severe if impacts of multiple threats are additive or synergistic (greater impact than the sum effect) (Hof et al. 2011). Climate-induced reductions in water-depth can increase exposure of amphibian eggs to UV-B radiation and disease outbreaks (caused by *Saprolegnia ferax*) that cause amphibian embryo mortality (Blaustein & Wake 1990; Kiesecker, Blaustein & Belden 2001). Impacts on amphibians may also increase if chemical pollutants (like pesticides) combine with other threats including predators (Relyea 2003; Sih, Bell & Kerby 2004; Relyea & Diecks 2008), UV radiation (Long, Saylor & Soule 1995; Blaustein & Kiesecker 2002) or habitat loss (Wake 2012). In some cases, threats may only impact species when combined with other factors (e.g. UV) (Long, Saylor & Soule 1995; Blaustein & Kiesecker 2002), whilst other threats may act independently but not increase when combined with additional threats (e.g. salinity and introduced predators) (Karraker, Arrigoni & Dudgeon 2010). Conceivably, some threats may also combine to be antagonistic (i.e. the combined impact is less than the sum of the individual impacts).
individual impacts). For example, climate change could cause pool warming and drying (due to reduced rainfall and increasing temperature), but when combined, these effects may be antagonistic, and permit faster tadpole development due to warmer water temperatures and increased food availability (O'Regan, Palen & Anderson 2013). The responses of populations and species to multiple threats can therefore be difficult to predict and may vary spatially and temporally (Blaustein & Kiesecker 2002; Blaustein et al. 2012). The combined impact of multiple drivers are anticipated to increase amphibian declines and exacerbate species future extinction risk (Hof et al. 2011). Investigations are required to evaluate the potential synergistic effects of multiple factors upon species (Wake 1991) and to determine the conditions under which threats act alone or together (Collins & Storfer 2003).

1.1.3 Chytrid: a pervasive problem for amphibian conservation

Chytrid fungus, Batrachochytrium dendrobatidis, (hereafter ‘chytrid’) is regarded as a major threat to amphibians (Berger et al. 1998). The fungal pathogen is implicated in declines of almost one quarter of threatened or extinct IUCN listed amphibians (Heard, Smith & Ripp 2011) and may have already caused declines and extinctions of around 200 species (Skerratt et al. 2007). More than 700 amphibian species (Bosch et al. 2015) have tested positive for chytrid in at least 52 countries (Olson et al. 2013).

Chytrid is highly infectious and causes a disease in amphibians known as chytridiomycosis (Berger et al. 1998). The pathogen has a free-swimming zoospore life-stage (Figure 1.2) that can be transmitted via direct interactions with infected hosts (Rachowicz & Vredenburg 2004), and by contacting contaminated surfaces and water. Additionally, zoospores can survive on substrates and in water for extended periods (e.g. up to 3 months in sterile water) (Johnson & Speare 2003; Johnson & Speare 2005). Once in contact with a host, a zoospore encysts into an amphibian’s keratinized tissue (Longcore, Pessier & Nichols 1999; Berger et al. 2005) and matures to form a sporangium that asexually produces new zoospores (Figure 1.2). Zoospores are released from sporangia through discharge tubules and can either continue to infect the current host or are shed into the surrounding environment (Berger et al. 2005). Chytrid may be spread between sites via dispersing infected individuals (amphibians and other taxa) (Reeder, Pessier & Vredenburg 2012; McMahon et al. 2013; Brannelly et al. 2015; Kolby et al. 2015b), by researchers or recreational users of water
ways (Skerratt et al. 2007; Phillott et al. 2010) and potentially even aerially through rain water (Kolby et al. 2015a).

Chytridiomycosis manifests as swelling of the epidermis and thickening of the keratin layer (hyperkeratosis), abnormal multiplication of skin cells (hyperplasia) and skin erosion or ulceration (Berger et al. 1998; Nichols et al. 2001; Berger et al. 2005). Individuals can become lethargic and reduce food intake (inappetance) (Berger et al. 2009). The pathogen can impair the host’s osmoregulation and when infected individuals fail to mount an effective immune response, they die of cardiac arrest (Voyles et al. 2007; Voyles et al. 2009; Campbell et al. 2012; Salla et al. 2015). Death can occur within 24 hrs of exposure (Searle et al. 2011; Gahl, Longcore & Houlanah 2012).

The duration of most amphibian populations association with chytrid is unknown (e.g. Phillips et al. 2012). Two hypotheses can explain the emergence of chytridiomycosis in some amphibian populations. The “Novel Pathogen Hypothesis” suggests epizootics (disease in wild populations) emerged following the expansion or spread of a highly virulent strain of the pathogen (Rachowicz et al. 2005). The “Endemic Pathogen Hypothesis” suggests epizootics emerged following changes in the immunology, ecology and/or behaviour of the host or pathogen that favoured the pathogen (Rachowicz et al. 2005). In doing so the balance may have tipped from a relatively benign association towards a pathogenic relationship (Rachowicz et al. 2005). Genetic analyses support both hypotheses with widespread hyper-virulent strains (B. dendrobatidis Global Pandemic Lineage: Bd-GPL) and enzootic strains being discovered (reviewed in James et al. 2015). Emerging evidence indicates that in many regions chytrid introductions have occurred (particularly in Australia, Panama and California) and hence the pathogen is novel, but in some cases chytrid has had a long-term association with amphibians (Rosenblum et al. 2013). Determining optimal approaches to manage the threat of chytrid infection requires that the spread of chytrid and the cofactors that exacerbate chytrid impacts are resolved (Venesky et al. 2014).
Figure 1.2 Scanning electron micrograph images of Chytrid fungus (*Batrachochytrium dendrobatidis*) produced in culture; a single zoospore (left) and a cluster of sporangia with rhizoids spreading over surface (right). Some zoospores can be seen inside the sporangia discharge tubes (arrow). Cultures produced as discussed in Chapter 3, Images by S. Crawford (Biosciences, University of Melbourne) and M. West.
Chytrid may not be the primary cause of some amphibian declines despite the pathogen’s detection (Heard, Smith & Ripp 2011). Diagnosis of chytridiomycosis in dead or dying amphibians can indicate the pathogen’s association with population decline but other factors could cause stress and exacerbate disease impacts or be a more potent threat (McCallum 1994). Chytrid can cause rapid population declines of some species at some sites, (e.g. Lips et al. 2006; Gillespie et al. 2014) but other species and populations can experience slower rates of decline or may even persist despite chytrid presence (Phillott et al. 2013; Scheele et al. 2015). Individual and population responses to chytrid can vary both between and within species (Blaustein et al. 2005; Briggs et al. 2005; Tobler & Schmidt 2010). Understanding why species and populations respond differently to chytrid is a key priority to manage the threat (Collins 2010; Wells 2010; James et al. 2015).

Population-level disease outcomes could be influenced by site-specific host, pathogen and environment interactions, a concept referred to as the ‘disease triangle’ (James et al. 2015). Essentially, an amphibian’s infection risk and ability to survive and recover if infected can be influenced by its behaviour or immune responses, the pathogen’s virulence, and environmental conditions to which the amphibian host and pathogen are both exposed (Tobler & Schmidt 2010; Gahl, Longcore & Houlanah 2012; Doddington et al. 2013; Daskin et al. 2014; Gervasi et al. 2014; Heard et al. 2014). Temperature, humidity and pH can influence the growth and survival of chytrid (Johnson & Speare 2003; Piotrowski, Annis & Longcore 2004; Johnson & Speare 2005). Notably, chytrid can grow and reproduce at temperatures of 4-25°C with 17-25°C being optimal, but mortality occurs at or above 30°C (Piotrowski, Annis & Longcore 2004). Ambient temperatures also can limit amphibian immune responses with host antifungal activity reduced in cool conditions (Daskin et al. 2014). Amphibian behaviour and microhabitat selection is important as it influences the microclimate they experience and infection risk is reduced when hosts spend more time above the pathogen’s optimal growth range (Rowley & Alford 2007a; Rowley & Alford 2013). These host-pathogen-environment interactions determine the fate of species when exposed to chytrid in the wild. For example, warm and slightly saline environments reduce the probability and intensity of infections in Growling Grass Frogs (Litoria raniformis) (Heard et al. 2014).
Sympatric species that can tolerate chytrid and carry high infection loads can influence the population-level outcomes of co-occurring species that are intolerant of the pathogen (McCallum 1994; Reeder, Pessier & Vredenburg 2012; Brannelly et al. 2015). Chytrid-tolerant species may act as a reservoir host and drive declines of more susceptible species by maintaining a constant source of infection even as the susceptible species population continues to decline (Fisher et al. 2012; McCallum 2012). Despite the suspected role of reservoir hosts in amphibian declines (e.g. Retallick, McCallum & Speare 2004; Woodhams 2008; Hunter et al. 2010; Stockwell, Clulow & Mahony 2010; Reeder, Pessier & Vredenburg 2012; Scheele et al. 2015) evidence of their true effects is lacking for most species. A highly virulent pathogen or one with a long-lived infectious stage can drive extinction when population densities are high (Fisher et al. 2012; McCallum 2012; Gillespie et al. 2014). Disease outcomes may result from density-dependent host-pathogen dynamics (Rachowicz & Briggs 2007; Briggs, Knapp & Vredenburg 2010), regardless of whether the hosts are conspecific or heterospecific. However, susceptible species may persist if they occur at, or decline below, a host-population density threshold for pathogen persistence (Briggs, Knapp & Vredenburg 2010; Fisher et al. 2012; McCallum 2012).

Populations infected with chytrid may be at greater risk of decline if adversely effected by additional threats (Phillott et al. 2013). Most species are likely to be influenced by multiple threats (Bielby et al. 2008; Heard, Smith & Ripp 2011). However, there are few clear examples of how chytrid interacts with other threats, and the resultant impact on species. Whilst chemical contaminants (like herbicides or salt) can kill amphibians (Mann, Bidwell & Tyler 2003; Kearney, Byrne & Reina 2012), in some instances such contaminants may reduce chytrid impacts by harming the pathogen more than the host (Bramwell 2011; Gahl, Pauli & Houlahan 2011; Heard et al. 2014; Heard et al. 2015; Stockwell, Clulow & Mahony 2015). Similarly, the impact of chytrid could either become more or less severe under climate change if host-pathogen dynamics are altered by shifts in ambient temperatures and precipitation (reviewed in Blaustein et al. 2010). Chytrid-infected invasive amphibians may have severe impacts on species if they transmit chytrid and eat native amphibians (Garner et al. 2006).

Introduced fish can severely impact amphibian populations and are implicated in species declines (Gillespie 2001b; Matthews et al. 2001; Vredenburg 2004; Knapp 2005). The
impact of introduced fish could be particularly severe for species that are also influenced by chytrid, as the threats may simultaneously affect different life stages. For example, introduced fish can reduce amphibian larval survival (Gillespie 2001b; Hunter et al. 2011), whereas chytrid impacts can be most severe following metamorphosis (Berger, Spear & Hyatt 1999; Garner et al. 2009). The combined (synergistic) effects of chytrid and introduced fish could therefore accelerate species decline. Alternatively, these threats may have opposing (antagonistic) effects and could reduce chytrid impacts if disease dynamics are influenced by host population density (e.g. Rachowicz & Briggs 2007). Unravelling such interactions is crucial to understanding the population-level impacts of chytrid and introduced fish for species afflicted by both threats.

Some populations may continue to persist despite chytrid infection provided some adult amphibians survive and successfully reproduce (Briggs et al. 2005). Several authors have recently hypothesized that infected populations may be able to compensate for disease induced mortality by bolstering recruitment (Muths, Scherer & Pilliod 2011; Tobler, Borgula & Schmidt 2012; Phillott et al. 2013; Scheele et al. 2015). Amphibian recruitment rates can vary between species, partially because amphibian species vary greatly in fecundity (e.g. Gillespie 2011a). However, recruitment rates can also vary intraspecifically for environmental or evolutionary reasons, as can growth, maturation and survival rates (Morrison & Hero 2003). Understanding interspecific and intraspecific variation in life history and population processes may provide important insights into the impacts of threats on species (Sæther, Ringsby & Røskaft 1996), including the effect of disease on amphibians (Morrison & Hero 2003; Wells 2010; Murray et al. 2011; Murray & Skerratt 2012; Scheele et al. 2015).

Despite the number of species listed at risk from chytrid, the actual role of the pathogen in the decline of most species remains unknown (Heard, Smith & Ripp 2011). In addition, many ‘enigmatic’ amphibian declines are suspected to be caused by chytrid (Lotters et al. 2009). Chytrid is often assumed to be the cause of decline without actually quantifying its impact (McCallum 2005; Phillips et al. 2012; Riley, Berry & Roberts 2013). Conversely, many non-threatened species are presumed to be unaffected by the pathogen when declines have not been observed, or when models indicate low habitat suitability for the pathogen (Riley, Berry & Roberts 2013). Unless the effect of chytrid upon populations is quantified
the pathogens role in decline could be either over- or under-estimated. Other threats may also be contributing to decline, or indeed be more important drivers of declines; a failure to consider/evaluate their role in declines may lead to poor conservation and management decisions (McCallum 2005; Gardner, Barlow & Peres 2007).
Figure 1.3 An adult female Spotted Tree Frog, *Litoria spenceri* (left), and a male and female Lesueur’s Frog, *L. lesueurii* in amplexus (right). Male *L. spenceri* are similar in appearance to females but are smaller. Photos taken by Michael Williams (© It’s A Wildlife).
1.2 Case Study: Sympatric frog species with suspected differing responses to a fungal pathogen

In this thesis, I seek to resolve key knowledge gaps about the role of chytrid in amphibian declines by studying two Australian frog species, one threatened and one non-threatened, that are thought to have differing responses to the pathogen (e.g. Kriger & Hero 2006; Gillespie et al. 2014). The focal species are the critically endangered Spotted Tree Frog (*Litoria spenceri*) (Hero et al. 2004) and the non-threatened Lesueur’s Frog (*L. lesueurii*), both of which occur in mountain streams of north-eastern Victoria and southern New South Wales (Figure 1.3). These species were selected as they co-occurred at some sites and I hypothesised *L. lesueurii* to be a reservoir host of chytrid for *L. spenceri*. In addition, existing detailed distributional, life history and demographic research undertaken particularly for *L. spenceri* presented a rare opportunity to clarify species responses to chytrid, and assess the extent to which interactions with other cofactors influence population dynamics.

*Litoria spenceri* is predominately restricted to north-west draining mountain streams from central Victoria to Mount Kosciusko, south-eastern Australia, at elevations of 300 - 1110 m above sea level (Gillespie & Hollis 1996) (Figure 1.4). *Litoria lesueurii* is sympatric with *L. spenceri* at some sites but occurs over a broader range throughout south-eastern Australia (north-west of Melbourne in Victoria to the Hunter River catchment in New South Wales) (Donnellan & Mahony 2004) (Figure 1.4). Both species breed annually in streams (Figure 1.5) generally from November – December (Watson et al. 1991; Gillespie 2001a). Adult female *L. spenceri* produce less eggs (mean: 528 eggs; range 116-938) than *L. lesueurii* (mean: 1510 eggs; range 427-2771) (Gillespie 2011a). *Litoria spenceri* eggs are deposited under rocks in fast flowing sections of streams while *L. lesueurii* is able to deposit eggs within streams and stream-side pools (Gillespie 2001a; Anstis 2013). Tadpoles of both species develop within the stream or connected side pools (Hero, Watson & Gillespie 1995; Gillespie 2001a; Gillespie 2011a; Anstis 2013). Metamorphosis occurs within three months, by March or April of each year (Hero, Watson & Gillespie 1995; Gillespie 2010; Gillespie 2011b; Anstis 2013). Following metamorphosis, juvenile *L. spenceri* remain in close proximity to the stream, whereas *L. lesueurii* disperse away from the stream to surrounding
forest (Gillespie 2001a; Rowley & Alford 2007b; Gillespie 2011b). *Litoria spenceri* mature more slowly (3-6 years versus 2-4 years) but live longer (12-16 years versus 9-12 years) than *L. lesueurii* (Gillespie 2010; Gillespie 2011b; Gillespie 2011a; G. Gillespie unpublished data).

Figure 1.4 Comparative distributions of *L. spenceri* and *L. lesueurii*, both species are restricted to south-eastern Australia.
Figure 1.5 Examples of the stream habitats used by *L. spenceri* and *L. lesueurii*; Howqua River (left) and Snowy Creek (right), Victoria, Australia. Photos by M. West.
*Litoria spenceri* is currently listed as Endangered under the Australian Environment Protection and Biodiversity Conservation (EPBC) Act 1999 (Gillespie & Robertson 1998) and Critically Endangered on the IUCN Red List of Threatened Species (Hero *et al.* 2004). The species’ threat status was determined following its non-detection at some historic sites during surveys conducted from 1988-1990 (Hero 1990; Watson *et al.* 1991) and 1991-1997 (Gillespie & Hollis 1996; Gillespie 2001a). Despite extensive surveys during the latter period, *L. spenceri* was only discovered in 16 of the 64 streams surveyed (Gillespie & Hollis 1996; Gillespie 2001a). This included six streams at which the species had not previously been observed, but *L. spenceri* was not observed at four streams with historical records (Gillespie & Hollis 1996). Importantly, *L. spenceri* was considered to occur at a low abundance at all but one stream (Bogong Creek in NSW) during the 1991-1997 surveys (Gillespie & Hollis 1996; Gillespie *et al.* 2014). The IUCN Red List justification for listing *L. spenceri* is that the current area of occupancy is less than 10 km², its distribution is severely fragmented and there is continuing decline in the area of occupancy, quality of its habitat, the number of subpopulations and the number of mature individuals (Hero *et al.* 2004).

*Litoria lesueurii* is non-threatened in Australia and listed as Least Concern on the IUCN Red List of Threatened Species (Hero, Meyer & Clarke 2004). The species status under the IUCN Red List is based upon its wide distribution, tolerance of habitat modification, presumed large population size and because it is unlikely to be declining fast enough to qualify for listing in a more threatened category (Hero, Meyer & Clarke 2004).

*Litoria spenceri* has historically been recorded at 50 sites in 23 streams in south-eastern Australia (Gillespie & Hollis 1996; Gillespie 2001a; Gillespie & Clemann in review). Occurrence records at these sites now span a 55 year period (1958 - 2013). Observational data were sporadic up until 1991 when regular surveys commenced at many of the known historic sites. Intensive mark-recapture surveys were commenced at three sites, Still Creek, Bogong Creek and Taponga River in 1992, 1993 and 1994 respectively to understand population dynamics and investigate the casual factors linked to *L. spenceri* decline (Gillespie *et al.* 2014; Todd *et al.* in prep.). Mark-recapture surveys at Still Creek and Taponga River are ongoing (currently 23 and 24 years respectively). *Litoria lesueurii* has
been recorded at some sites during surveys for *L. spenceri* and often at a high abundance (Watson *et al.* 1991; Gillespie 2001a).

Multiple threatening processes have been implicated in the decline of *L. spenceri*, however there currently is no evidence that they have adversely effected *L. lesueurii*. Introduced Brown Trout (*Salmo trutta*) and Rainbow Trout (*Oncorhynchus mykiss*) have been released into streams and lakes throughout south-eastern Australia since the late 1800’s and introductions became more frequent and widespread commencing in the 1940’s (Jackson 1981; Jackson *et al.* 2004). Introduced trout predation have been demonstrated to significantly reduce the survival of *L. spenceri* tadpoles, but trout do not appear to predate upon *L. lesueurii* tadpoles (Gillespie 2001a; Gillespie 2001b). The impact of introduced trout is also in contrast to that of native fish which do not appear to be significant predators of *L. spenceri* tadpoles (Gillespie 2001b). Introduced trout now occur at all but the historic *L. spenceri* site on Bogong Creek in New South Wales. Habitat disturbances including eductor dredging, logging and road construction have occurred within or adjacent to many of the historic *L. spenceri* sites (Watson *et al.* 1991; Gillespie 2001a). These disturbances could influence breeding success of *L. spenceri* by either direct disturbance of oviposition sites or through increased stream sedimentation. Increases in sedimentation can reduce *L. spenceri* tadpole growth and development (Gillespie 2002). Eductor dredging is now banned and other activities that could disturb *L. spenceri* habitat are regulated under State and Federal legislation (particularly the Victorian Flora and Fauna Guarantee Act 1988, New South Wales Threatened Species Conservation Act 1995, New South Wales National Parks and Wildlife Act 1974, and Environment Protection and Biodiversity Conservation Act 1999). Herbicides that are known to be toxic to frogs (Mann, Bidwell & Tyler 2003) have been used within and adjacent to *L. spenceri* habitat primarily to supress weeds such as Blackberry (*Rubus fruticosus* aggregate) (Gillespie & Clemann in review). Herbicides may still be used in *L. spenceri* habitat, however management authorities are now generally aware of the risk and a revised National Recovery Plan for Spotted Tree Frogs recommends against herbicide use unless undertaken in a controlled manner (Gillespie & Clemann in review).

Chytrid is believed to have caused the rapid decline of *L. spenceri* at Bogong Creek in 1996 whilst being monitored during a mark-recapture study (Gillespie & Marantelli 2000;
Dead frogs found at Bogong Creek in 1996 were diagnosed with chytridiomycosis (Berger et al. 1998; Gillespie & Marantelli 2000). Sampling effort was sufficient to indicate that chytrid was unlikely to have been present prior to 1996 and no other threats were recorded at the site during the decline (Gillespie et al. 2014). The last observed wild frog was removed from the site to establish a captive breeding program in 1999 (Gillespie & Marantelli 2000; Gillespie et al. 2014). Despite extensive surveys conducted in subsequent years, no *L. spenceri* were detected (Gillespie et al. 2014). A dedicated reintroduction program was initiated at this site in 2005, but this has proved problematic due to further chytrid outbreaks (D. Hunter pers. com.). Chytrid has been detected at other streams (G. Gillespie and M West unpublished data), although the pathogens impact upon other *L. spenceri* populations has not been assessed.

The impact of chytrid upon *L. lesueurii* is unclear. *Litoria lesueurii* is now considered to belong to a complex of three closely related species; which include Wilcox’s Frog (*L. wilcoxii*) in central-eastern and north-eastern Australia (from the Hawkesbury-Nepean River system to far North Queensland) and the Jungguy Tree Frog (*L. jungguy*) in north-eastern Australia (predominately occurring in the Barron and Murray River catchments in mid-east to northern Queensland) (Donnellan & Mahony 2004). All three species within the *L. lesueurii* complex are hypothesised to be reservoir hosts for chytrid as despite harbouring chytrid infections, they are not known to have suffered declines (Retallick, McCallum & Speare 2004; Kriger & Hero 2006; Kriger, Pereoglou & Hero 2007). *Litoria wilcoxii/jungguy* were not observed to decline during one study despite a reported chytrid prevalence of 28% (Retallick, McCallum & Speare 2004). Another mark-recapture study suggested that *L. wilcoxii* not only survive infections but they can also clear their infections entirely (Kriger & Hero 2006). Sampling throughout the range of all species within the complex, found that chytrid was wide spread and that infection load was highest at low elevations (Kriger, Pereoglou & Hero 2007). As a result frogs in temperate areas were considered to be at a higher risk of chytrid-associated mortality compared to tropical areas (Kriger, Pereoglou & Hero 2007). Another related species, the Booroolong Frog (*L. booroolongensis*) (Donnellan & Mahony 2004), found in central-eastern Australia (western fall of the Great Dividing Range in New South Wales), is suspected to have suffered chytrid related declines, although the impact of the pathogen has not yet been assessed and other threats including introduced fish are also implicated (Hunter 2012).
Introduced trout and chytrid are currently considered the primary ongoing threats to *L. spenceri* (Gillespie & Clemann in review). However, the historic, current and future impact of these threats upon *L. spenceri* populations is unclear, particularly given potential multiplicative effects and the possibility that *L. lesueurii* represents a significant reservoir host for chytrid. In this thesis, I sought to address these knowledge gaps.

### 1.3 Thesis Aims and Structure

The specific aims of this thesis were:

1. To assess site occupancy dynamics of *L. spenceri* and *L. lesueurii* over the last six decades, and evaluate relationships between the persistence of *L. spenceri* populations and spatio-temporal variability in the occurrence of chytrid fungus and *L. lesueurii*;

2. To evaluate the accuracy (diagnostic sensitivity and specificity) of a polymerase chain reaction (PCR) diagnostic test to detect chytrid, to facilitate research on chytrid infection dynamics of *L. spenceri* and *L. lesueurii*;

3. To compare the individual-level responses of *L. spenceri* and *L. lesueurii* to chytrid, and to quantify the probabilities of survival of these species relative to their infection state, becoming infected and recovering from an infection, and;

4. To examine the population-level consequences of chytrid and introduced trout upon *L. spenceri* and *L. lesueurii*.

In Chapter 2, I compare *L. spenceri* and *L. lesueurii* population trends across 49 sites using a two-species dynamic occupancy model and 55 years of survey data collected between 1958 and 2012. These data were used to evaluate evidence for *L. spenceri* decline and to develop hypotheses about factors that may have had a causative role in historical population extinctions.
In Chapter 3, I assess the diagnostic sensitivity and specificity of a PCR method to detect chytrid fungus on individual frogs. I undertook this assessment in order to understand the performance of the diagnostic method and its ability to accurately assess the presence of the pathogen in samples. The diagnostic accuracy of this approach was an important consideration for analyses in subsequent chapters.

In Chapter 4, I quantify the impact of chytrid fungus on the survival rates of *L. spenceri* and *L. lesueurii* by conducting a mark-recapture study over a six year period at two sites and over a four year period at two further sites. I assessed the infection state of each frog each time it is captured using the PCR method validated in Chapter 3 and constructed a multistate mark-recapture model to estimate survival probabilities for each species in the two infection states (as well as the probabilities of transitioning between infection states). In this chapter I also consider and evaluate four potential sources of bias: 1) misclassification of disease state, 2) the influence of disease state upon an individual’s detection probability, 3) impact of marking on survival or capture probabilities, and 4) the capacity of individuals to recover from an infection.

In Chapter 5, I evaluate the population-level impact of chytrid fungus and introduced trout upon *L. spenceri* and *L. lesueurii* populations based on demographic rates estimated in Chapter 4. I construct multi-infection-state deterministic matrix population models to compare the population growth rate of each species across different sites. In turn, I evaluate hypothetical management strategies that may be considered to conserve *L. spenceri*, either by mitigating mortality rates or bolstering recruitment.

In Chapter 6, I discuss the general findings of the thesis and place them in context of their contributions to existing knowledge. I make recommendations for future research and highlight species management opportunities arising from this thesis.
Evaluating long term patterns of population change and multiple factors linked to decline in sympatric frog species, using 55 years of data
Chapter 2. Evaluating long-term patterns of population change and multiple factors linked to decline in sympatric frog species, using 55 years of data

2.1 Introduction

Identifying causes of species decline is often difficult because multiple factors influence changes in abundance, and some apparent declines could be natural fluctuations (Pechmann et al. 1991; Pechmann & Wilbur 1994). Long-term monitoring programs are critical to distinguish between the role of natural and threatening processes and to provide evidence when threats are suspected to drive decline (Pechmann et al. 1991; Lindenmayer et al. 2012). However, this is challenging for many species. Species monitoring programs are often only initiated after significant population changes have occurred. Historic sampling data, such as inventory surveys or incidental sightings, are usually fragmented or not systematically recorded, and are not easily incorporated into conventional population trend analyses (Tingley & Beissinger 2009; Lindenmayer et al. 2012). An inability to use such data may significantly truncate time frames over which species trends can be assessed, thus contributing to uncertainty in population change and limiting interpretation of causes. Recent development of statistical approaches for dealing with uncertainty in site occupancy over time and missing observational data (MacKenzie et al. 2002; Royle & Kery 2007) provide opportunities to evaluate long term population trends using historic fragmented data on species occurrence.

Effective threatened species management can depend upon spatially-explicit knowledge of both the pattern of threatened species population change and the underlying causes (Evans et al. 2011). This is because factors impacting populations may vary across a species’ range (e.g. Blaustein et al. 2010; Savage, Sredl & Zamudio 2011; Doddington et al. 2013) and interactive effects amongst multiple factors on populations can be complex (Blaustein & Kiesecker 2002; Hof et al. 2011; Blaustein et al. 2012). Failing to consider combined
impacts of multiple processes and species interactions can bias forecasts of population viability (Sabo 2008), or lead to erroneous conclusions about underlying causes of change.

Identification of factors influencing population dynamics is crucial for amphibian species potentially impacted by Chytrid Fungus (*Batrachochytrium dendrobatidis*) because few options currently exist to mitigate this pathogen (McCallum 2012; Scheele *et al.* 2014b). Chytrid can cause rapid population decline (Scherer *et al.* 2005; Lips *et al.* 2006; Schloegel *et al.* 2006; Ryan, Lips & Eichholz 2008; Briggs, Knapp & Vredenburg 2010; Gillespie *et al.* 2014). But some populations appear to persist despite suffering chytrid infections (Muths, Scherer & Pilliod 2011; Phillott *et al.* 2013). Population responses to chytrid can be influenced by factors (like temperature or sympatric species) that limit pathogen growth, survival and transmission, or that affect host infection, recovery or survival rates (James *et al.* 2015). Understanding the relative impact of chytrid on population demography will enable the development of better-informed management responses (e.g. Heard *et al.* 2014).

Some susceptible amphibian populations could be driven to extinction if pathogen transmission is maintained by sympatric amphibian species that are more tolerant of chytrid (e.g. Retallick, McCallum & Speare 2004; Woodhams 2008; Hunter *et al.* 2010; Stockwell, Clulow & Mahony 2010; Reeder, Pessier & Vredenburg 2012; Scheele *et al.* 2015). In fact, both amphibian and non-amphibian species (such as crayfish, *Procambarus* spp.) could be pathogen-host-reservoirs for chytrid if they persist at sites despite harbouring infections (Reeder, Pessier & Vredenburg 2012; Brannelly *et al.* 2015). However, the role of reservoir hosts in most amphibian declines has not been evaluated. Sympatric amphibian species could influence each other’s population dynamics via other interactions including competition or predation. Evaluating these types of interactions is important when contemplating management solutions for declining species.

I suspected that two mountain stream breeding frog species in south-eastern Australia have different responses to chytrid, and were interested to evaluate if either chytrid and/or the frogs interactions with each other has influenced their extinction risk and distribution. The Spotted Tree Frog (*Litoria spenceri*) is a Critically Endangered species (Gillespie & Robertson 1998), and Lesueur’s Frog (*Litoria lesueurii*), is a non-threatened species that occurs sympatrically with *L. spenceri* at some sites. *Litoria spenceri*’s population decline
was initially linked to introduced trout species and historic habitat changes (Watson et al. 1991), then an epidemic chytrid infection in 1996 caused the rapid decline and local extinction of one population (Gillespie et al. 2014). The impact of chytrid upon other \textit{L. spenceri} populations has not been assessed. Chytrid associated declines have not been reported for \textit{L. lesueurii} or taxa within the same species complex, (Retallick, McCallum & Speare 2004; Kriger & Hero 2006). Consequently \textit{L. lesueurii} is suspected as a pathogen-host-reservoir. \textit{Litoria spenceri} and \textit{L. lesueurii} also seem to respond differently to introduce trout (Gillespie 2001b). But the impact of trout upon wild populations has been difficult to assess, as trout occur at all but the one historic \textit{L. spenceri} site at which the chytrid-linked extinction was recorded (Gillespie et al. 2014).

Records for \textit{L. spenceri} have now been collected over a 55 year period (1958 -2012). Data prior to the decline is highly fragmented. Although, survey effort increased in 1991-2012 when broad-scale and intensive mark-recapture programs were initiated to monitor \textit{L. spenceri} populations and evaluate factors linked to decline. Data on \textit{L. lesueurii} have also been recorded during surveys.

Dynamic occupancy models have been developed to assess spatially-explicit and temporal changes in species distributions (the presence or absence of a species across sites and through time) driven by extinction and colonization events (Royle & Kery 2007). An advantage of occupancy modelling is that the probability of species occurrence is assessed against the probability of detecting the species given the survey effort (MacKenzie et al. 2003). Previous assessments of \textit{L. spenceri} have suspected decline (Watson et al. 1991; Gillespie & Hollis 1996) but have not accounted for imperfect detection of frogs during surveys. Here, I extend this modelling method to assess temporal trends in site occupancy of \textit{L. spenceri} and \textit{L. lesueurii}, and examined the influence of chytrid and/or the presence of each frog species upon their probability of extinction. Additionally, I assessed three colonization scenarios that could influence \textit{L. spenceri} occupancy at sites; by assuming there was either constant, spatially-linked, or no colonization. The occupancy of both frog species was examined concurrently over the 55-year data period using a two-species dynamic occupancy model. The parameterized models were then used to predict changes in occupancy for an additional 45 years.
2.2 Methods

2.2.1 Frog survey data

I compiled records for both *L. spenceri* and *L. lesueurii* at all sites where *L. spenceri* has been historically reported. Accurate location records of *L. spenceri* have been reported since December 1958 (Watson et al. 1991; Gillespie & Hollis 1996). During the 1960’s, 1970’s and early 1980’s the species was observed sporadically. From the late 1980’s to 1996 targeted systematic surveys were undertaken throughout the entire potential range of *L. spenceri*. From 1991 – 2012 population monitoring was undertaken at most known sites, with intensive mark –recapture studies at some sites. *Litoria spenceri* has been recorded at 50 sites on 23 streams in south-eastern Australia (Gillespie & Hollis 1996; Gillespie 2001a; Gillespie & Clemann in review). Records from 49 sites were included in this study as one site was converted to a reservoir in 1982 following a single recorded observation in 1971 (Watson et al. 1991). In total, 1381 surveys were conducted at the 49 sites over the 55-year period (Hero 1990; Watson et al. 1991; D. Hunter and M. West unpublished data; Ehmann, Ehmann & Ehmann 1992; Gillespie & Hollis 1996; Gillespie 2001a; Todd et al. in prep.). *Litoria spenceri* was detected on 948 occasions, not detected on 431 occasions and not recorded on two occasions (when *L. lesueurii* were recorded). *Litoria lesueurii* was detected on 320 occasions, not detected on 976 occasions and not recorded on 84 occasions.

2.2.2 Chytrid records

Chytrid was first identified at a *L. spenceri* site in February 1996 (Berger et al. 1998; Gillespie et al. 2014). The timing of arrival of chytrid at this site is known precisely because the population had been intensively monitored prior to and during the pathogens arrival (Gillespie et al. 2014). Chytrid was subsequently detected at most other sites in 1997, 1998 or during 2009-2012. The precise timing of arrival of the pathogen at these sites is unknown. Chytrid has not yet been confirmed in one stream with two sampling sites. Chytrid was assumed continuously present at each site following its first detection. I also examined the potential presence of chytrid at sites prior to the first detection. Records for other frog species in south-eastern Australia suggest that chytrid could have been present since at least the late 1970’s when other frog declines appear to have commenced (Osborne, Hunter &
Hollis 1999; Hunter et al. 2010; Scheele et al. 2014a). Chytrid was first detected in north-eastern Australian frogs in 1978 (Berger et al. 2009) and it could have been present at L. spenceri sites prior to the 1980’s.

2.2.3 Multi-species dynamic occupancy model

Dynamic occupancy models evaluate changes in species’ site occupancy over time (Royle & Kery 2007). The method requires repeated surveys at multiple sites within sequential closed periods (usually a breeding or core active season), during which the detection or non-detection of species is recorded (Royle & Kery 2007). The closed period is defined as a discrete interval during which the species occupancy status is assumed to be constant, and closed to extinction and colonization events (MacKenzie et al. 2002). In this study the closed period was October – April when the two frog species were active along streams and breeding (Gillespie 2011a). Repeated surveys were conducted within 55 closed periods (summer seasons) between 1958 -2012 at the 49 known historic sites. Observations of site occupancy were modelled as a function of two processes; the extinction and colonization probability at sites between years, and the species detection probability during each repeat survey in each year given the species is extant at the site.

Multiple factors were examined that could influence species extinction and colonization probabilities. Candidate models compared the potential effect of the presence of Bd and/or the sympatric species upon the probabilities of species extinction (Table 2.1). Each of the models was compared under three different assumptions of L. spenceri colonization: 1) no colonization, 2) spatially-linked colonization and 3) constant colonization. Spatially-linked colonization assumed that sites could only be colonized by L. spenceri if a genetically related population was extant at a neighbouring site within a common stream catchment. Genetic relationships between populations have been previously assessed by allozyme and mtDNA analyses and 3 genetic groups have been identified (G. Gillespie and S. Donnellan, unpublished data). Litoria lesueurii colonization probability was assumed constant at all sites, as they are highly dispersive and widely distributed at lower elevation sites below historic L. spenceri sites (Donnellan & Mahony 2004). Models concurrently evaluated the site occupancy of L. spenceri and L. lesueurii as well as their probabilities of extinction and colonization.
Table 2.1 Combinations of factors evaluated by multi-species dynamic occupancy models to estimate the probability of extinction of *Litoria spenceri* and *L. lesueurii*. Bd: chytrid fungus; Symp: sympatric species.

<table>
<thead>
<tr>
<th>Model</th>
<th>Factors influencing extinction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bd only</td>
<td>Occurrence of Bd; no influence of other factors</td>
</tr>
<tr>
<td>Symp only</td>
<td>Occurrence of the sympatric species; no influence of other factors</td>
</tr>
<tr>
<td>Bd, Symp</td>
<td>Occurrence of both Bd and the sympatric species</td>
</tr>
</tbody>
</table>

The detection history (*Y*) of each species’ (*s*) at each site (*i*), in each year (*t*), and for each repeated survey (*k*) was modelled as a Bernoulli distribution:

\[ Y_{s,i,t,k} \sim \text{Bernoulli}(Z_{s,i,t} \times d_{F,i,t,k}) \]  

where the probability of observation was assumed dependent on the site being occupied (*Z_{s,i,t} = 1*) and the species being detected. The probability of detecting the species (*d_{F,i,t,k}*) varied with the type of survey (*F*) that was undertaken at the site (*i*) during a repeat survey (*k*) in a closed survey period (*t*). Survey effort was greater in when conducted after 1990 compared to earlier (Gillespie and Hollis 1996) and so all models assumed that detection rates could be different for surveys conducted during 1958 – 1989 compared to 1990 - 2012.

Models were compared assuming that either *L. spenceri* occupied all sites in the first year (*Z_{i,1} = 1*), or that the initial occupancy state was unknown except at one site when *L. spenceri* had been detected. Initial occupancy status of *L. lesueurii* was assumed unknown in all model scenarios. When unknown, initial occupancy probability was estimated from an uninformative uniform prior distribution. In subsequent years (*t =2…55*), the occupancy state (*Z*) of each species (*s*) at each site (*i*) in each year (*t*) was informed by the data and modelled as a draw from a Bernoulli distribution:
where the probability that each site \((i)\) was occupied in year \(t\) equaled the probability of remaining extant \((1-E_{s,i,t})\) if the site was occupied \((Z_{s,i,t-1} = 1)\) in the previous year \((t-1)\), or the probability of annual colonization \(C_{s,i,t}\) if the site was not occupied in the previous year \((Z_{s,i,t-1} = 0)\). In all models, \(L. lesueurii\) \((s=2)\) colonization probability \(C_{s,i,t}\) was assumed constant. Constant colonization probability for both species was drawn from an uninformative prior distribution \([0,1]\). When no colonization was assumed for \(L. spenceri\) \((s=1)\) \(C_{s,i,t}\) was 0. \(Litoria spenceri\) colonization was assumed the same across all sites, except in the model version assuming colonization was only possible when spatially-linked populations were occupied. Spatially-linked colonization was modeled as a logistic function:

\[
\text{Logit}(C_{s,i,t}) = \alpha + \beta \times G_{i,t}
\]

where \(G_{i,t}\) was the mean proportion of spatially-linked sites, to site \(i\), that were occupied in each year \((t)\), and the intercept term \((\alpha)\) and slope term \((\beta)\) were drawn from a uninformative normal distribution. This approach is also known as auto-logistic modelling (e.g. Yackulic et al. 2012; Eaton et al. 2014)

The probability of annual extinction \(E_{s,i,t}\) of each frog species could vary spatially and temporally, depending on one or more factors:

\[
E_{s,i,t} = E_{s,i,t} \left[ Z_{s,i,t-1}, B_{i,t-4} \right]
\]

The annual extinction probability \(E_{s,i,t}\) of species \(s\) at site \(i\) in year \(t\) depended on the presence of the sympatric species \((Z_{s,i,t-1} = 1)\) in the previous year \((s - 3)\), taking values of 2 or 1 when \(s\) is 1 or 2, respectively) and the presence of chytrid \((B_{i,t-4})\) after a 4-year time lag \((t-4)\). This time lag was selected as the local extinction of \(L. spenceri\) occurred 4 years after chytrid arrived in the only known case (Gillespie et al. 2014).

When the presence of chytrid at sites was unknown, it was modelled in each time step as a Bernoulli distribution:
where the probability that the site was actually infected was assumed to be a function of the annual probability of infection ($V_t$) if the site was previously uninfected ($B_{i,t-1} = 0$), and 1 otherwise. In this formulation, sites did not recover from an infection. I constructed models initially assuming chytrid was absent at all sites prior to 1996 (the first known chytrid detection at a site), and following 1996, the probability of sites becoming infected was informed by the known infection record. Alternative models were constructed to assess the influence of chytrid if present at sites prior to 1996. To do this, chytrid was assumed present at 45 of the 49 sites since 1965, 1975 or 1985. For the other four sites, two analysis indicated chytrid was absent at two sites (those at Bogong Creek) prior to 1996 (Gillespie et al. 2014), while chytrid has not yet been detected at two other sites and so the pathogen presence was assumed unknown. Models that only evaluated the impact of the sympatric species were equivalent to an assumption that chytrid was always present at all sites and had a constant effect or had no effect.

The multi-species dynamic occupancy model was constructed in a Bayesian framework in R (Version 3.2.1) (R Core Team 2013). Model analysis was performed with JAGS (Version 3.4.0) using packages R2jags (Version 0.5-6) and rjags (Version 3-15) and results were summarized using jags tools (Version 1.3). JAGS is a program for Bayesian analyses which combines prior knowledge and data to obtain samples from a posterior distribution using a Markov Chain Monte Carlo (MCMC) algorithm. The relationship between the prior knowledge and the data is defined by the model. Uninformative priors (drawn from a uniform distribution between 0 and 1) were assumed for all estimated probabilities of colonization and extinction. All models were performed with three replicate Markov chains that were each run for 300,000 MCMC iterations. The Markov chains were checked for convergence ensuring that the potential scale reduction factor (Rhat) values were less than 1.1. Parameters were estimated from the posterior distribution after discarding the first 150,000 iterations.
2.2.4 Predicted future change in species site occupancy

Following the assessment of the 55-year historic period (1958-2012), future changes in site occupancy were predicted for an additional 45 years (2013-2057); a total period of 100 years. Future changes in occupancy were modelled according to the same processes as in the previous model, with potential further colonization by *L. lesueurii*, ongoing chytrid presence and with subsequent effects on colonization and extinction probabilities.

2.3 Results

2.3.1 Historic change in site occupancy

All models predicted a similar historic decline in the number of sites occupied by *L. spenceri* assuming that all 49 sites were initially occupied (Table 2.2). By 2012, modelled estimates of mean *L. spenceri* occupancy were between 23 and 28 sites, representing a decline of between 47.2 and 57.1% since 1958. Mean declines in *L. spenceri* occupancy were most severe when models assumed chytrid could influence extinction probability and when either no, or spatially-linked, colonization was assumed (Table 2.2). The mean decline in *L. spenceri* occupancy was least severe when only the presence of *L. lesueurii* was assumed to influence probability of extinction at sites (Table 2.2). Mean probability of occupancy by *L. spenceri* in 2012 was estimated to be 0.502 (95%CI 0.408 – 0.592) assuming that both chytrid and *L. lesueurii* influenced the probability of extinction and that colonization was spatially-linked. Models assuming that initial *L. spenceri* occupancy status was unknown also predicted a similar mean probability of occupancy (mean 0.510, 95%CI 0.428 – 0.612) by *L. spenceri* in 2012. Despite assuming that initial *L. spenceri* occupancy status was unknown, initial occupancy was estimated to be high (mean 0.880, 95%CI 0.612 – 100%, assuming constant colonization).

All models predicted a similar expansion in the number of sites that *L. lesueurii* occupied (Table 2.2). Initial (1958) *L. lesueurii* occupancy was uncertain. Although, *L. lesueurii* was estimated to initially occupy a mean of 15 sites when only the presence of *L. spenceri* influenced its extinction probability, and around 20 - 23 sites when models included an
effect of chytrid (Table 2.2). In all models, mean *L. lesueurii* occupancy increased over time, and by 2012, *L. lesueurii* was estimated to occupy 32 - 34 sites (Table 2.2). Increasing *L. lesueurii* occupancy coincided with declining *L. spenceri* occupancy.

### 2.3.2 Detection probability

Detection probabilities differed between the species and for each of the two survey periods (Table 2.3). Between 1958 and 1990 the estimated probability of detection of *L. spenceri* was influenced by the assumptions underpinning probability of colonization. The probability of *L. spenceri* detection was lowest when no colonization was assumed compared to other assumptions of colonization (Table 2.3).

### 2.3.3 Annual infection probability

Estimates for the annual probability of infection of sites by chytrid were consistent across models, with a mean of approximately 0.266 (95% CI: 0.183 – 0.368).

### 2.3.4 Factors influencing annual extinction probability

The probability of *L. spenceri* extinction was higher when chytrid was present at sites compared to when chytrid was absent (Figure 2.1), for models assuming chytrid arrived at sites from 1996. *Litoria spenceri* extinction probability also increased when *L. lesueurii* was present at sites (Figure 2.1). Models examining multiple factors found that *L. spenceri* extinction probability was highest when both chytrid and *L. lesueurii* occurred at sites compared to when only one of these factors was present, or both factors were absent at sites (Figure 2.1). This trend was consistent regardless of the assumed timing of arrival of chytrid at sites (Figures 2.1 and 2.2), or of the colonization assumptions (Figure 2.1), and regardless of the assumptions of initial *L. spenceri* occupancy (see Appendix 1). Chytrid and *L. lesueurii* were predicted not to independently influence *L. spenceri* extinction when models assumed chytrid was present at sites prior to 1996 (Figure 2.2).

*Litoria lesueurii* extinction was lowest when either chytrid was present or *L. spenceri* was absent, assuming chytrid arrived at sites at or following 1996. However, the influences of chytrid and *L. spenceri* upon the probability of *L. lesueurii* extinctions were uncertain
(Figure 2.1). The impact of chytrid and *L. spenceri* upon *L. lesueurii* extinction probability was also uncertain assuming chytrid arrived at sites prior to 1996 (Figure 2.2).

**Figure 2.1** Factors influencing the probability of extinction of *Litoria spenceri* and *L. lesueurii*. Plot panels compare each species probability of extinction for three different models (shown in each column) that assume the presence (+) or absence (-) of one or more factors: “Bd”: chytrid fungus (*Batrachochytrium dendrobatidis*) and/or “symp”: sympatric frog species (either *L. spenceri* or *L. lesueurii*). Points and lines represent the mean and 95% credible interval estimates. Results are shown for models that assume: no *L. spenceri* colonization (black solid lines); spatially-linked *L. spenceri* colonization (grey dashed lines); constant colonization (grey solid lines).
Figure 2.2 Factors influencing the probability of extinction of *Litoria spenceri* and *L. lesueurii* assuming chytrid fungus (*Batrachochytrium dendrobatidis*) was present at sites prior to 1996, arriving in either 1965, 1975 or 1985. Plot panels compare the species probability of extinction assuming the presence (+) or absence (-) of one or more factors: “Bd”: chytrid fungus and/or “symp”: sympatric frog species (either *L. spenceri* or *L. lesueurii*). Points and lines represent the mean and 95% credible interval estimates. Results are shown assuming no *L. spenceri* colonization.
Table 2.2 Estimated mean current (2012) and future (2057) proportion of the 49 historic *Litoria spenceri* sites occupied by *L. spenceri* and *L. lesueurii* under varying assumptions of extinction and colonization. The 95% credible interval for the estimated proportion of occupied sites is shown in parenthesis. The model Deviance Information Criterion (DIC) is also shown.

<table>
<thead>
<tr>
<th>Model</th>
<th>Species extinction influenced by:</th>
<th><strong>L. spenceri</strong></th>
<th><strong>L. lesueurii</strong></th>
<th><strong>Model DIC</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Site Occupancy</strong></td>
<td><strong>Site Occupancy</strong></td>
<td><strong>Site Occupancy</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>at 2012</td>
<td>at 2057</td>
<td>at 2012</td>
</tr>
<tr>
<td></td>
<td>Assuming no colonization of sites by <em>L. spenceri:</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bd only</td>
<td>0.472 (0.387 – 0.551)</td>
<td>0.029 (0.0 – 0.122)</td>
<td>0.684 (0.571 – 0.796)</td>
<td>0.656 (0.408 – 0.877)</td>
</tr>
<tr>
<td>Symp only</td>
<td>0.562 (0.490 - 0.612)</td>
<td>0.355 (0.224 – 0.490)</td>
<td>0.661 (0.571 – 0.775)</td>
<td>0.628 (0.428 – 0.816)</td>
</tr>
<tr>
<td>Bd, Symp</td>
<td>0.484 (0.408 – 0.571)</td>
<td>0.030 (0.0 - 0.122)</td>
<td>0.677 (0.571-0.775)</td>
<td>0.698 (0.408 – 0.939)</td>
</tr>
<tr>
<td>Assuming spatially-linked colonization by <em>L. spenceri:</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bd only</td>
<td>0.489 (0.408 – 0.592)</td>
<td>0.115 (0.0 - 0.531)</td>
<td>0.685 (0.592 – 0.796)</td>
<td>0.657 (0.428 – 0.877)</td>
</tr>
<tr>
<td>Symp only</td>
<td>0.560 (0.470 – 0.653)</td>
<td>0.379 (0.082 – 0.816)</td>
<td>0.657 (0.551 – 0.755)</td>
<td>0.591 (0.388 – 0.796)</td>
</tr>
<tr>
<td>Bd, Symp</td>
<td>0.502 (0.408 – 0.592)</td>
<td>0.124 (0.0 - 0.551)</td>
<td>0.675 (0.571 -0.776)</td>
<td>0.698 (0.428 - 0.939)</td>
</tr>
<tr>
<td>Assuming constant colonization by <em>L. spenceri:</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bd only</td>
<td>0.503 (0.408 – 0.592)</td>
<td>0.445 (0.224 – 0.673)</td>
<td>0.685 (0.571 – 0.796)</td>
<td>0.654 (0.428 – 0.878)</td>
</tr>
<tr>
<td>Symp only</td>
<td>0.580 (0.490 – 0.673)</td>
<td>0.697 (0.531 – 0.587)</td>
<td>0.654 (0.551 – 0.755)</td>
<td>0.545 (0.367 – 0.714)</td>
</tr>
<tr>
<td>Bd, Symp</td>
<td>0.517 (0.428 – 0.612)</td>
<td>0.446 (0.224 – 0.673)</td>
<td>0.675 (0.571 -0.775)</td>
<td>0.642 (0.408 – 0.857)</td>
</tr>
</tbody>
</table>
Table 2.3 Estimated probability of detection and colonization of *Litoria spenceri* and *L. lesueurii* in 2012 under varying modelled scenarios. The 95% credible interval for estimates is shown in parenthesis.

<table>
<thead>
<tr>
<th>Model</th>
<th><em>L. spenceri</em></th>
<th><em>L. lesueurii</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Pr (Detection)</em></td>
<td><em>Pr (Colonization)</em></td>
</tr>
<tr>
<td><strong>Assuming no colonization of sites by <em>L. spenceri</em></strong>:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bd only</td>
<td>0.458 (0.364 – 0.554)</td>
<td>0.845 (0.822 – 0.868)</td>
</tr>
<tr>
<td>Symp only</td>
<td>0.460 (0.367 – 0.557)</td>
<td>0.844 (0.822 – 0.866)</td>
</tr>
<tr>
<td>Bd, Symp</td>
<td>0.458 (0.363 – 0.554)</td>
<td>0.845 (0.822 – 0.867)</td>
</tr>
<tr>
<td><strong>Assuming spatially-linked colonization by <em>L. spenceri</em></strong>:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bd only</td>
<td>0.738 (0.598 – 0.853)</td>
<td>0.850 (0.828 – 0.871)</td>
</tr>
<tr>
<td>Symp only</td>
<td>0.744 (0.612 – 0.855)</td>
<td>0.850 (0.828 – 0.871)</td>
</tr>
<tr>
<td>Bd, Symp</td>
<td>0.738 (0.606 – 0.851)</td>
<td>0.850 (0.828 – 0.871)</td>
</tr>
<tr>
<td><strong>Assuming constant colonization by <em>L. spenceri</em></strong>:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bd only</td>
<td>0.745 (0.610 – 0.861)</td>
<td>0.851 (0.828 – 0.875)</td>
</tr>
<tr>
<td>Symp only</td>
<td>0.759 (0.632 – 0.867)</td>
<td>0.850 (0.827 – 0.872)</td>
</tr>
<tr>
<td>Bd, Symp</td>
<td>0.749 (0.614 – 0.859)</td>
<td>0.850 (0.827 – 0.872)</td>
</tr>
</tbody>
</table>

* Logit \( C_{s,i,t} \) = -7.69 + 6.56*G, if all neighbouring spatially-linked sites are occupied (G=1) mean colonization probability: 0.203 (0.039 – 0.505)

** Logit \( C_{s,i,t} \) = -7.98 + 6.24*G, if all neighbouring spatially-linked sites are occupied (G=1) mean colonization probability: 0.273 (0.066 – 0.615)

*** Logit \( C_{s,i,t} \) = -7.64 + 6.15*G, if all neighbouring spatially-linked sites are occupied (G=1) mean colonization probability: 0.212 (0.044 – 0.562)

Where \( C_{s,i,t} \) = probability of colonization and \( G \) = mean proportion of spatially-linked neighbouring sites that are occupied.
2.3.5 Annual colonization probability

Mean annual colonization probability estimates appeared higher for *L. lesueurii* than for *L. spenceri* when species colonization was assumed to be constant (Table 2.3), but the 95%CI estimates overlapped and so differences were uncertain.

The probability of spatially-linked *L. spenceri* colonization was similar for all models regardless of the factors influencing extinction probability (Table 2.3). The probability of annual *L. spenceri* colonization was estimated to be 0.212 (95% CI: 0.044 – 0.562) if all spatially-linked sites were occupied, when assuming that both the presence of chytrid and *L. lesueurii* influence *L. spenceri* extinction probability.

2.3.6 Future change in occupancy

All models predicted that *L. spenceri* site occupancy will continue to decline beyond 2012 when either no colonization or spatially-linked colonization was assumed (Table 2.2). Future declines were most severe and more likely under an assumption of no colonization when both chytrid and *L. lesueurii* were present (Figure 2.3). Although similar declines were predicted by all models that included an effect of chytrid (Table 2.2). *Litoria spenceri* declines were least severe when only *L. lesueurii* influenced the species probability of extinction (Table 2.2).

The analysis indicates that *L. spenceri* may occupy only 25% of historic sites by 2022 and become extinct at all sites as early as 2035, assuming both chytrid and *L. lesueurii* are present (Figure 2.3). However, the risk of a future decline in *L. spenceri* occupancy was reduced under an assumption of constant colonization compared to other types of colonization (Table 2.2). If colonization is constant, site occupancy could even stabilize or increase in future (Figure 2.3). However predicted future occupancy may be overly optimistic when constant colonization is assumed, as extinction is not possible if colonization probability is > 0.

Site occupancy by *L. lesueurii* was generally predicted to stabilize between 2013 and 2057 (Figure 2.3, Table 2.2), but, future site occupancy was uncertain.
Figure 2.3 Estimated historic (1958-2012) and predicted future (2013-2057) change in proportion of sites occupied by *L. spenceri* and *L. lesueurii* under three different assumptions of colonization by *L. spenceri*: A) no colonization; B) colonization occurs between sites within a major stream catchment that are spatially-linked; C) constant probability of colonization. Colonization by *L. lesueurii* was always assumed to be constant. The probability of extinction of both frog species was assumed to be influenced by chytrid and the other frog species when present at the sites. Black lines and green shading indicate mean and 95% CI estimated occupancy changes for *L. spenceri*. Blue lines and shading indicate mean and 95% CI estimated occupancy changes for *L. lesueurii*. 
2.4 Discussion

My analysis reveals an approximate 50% decline in the proportion of sites that *L. spenceri* occupied by 2012, and that future declines are likely. The findings support previous reported concerns of a decline in distribution and number of populations of *L. spenceri* (Watson *et al.* 1991; Gillespie & Hollis 1996; Hunter & Gillespie 1999; Gillespie 2001b). Even when initial *L. spenceri* occupancy status was assumed unknown, models still suggested that 43 - 61% (mean 51%) of sites are currently occupied. My results also demonstrate that the observed decline in occupancy of *L. spenceri* coincided with an expansion in the proportion of sites occupied by *L. lesueurii* and in the detection of chytrid at historic sites. These findings suggest that the decline of *L. spenceri* could be influenced by the presence of chytrid, the presence of *L. lesueurii*, or both factors acting in concert.

Chytrid has already been directly linked to the decline and extirpation of a *L. spenceri* population at one site (Gillespie *et al.* 2014). Importantly, this chytrid-linked decline occurred in the absence of other threatening processes (such as introduced trout) and in the absence of *L. lesueurii* (Gillespie *et al.* 2014). However, long-term monitoring indicates that other *L. spenceri* populations can persist for periods of at least 14 years with chytrid (Todd *et al.* in prep.). Differences in the response of *L. spenceri* between sites could be driven by site-specific factors such as the thermal properties of the site, the total density of amphibian hosts and the presence of other threats or sympatric species. For instance, species’ thermoregulatory opportunities and behaviour can influence host defences against chytrid (Rowley & Alford 2013; Daskin *et al.* 2014). Thermal conditions can restrict chytrid growth and survival, with the pathogen dying above 30°C (Piotrowski, Annis & Longcore 2004). Given this, I would expect *L. spenceri* extinction probability to be lowest at sites when temperatures are frequently above 30°C. However despite some thermal differences between the known 49 *L. spenceri* sites, I have not yet found a clear influence of temperature upon changes in *L. spenceri* occupancy (see Appendix 1). Given the clear relationships that have been identified between chytrid and temperature additional research is warranted.

*Litoria spenceri* extinction risk was most severe when *L. lesueurii* and chytrid were both present at sites, which could indicate an additive or synergistic interaction between these factors. This interaction could involve competition (Gillespie 2001a; Gillespie 2002) or
predation, but clear evidence is lacking. Alternatively, the apparent interaction could occur if *L. lesueurii* is tolerant of chytrid and maintains a pathogen-reservoir regardless of *L. spenceri*’s density. My results support this, as *L. lesueurii* site occupancy was positively associated with chytrid, which suggests that *L. lesueurii* population can tolerate chytrid. This is further supported by other studies that suggest closely related species to *L. lesueurii* (*Litoria wilcoxii* and *Litoria jungguy*) could act as pathogen-host-reservoirs and may have been involved in the decline of other species like Eungella Torrent Frog (*Taudactylus eungellensis*) and Eungella Gastric-Brooding Frogs (*Rheobatrachus vitellinus*) in Queensland, Australia. (Retallick, McCallum & Speare 2004; Kriger & Hero 2006). Additional research is required to clarify these associations and evaluate the demographic impact of chytrid on both *L. spenceri* and *L. lesueurii*.

Behavioural (Rowley & Alford 2009), immunological (Woodhams *et al.* 2007) and demographic differences could influence amphibian species ability to cope with chytrid, and may explain the contrasting associations of *L. spenceri* and *L. lesueurii* with chytrid. Species behaviour could be important if they spend more time in water or cool, moist microclimates. At my study sites *L. lesueurii* disperse away from streams after breeding whereas *L. spenceri* is known to remain close to streams throughout the year (Gillespie 2001a). When *Litoria wilcoxii*, a genetically related and behaviourally similar species to *L. lesueurii*, was tracked, it was found to spend less time in water and to be less susceptible to chytrid than other species to which it was compared (Rowley & Alford 2007a). The extent to which behavioural differences or potential immune defences influence the response of *L. spenceri* and *L. lesueurii* to chytrid has not been assessed. Some species appear to tolerate chytrid infection if recruitment rates can offset pathogen-induced adult mortality (Muths *et al.* 2011; Scheele *et al.* 2015). *Litoria lesueurii* has a higher recruitment potential than *L. spenceri*, with *L. lesueurii* producing three times more eggs than *L. spenceri* (Gillespie 2011a). In addition, the presence of introduced trout, which occur at all but one historic *L. spenceri* site, could further exacerbate differences in recruitment. Whilst trout appear to have little impact upon *L. lesueurii* tadpole survival, trout can significantly reduce *L. spenceri* tadpole survival (Gillespie 2001b).

Interactions between multiple factors are often neglected in species decline but need to be evaluated to effectively prioritize conservation efforts (Blaustein *et al.* 2011; Hof *et al.*
My results indicated that *L. spenceri* extinction risk is likely to be influenced by complex interactions between multiple factors, including chytrid and sympatric species. Other site-specific factors, including the presence of introduced trout species, other sympatric frog species, habitat variability and climatic conditions could also influence *L. spenceri* population dynamics. Trout could not be explicitly examined in this study because they were present at all but one site. Similarly, the evaluation of other factors (such as climate change) that could influence *L. lesueurii* expansion were beyond the scope my project.

The analysis was able to handle missing data and unknown state variables for the *L. spenceri* and *L. lesueurii* data sets. The analysis is limited by an absence of known unoccupied sites which could mean that *L. spenceri* site occupancy only can change in one direction (decline). But current *L. spenceri* occupancy estimates were similar regardless of the species assumed initial occupancy state, and models that assumed all sites were initially occupied were more parsimonious. The changes in *L. spenceri* occupancy do not appear to have been offset by their colonization of previously un-occupied areas. An assumption that sites were initially occupied may be justified particularly as *L. spenceri* is considered to have a low dispersal rate (Gillespie & Hollis 1996; Gillespie 2001a; G. Gillespie and M West unpublished data) and is not known to recolonize sites following a localized extinction (Gillespie *et al.* 2014).

The ability of *L. spenceri* to recolonize sites naturally appears increasingly unlikely given that *L. spenceri* occupancy has already declined by around 50%. Future declines in *L. spenceri* site occupancy were predicted to be least severe when colonization was assumed to be constant. Further modelling may reveal management solutions for *L. spenceri* involving translocation to maintain site colonization. In contrast to *L. spenceri*, *L. lesueurii* are capable of dispersal over greater distances and they are regularly encountered away from the stream (Gillespie & Hollis 1996; Rowley & Alford 2007). The species’ contrasting dispersal behaviours may help to explain their differences in extinction risk.

The duration of most amphibian populations association with chytrid remains unknown (e.g. Rachowicz *et al.* 2005; Phillips *et al.* 2012). There is ongoing debate regarding whether the emergence of chytridiomycosis has resulted from a novel epidemic infection or an existing
endemic association and change in the pathogen’s impact (Venesky et al. 2014). Chytrid sampling has only been sufficient to determine the time of chytrid arrival (1996) at two *L. spenceri* sites (Gillespie et al. 2014). When I tested assumptions that chytrid arrived at other sites prior to 1996, chytrid and *L. lesueurii* did not independently influence *L. spenceri* extinction. Instead, the analysis suggested that *L. spenceri* extinction only increased when both chytrid and *L. lesueurii* were present at sites. This could indicate that an expansion in *L. lesueurii* occupancy may have exacerbated chytrid impacts upon *L. spenceri*. Chytrid may have been present at some *L. spenceri* sites prior to 1996 which is consistent with other observations in south-eastern Australia. Chytrid has been implicated in other species declines in this region since the 1980’s (e.g. Hunter et al. 2010). Models assuming chytrid infection commenced in 1996 were ranked higher (lower DIC values) than pre-1996 arrival models, but I cannot yet resolve the timing of arrival of chytrid at most sites.

Long-term studies are important for making informed conservation management decisions (Lindenmayer et al. 2012) and to permit a broader understanding of the processes that drive population decline (Caughley 1994). Population change can be difficult to assess when occurrence data prior to suspected declines is sparse or incomplete (Tingley & Beissinger 2009). But my approach, combining both historic observational records and more intensively collected survey data, could be useful to assess other species where mixed data types exist and the population decline or causes of change are uncertain. Hypotheses about factors influencing species extinction and colonization of sites can be generated and compared using dynamic occupancy assessments that are underpinned by models of the processes that drive population change.

I hypothesize that the decline of *L. spenceri* is most severe at sites when the presence of less-susceptible host reservoir species (or total host density) facilitate chytrid persistence and maintain chytrid transmission. Efforts to conserve *L. spenceri* may be most effective at sites where sympatric species (potential pathogen-host-reservoirs) are absent. If both chytrid and the presence of *L. lesueurii* together drive the decline of *L. spenceri*, actions to reduce the impact of chytrid or to reduce the occurrence of *L. lesueurii* may limit further declines and perhaps promote recovery.
Imperfect disease classification: can PCR tests accurately detect wildlife pathogens?
Chapter 3. Imperfect disease classification: can PCR tests accurately detect wildlife pathogens?

3.1 Introduction

Genetic identification tests using the polymerase chain reaction (PCR) method have become an important tool to detect pathogens and assess the impact of disease on wildlife. However, PCR test results can be imperfect which may result in a pathogen’s presence being misclassified. Misclassification can lead to inaccurate assessments of disease impact and inappropriate management intervention (McClintock et al. 2010; Miller et al. 2012). For instance, a failure to account for false negative results could be particularly devastating for populations recently exposed to a highly virulent disease, and severe declines may occur unless appropriate management intervention is initiated (OIE 2010; OIE 2012). The accuracy of PCR test results must be understood, particularly as disease is increasingly linked to wildlife declines and options to mitigate disease impacts are often limited (Scheele et al. 2014b; Langwig et al. 2015).

Evaluating sensitivity and specificity is a primary step when developing diagnostic tests (OIE 2010; Skerratt et al. 2011; OIE 2012). Diagnostic sensitivity is the proportion of known infected (true positive) reference samples that test positive and diagnostic specificity is the proportion of known uninfected (true negative) samples that test negative (Saah & Hoover 1997). Whilst other validation steps are also important (outlined in OIE 2012), diagnostic sensitivity and specificity should be assessed to provide confidence in test accuracy and permit inferences to be drawn from the results (OIE 2012). These terms should not be confused with analytic sensitivity and specificity which respectively refer to the minimum concentration of a target that can be detected and the PCR test’s ability to distinguish the target DNA sequence from other potential non-target DNA sequences in a sample (Saah & Hoover 1997). Diagnostic sensitivity can be influenced by an individual’s stage of infection, sampling methodology, degradation of the target DNA sequence prior to testing, a failure to extract DNA, concentrations of the target DNA sequence in the sample
that are below the test detection limit (poor analytic sensitivity), contaminants that inhibit the PCR test performance, and methodological problems during laboratory processing (poor repeatability) (Greiner & Gardner 2000; Van Sluys et al. 2008). Diagnostic specificity can be influenced by cross-reactivity with other substances in the sample (poor analytic specificity), accidental contamination of the sample with target DNA in the laboratory, or products within the sample that could indicate a previous exposure to the pathogen (including maternal antibodies) (Greiner & Gardner 2000). Consequently, diagnostic sensitivity and specificity may need to be defined for a particular set of conditions, populations or species. Furthermore the handling of equivocal test results can influence diagnostic sensitivity and specificity (Greiner & Gardner 2000). When samples are tested in triplicate, equivocal results indicate that replicate tests do not agree and that at least one replicate produced a false negative or a false positive result (Hyatt et al. 2007). Consequently the actual presence of the target DNA in the sample is uncertain.

Diagnostic methods have been developed to test amphibians for chytrid fungus (*Batrachochytrium dendrobatidis*) (hereafter chytrid). Chytrid, a fungal pathogen causing chytridiomycosis (Berger et al. 1998; Longcore, Pessier & Nichols 1999), is linked to amphibian declines and extinctions worldwide (e.g. Daszak et al. 1999; Lips 1999; Gillespie et al. 2014). Amphibians become infected with chytrid if a free swimming zoospore embeds into their keratinized tissue (Berger et al. 1998). Zoospores then mature through several stages to form sporangia which ultimately release new zoospores (Berger et al. 2005). Histological analysis can be used to detect chytrid within the keratinized tissue, although the method is invasive, requiring animal tissue, and diagnosis is relatively slow (Berger et al. 1998; Skerratt et al. 2011). More rapid and less invasive diagnostic PCR methods include conventional (Annis et al. 2004; Garland, Wood & Skerratt 2011) and nested PCR methods (Goka et al. 2009) that permit qualitative assessment and real-time PCR methods (Boyle et al. 2004; Hyatt et al. 2007; Kosch & Summers 2013) that permit quantitative assessment of chytrid DNA in a sample. Some validation of the published chytrid PCR methodologies has been undertaken, including assessments of analytic sensitivity and specificity (e.g. Hyatt et al. 2007; Goka et al. 2009). However, the diagnostic sensitivity and specificity of these PCR tests have been overlooked or inadequately assessed (Skerratt et al. 2011).
By definition, assessments of diagnostic sensitivity and specificity should be conducted using samples where the target’s presence (such as the pathogen DNA) is known with certainty. Assessments are often undertaken using deliberately infected animals, although this approach cannot guarantee actual presence or absence of the target DNA within the collected sample. For instance, a Taqman quantitative PCR method (Boyle et al. (2004) (hereafter Taqman qPCR) did not always detect chytrid in deliberately exposed individuals, which could indicate a failure to sample the pathogen or a false negative qPCR result (Hyatt et al. 2007). Recognizing that additional validation was required, Skerratt et al. (2011) estimated diagnostic sensitivity and specificity of two chytrid diagnostic methods using a Bayesian latent class analysis that had been used to test wild collected samples. This approach relies on the comparison of at least two tests assuming neither is perfect (Enøe, Georgiadis & Johnson 2000; Branscum, Gardner & Johnson 2005). However the approach may not accurately assess diagnostic sensitivity or specificity as the presence and concentration of the target DNA is not known. An alternative approach to evaluate diagnostic sensitivity and specificity is to deliberately spike samples with target (pathogen) DNA.

The cost of diagnostic testing limits research on impacts of diseases such as chytridiomycosis on wildlife, particularly as studies often require large sample sizes to achieve acceptable statistical power (Kriger, Hero & Ashton 2006). Published diagnostic PCR methods usually test samples in triplicate. Singlicate testing has been suggested as an option to reduce cost (Kriger, Hero & Ashton 2006). However, triplicate testing can minimize the risk of failed PCR reactions and misclassifying samples by comparing the results between each replicate test. The use of triplicate testing and correct interpretation of equivocal results (when replicate results differ) may be important and could help to reduce the risk of misclassifying samples.

Here I conduct preliminary assessments of diagnostic sensitivity and specificity of four PCR tests for chytrid, and assess the diagnostic sensitivity and specificity of the preferred method using samples that were either spiked with chytrid zoospores or a control. In addition, I evaluate how equivocal results should be interpreted when assessing the risk or impact of disease on wildlife.
3.2 Methods

3.2.1 Production and quantification of zoospores

Chytrid cultures were produced by seeding sterile TGhL agar plates (8g tryptone, 2g gelatin hydrolysate, 2g lactose, 10g bacteriological agar and 1000ml double-distilled water (ddH₂O)) with zoospores from chytrid isolates supplied by James Cook University. Preliminary experiments used an Abercrombie isolate (AbercrombieR-Lbooroologensis-09-LB-1) and experiments 1 and 2 used a Waste Point isolate (Litoria verreauxii-2013-LB,RW-2). Plates were incubated at 22 °C for 6-7 days. Plates were flooded with 2 ml ddH₂O to stimulate release of zoospores from sporangia. Resultant zoospore solutions were collected after 30 minutes. The zoospore solutions from eight plates of the same isolate were combined. Zoospore solutions were filtered using a 10 μm isopore filter (Millipore) to remove larger particles like sporangia. The zoospores were mixed gently by slow pipetting to ensure an even dispersal of zoospores within the solution, and 10 x 10 μl samples were counted on a haemocytometer to estimate the concentration of zoospores in the solution for each isolate (Abercrombie mean 10578 zoospores/μl; range: 9050 – 12650; Waste Point mean 8322 zoospores/μl; range: 7050 – 9325). Stock solutions for each isolate were serially diluted with ddH₂O prior to swab sample preparation.

3.2.2 DNA extraction

Samples were extracted using a Chelex/Proteinase K methodology (hereafter Chelex method): 200 μl of 5% Chelex 100 molecular biology grade resin (Bio-Rad) in ddH₂O and 3 μl of Proteinase K, recombinant PCR grade (Roche) was added to each sample contained within a 1.5 ml microtube. The microtube was incubated at 56 °C for 60 min then at 95 °C for 15 min with periodic vortexing. The microtube containing the extract solution and swab was stored at -20 °C. Prior to PCR, extractions were centrifuged at 10,000 g for 2 min and supernatant from just above the Chelex resin precipitate was used for PCR amplification.
3.2.3 PCR methods

The Taqman qPCR methodology has been previously described by Boyle et al. (2004) and Hyatt et al. (2007). Taqman qPCR assays were conducted using a Roche LightCycler 480 system (Roche Diagnostics Australia, Castle Hill, New South Wales, Australia) in a 384-well format. The primers and probe sequences for chytrid detection were; forward ITS1-3 Chytr (‘5-CCTTGATATAATACAGTGCCATATGTC-3’), reverse 5.8S Chytr (‘5-AGCCAAGA- GATCCGTTGTCAA-3’), and the Chytr MGB2 probe (5’- 6FAM CGAGTCGAACAAAT MGBNFQ-3’). A total 25 μl reaction volume containing 12.5 μl 2 × Taqman Master Mix (Applied Biosystems), qPCR primers at a concentration of 900 nM, the MGB probe at 250 nM and 5μl of DNA. DNA samples were either diluted 1:10 or 1:15 (hereafter referred to as Taqman 1:10 qPCR or Taqman 1:15 qPCR respectively). Samples were analysed in triplicate. PCR amplification conditions were 10 minutes at 95 °C, followed by 50 cycles of 15 seconds at 95 °C and 1 minute at 60 °C.

The Qiagen qPCR methodology was a modification of the Taqman qPCR methodology; the reaction volume was 10 μl and contained the Qiagen multiplex PCR Master Mix (Qiagen) instead of the Taqman Master Mix above. The reaction mix consisted of 5 μl of 2x Qiagen multiplex PCR Master Mix (Qiagen), PCR primers at a concentration of 900 nM, the MGB probe at 250 nM and 2 μl of DNA. PCR amplification conditions were 15 minutes at 95 °C, followed by 50 cycles of 15 seconds at 95 °C and 1 minute at 60 °C.

A standard curve of 100, 10, 1 and 0.1 chytrid zoospores (Abercrombie chytrid isolate) was included in triplicate on each qPCR plate along with a negative control of ultrapure water. The reaction efficiency and number of zoospores was calculated using the Absolute Quantification module of the LightCycler 480 1.5.1 software package. The efficiency of the qPCR was always 95-100% across all assay plates.

The Nested PCR methodology followed Goka et al. (2009) except using 2 μl of DNA sample and a different DNA polymerase and reaction buffer. I added 0.75 units of Immolase Hot-Start thermostable DNA polymerase and 1 x Immolase reaction buffer (Bioline Australia Pty Ltd) in the first 10μl reaction volume and 2.25 units of Immolase Hot-Start thermostable DNA polymerase and 1 x Immolase reaction buffer in the second 30 μl
reaction volume, instead of the Taq DNA polymerase (Amplitaq Gold) and 50 µl reaction volumes used by Goka et al. (2009).

All PCRs used the same standards supplied by the Australian Animal Health Laboratory (AAHL, CSIRO, Geelong, Australia) that had been produced from an Abercrombie chytrid isolate (Boyle et al. 2004).

3.2.4 Preliminary assessments of diagnostic sensitivity and specificity

Two preliminary experiments were conducted to compare the sensitivity and specificity of the four PCR methods. Experiments were performed using swabs that were spiked with a known concentration of zoospores.

3.2.4.1 Preliminary Experiment 1: Comparing diagnostic sensitivity and specificity of four PCR methods: spiked samples, no other contaminants.

A total of 50 swabs were split into five groups of ten swabs. Swabs in each group were spiked with either a control sample (sterile water, ddH₂O) or one of four different concentrations of harvested Abercrombie isolate zoospores (4, 10, 40 or 4000 zoospore/sample). The swabs were air dried for 20 minutes and kept at 4 °C for 10 days. To ensure consistency between PCR methods, all swabs were extracted using the Chelex method described above, and 40 µl aliquots of the supernatant from each extracted swab were dispensed into three microtubes. A random aliquot from each swab was sent to independent laboratories to perform analysis. Laboratory 1 performed the Qiagen qPCR and Nested PCR tests. Laboratory 2 performed the Taqman 1:15 qPCR, and Laboratory 3 performed the Taqman 1:10 qPCR.

The diagnostic sensitivity and specificity were then evaluated using a Bayesian approach to estimate the mean proportion and 95% credible interval (McCarthy 2007) of samples that were accurately determined to be either positive or negative. The observed proportion of samples that accurately tested positive or negative was assumed to be sampled from a binomial distribution. The prior for the proportion was a uniform distribution between 0 and 1.
3.2.4.2 Preliminary Experiment B: Comparing diagnostic sensitivity of four PCR methods: spiked samples, with natural contaminants present.

A total of 50 swabs were split into five groups of ten swabs. Swabs in each group were spiked with either a control sample (sterile water) or one of four different concentrations of Abercrombie isolate zoospores (8, 20, 80 or 8000 zoospore/sample, double the concentration of Pilot Experiment A). Swabs were prepared and extracted in the same manner as Pilot Experiment A, then 200 µl of a wild frog contaminate solution was added to each extraction. The contaminate solution contained additional DNA and other contaminants that naturally occur on wild collected samples and was produced by combining DNA extractions of swab samples from 100 wild frog swabs. The addition of the 200 µl contaminant solution meant that the final known concentration of zoospores in each sample was equal to samples in Pilot Experiment A.

Swabs from the wild frogs had been taken from Spotted Tree Frogs (Litoria spenceri) and Lesueur’s Frogs (L. lesueurii) during field surveys in November 2012. Both species are known to suffer chytrid infections and so it was possible that some samples contained chytrid. All solutions were likely to contain a range of other natural contaminants found on wild frogs. The swabs from the wild frogs were individually extracted using the Chelex method and 100 µl of the supernatant from each extract was combined to produce the wild frog contaminant solution.

A 40ul aliquot of each spiked sample containing contaminants was distributed to laboratories as per Pilot Experiment A.

Diagnostic sensitivity was then evaluated using the analysis of proportions described in Pilot Experiment A. Specificity was not evaluated as it was not known if the "wild frog DNA solutions" contained chytrid DNA.

3.2.5 Qiagen PCR diagnostic sensitivity and specificity

Pilot experiments indicated that the Qiagen qPCR may perform as well if not better than the other PCR methods tested. To clarify the diagnostic sensitivity and specificity of the combined Chelex extraction and Qiagen qPCR method, I conducted an experiment using a
larger sample size than had been used in preliminary experiments. Six groups of 30 sterile swabs were spiked with one of seven known concentrations of harvested Waste Point isolate zoospores (1, 4, 42, 420, 4200 or 42000 zoospores/sample) and 50 swabs were spiked with sterile water. The swabs were air dried for 40 minutes and kept at 4 °C for 10 days. Swabs were extracted using the Chelex/Proteinase K method and tested with the Qiagen qPCR method.

The PCR was performed using the AHHL-supplied Abercrombie chytrid isolate and a known concentration of synthetic ITS as standard controls. The synthetic ITS standard was a 10-fold dilution series, from 3,000,000 to 3 ITS copies, generated from a 473bp synthetic gene fragment of chytrid ITS strain CW34 (gBlocks® Gene Fragments, Integrated DNA technologies, Baulkham Hills, New South Wales, Australia). On each 384-well assay plate, a 7-point ITS standard, a 4-point Abercrombie chytrid spore standard (supplied by AAHL) and a no DNA template reaction (negative control) was included in triplicate. Quantitative PCR efficiencies were between 94-100% for all 384-well plates. The concentration of the test samples was determined using the Absolute Quantification module of the Roche LightCycler® 480 software package and expressed as the total copies of ITS or number of zoospores.

The diagnostic sensitivity and specificity was evaluated as described for Preliminary Experiment A.

3.3 Results

The preliminary experimental results are discussed in detail in the supplementary material (Appendix 2). The results suggested the diagnostic sensitivity and specificity of all methods varied relative to the number of zoospores that were added to samples. The diagnostic sensitivity of the Qiagen qPCR method appeared to be superior to the nested PCR and Taqman 1:15 qPCR, particularly at lower zoospore concentrations and when equivocal samples were defined as positive. The Qiagen qPCR method and the Taqman 1:10 qPCR method had similar diagnostic sensitivity. All methods had a similar diagnostic specificity.
3.3.1 Qiagen PCR diagnostic sensitivity and specificity

When equivocal samples were classified as negative, the diagnostic sensitivity of the Qiagen PCR method was lowest (mean: 0.188; 95%CI: 0.077-0.347) for samples containing approximately one zoospore, but increased when spiked samples contained higher numbers of zoospores (mean diagnostic sensitivity of 0.937-0.969 for samples containing 42 – 42000 zoospores).

The diagnostic sensitivity of the Qiagen method was improved by classifying equivocal samples as positive (Figure 3.1a versus 1b), particularly for samples that contained few zoospores (~ 1 zoospore; shift in mean diagnostic sensitivity from 0.189 to 0.842; 95%CI: 0.701- 0.943). Mean diagnostic sensitivity was 0.937 (95%CI: 0.838 – 0.991) when samples contained 4 zoospores, and 0.969 for 42 -42000 zoospores (42 zoospores 95% CI: 0.886 – 0.999; 42000 zoospores 95% CI: 0.891 – 0.999). The classification of the equivocal samples as positive ($n = 50$) had a minimal impact on the diagnostic specificity (diagnostic specificity when equivocal = negative: mean of 0.981, 95% CI of 0.931-0.999 versus equivocal = positive: mean of 0.961, 95% CI of 0.897-0.995). These results were very similar to the diagnostic sensitivity and specificity estimates generated during the Preliminary Experiments (Appendix 2).

The Qiagen qPCR method generally estimated a greater number of zoospores than were known to have been added to samples (Figure 3.2 a). A mean of 1.6 zoospores per spiked zoospore were estimated, for samples containing between 1 – 4200 zoospores (Figure 3.2 a). When samples had been spiked with 42000 zoospores (the highest number evaluated), less zoospores were estimated to be in the sample compared with the number added. The difference in the zoospore counts relative to the spiked concentrations likely suggests that some inhibition occurs at high concentrations; however, all of the samples ($n = 30$) spiked with this concentration were recorded as positive for all three replicates. The analysis estimated a mean of 218 copies of the ITS1 region per spiked zoospore, for samples containing between 1 – 4200 zoospores (Figure 3.2 b). There was significant variation in the estimated proportion of observed to expected zoospores or ITS1 copies per spiked zoospore and this could reflect differences in the number of spiked zoospores or differences in the efficiencies of the recovery of the zoospores from the swabs.
Figure 3.1 A comparison of the diagnostic sensitivity and specificity of the Qiagen qPCR method relative to the known concentration of chytrid fungus, *Batrachochytrium dendrobatidis*, zoospores in the sample and the interpretation of equivocal sample results as either negative or positive. In each plot the mean is shown by an open circle and the 95% credible interval is represented by the length of the line. Diagnostic sensitivity (Figure 3.1 a, b) was estimated for samples (n=30) spiked with six different concentrations of between approximately 1 and 42000 zoospores (shown above the mean). Diagnostic specificity (Figure 3.1 c, d) was estimated for samples (n=50) containing no zoospores and spiked with only sterile water (sp (specificity) shown above the mean).
Figure 3.2 Estimated number of a) zoospores and b) copies of the ITS1 region per chytrid fungus zoospore in swab samples. Swabs (n=180) were spiked with either 1, 4, 42, 420, 4200 or 42000 zoospores (shown on a log scale) and then extracted using a Chelex/ Proteinase K method. The number of zoospores and ITS copies per spiked zoospore was estimated following Qiagen PCR analysis that incorporated both an Abercrombie chytrid standard and a synthetic ITS standard. The open circle points indicate number of zoospores or ITS copies/ zoospore added to each of the spiked swabs (n=30 samples at each spiked zoospore concentration, total n=180 swab samples). Blue points indicate the mean number of zoospores or ITS copies/ spiked zoospore for groups of samples at each concentration of spiked zoospores. The red dotted lines indicate the estimated mean number of zoospores (~1.6) or ITS copies (~218) / spiked zoospore for spiked concentrations between 1 – 4200 zoospores. The results for the samples containing 42000 zoospores were not included in the mean estimated numbers as they appeared to estimate fewer copies than at the higher concentrations. Variation in the estimated zoospore count and ITS copy number/ spiked zoospore is likely to reflect both the variation in the number of zoospores added to each sample as well as the variation in the performance of the extraction and PCR methods.
3.4 Discussion

The results indicate that chytrid detection using PCR can be imperfect, as some misclassification of both positive and negative samples can occur. Few researchers have accounted for disease state misclassification (for examples see: McClintock et al. 2010; Miller et al. 2012; Heard et al. 2014). When ignored, the effect of chytrid on amphibians can be either over- or under-estimated (McClintock et al. 2010; Miller et al. 2012). The risk of misclassification was alluded to during initial validation of PCR methods (Hyatt et al. 2007) and retrospectively estimated using a Bayesian latent class analysis (Skerratt et al. 2011). However, neither of these assessments evaluated diagnostic sensitivity and specificity when the pathogen presence in samples was known with certainty. Diagnostic sensitivity was lowest when samples contained very low concentrations of zoospores and increased across an increasing range of biologically plausible concentrations of zoospores. Preliminary results suggest that the diagnostic sensitivity of the Qiagen qPCR and Taqman 1:10 qPCR methods were similar and both appeared superior to that of the Nested PCR and Taqman 1:15 qPCR methods, most notably when samples contained low concentrations of zoospores. The diagnostic specificity of all methods, however, were similarly high. These results may be confounded by differences between laboratories, or incompatibilities with the extraction methods, and additional comparisons are required. The Qiagen qPCR method with a Chelex extraction was preferred as it was cheaper than Taqman methods when samples are extracted using PrepMan Ultra (Applied Biosystems) (approximately AUD$25 versus AUD$35 - $50/sample) and appeared to have a higher diagnostic sensitivity than the Nested PCR method.

The risk of samples being misclassified can vary with the concentration of zoospores in the sample. Zoospore concentration may be positively correlated with the stage of infection of the host and individuals may shed less zoospores at low infection stages (Kriger et al. 2007). Misclassification risk may, therefore, be greatest for individuals in a low infection-state (Miller et al. 2012), although other factors including inefficient sampling (swabbing) techniques could also cause low concentrations of zoospores (Smith 2007). To help avoid this, standardized sampling techniques should be adopted (Skerratt et al. 2008). My results confirm that the risk of misclassification is highest when samples contain low concentrations of zoospores. The diagnostic sensitivity of the Taqman 1:10 qPCR method was previously
estimated to be 0.627-0.822 using latent class analysis (Skerratt et al. 2011), but this estimate did not account for the concentration of zoospores in samples. Instead, PCR methods usually define the analytic sensitivity of the test, (the lower limit to the reliability of the test) but this could mean ignoring some true positive individuals. Alternatively, PCR diagnostic sensitivity can be described across a range of biologically plausible concentrations. For example, the preliminary assessment indicates diagnostic sensitivity of the Taqman 1:10 qPCR method (when using a Chelex rather than PrepMan extraction) could be significantly less (0.003 - 0.288) for low positive samples (~4 zoospores/sample) than previously indicated by Skerratt et al. (2011). However, my results indicated that the Taqman 1:10 qPCR diagnostic sensitivity could be 0.698 - 0.975 for samples containing higher numbers of zoospores (400 - 4000 zoospores), which was similar to previous estimates using latent class analysis.

In practice, the actual concentration of zoospores (or target analyte) is never truly known when samples have been collected from wild individuals. The dilemma then is how best to account for the uncertainty in sample classification, particularly given that diagnostic sensitivity can vary with the concentration of the analyte (Greiner & Gardner 2000; Miller et al. 2012). One solution is to collect multiple swabs for each individual and compare diagnostic results (see McClintock et al. 2010; Miller et al. 2012), although whilst useful for small sample sizes, this approach can dramatically increase diagnostic costs for larger studies. A simple but conservative alternative could be to assume that the diagnostic sensitivity of the method is at least equal to the minimum estimated diagnostic sensitivity. The resultant assessment could then be compared and weighed up against assessments assuming maximum diagnostic sensitivity (or perfect detection). In this study, the minimum diagnostic sensitivity of the Qiagen qPCR method was 0.842 (95% CI 0.701 – 0.943) for samples with 1 zoospore when equivocal samples were defined as positive.

Our results support the classification of equivocal samples as positive, as has been previously advocated (Skerratt et al. 2011) and already implemented in some assessments of disease impact (e.g. Murray et al. 2009). During assessment of the Qiagen qPCR method the diagnostic sensitivity clearly improved by classifying equivocal samples as positive (particularly at low zoospore concentrations), and this did not appear to adversely impact the
diagnostic specificity. This finding is important because the classification of equivocal samples as positive can help to reduce the risk of a false negative diagnosis.

The concentration of zoospores as determined by PCR methods may not reflect the actual number of zoospores in samples, as identified in this study. Differences in the estimated number of zoospores between methods could reflect 1) variation in the concentration of zoospores added to samples, 2) inefficient extraction of DNA from the sample, or 3) differences in zoospore DNA content. Zoospores are difficult to count on a haemocytometer, due to their size and the depth of the haemocytometer counting chamber. But hemocytometer counts suggested that the number of zoospores added to samples most likely varied. To reduce variation between samples, the same person and same calibrated pipette was used to spike all samples and the stock solutions were thoroughly mixed prior to spiking. There has not yet been a rigorous assessment of the efficiency of chytrid DNA extraction methods. Extraction efficiency is known to vary widely between methods for other fungal species (Möhlenhoff et al. 2001; Fredricks, Smith & Meier 2005; Griffiths et al. 2006; Osmundson et al. 2013) and other pathogens (Fahle & Fischer 2000; McOrist, Jackson & Bird 2002; Amaro et al. 2008). If chytrid extraction methods are inefficient, the amount of DNA and number of zoospores in samples could be under-estimated. PCR methods all target the ITS1 DNA region for chytrid, but the number of ITS1 copies per zoospore can vary between chytrid strains (Longo et al. 2013). My experiment assessed samples that contained a different chytrid isolate (Waste Point) compared to the chytrid standard used (Abercrombie), which may explain why the observed and expected zoospore counts differed. If attempting to quantify the number of zoospores in a sample, qPCR methods should incorporate a known ITS1 standard and determine the number of ITS1 copies in the sampled strain (Longo et al. 2013). This was beyond the scope of my study, as I were primarily interested in the ability of the Qiagen qPCR to accurately diagnose samples as positive or negative.

Diagnostic sensitivity and specificity may differ for wild collected samples compared to sterile spiked samples. Recent publications have highlighted the importance of evaluating inhibition (e.g. Kosch & Summers 2013). However, the only way to guarantee that wild collected samples contain the target analyte is by deliberately spiking samples with a known concentration of chytrid zoospores. The preliminary experiments suggest that the Qiagen
qPCR method was not significantly affected by wild contaminants. The diagnostic sensitivity and specificity were similar for samples containing only chytrid zoospores compared to samples that also contained DNA and other naturally occurring wild contaminants.

While I did not assess all OIE validation steps, the Qiagen qPCR method is expected to meet or exceed the performance criteria of the Taqman qPCR and Nested PCR methods. For example, the analytic sensitivity is likely to be similar for the Qiagen and Taqman qPCRs as both use the same primers and should be able to distinguish chytrid from the same potential fungal contaminants as examined by Boyle et al. (2004). The analytic specificity of the Qiagen qPCR may be superior to the Taqman and Nested PCR methods as the Qiagen qPCR correctly diagnosed samples with the lowest concentrations of zoospores more often than the other methods during preliminary analyses. Clear differences between the Nested PCR and Qiagen PCR results at low zoospore concentrations exist as they were performed in the same laboratory. However, additional assessment is required to confirm that the differences in the Taqman qPCR results were not due to laboratory variation. The analytic sensitivity of methods can be influenced by the amount of template DNA solution that is added to a sample (Kerby et al. 2013). In my study the Qiagen qPCR and Taqman qPCR methods contained the same amount of template DNA. Kerby et al. (2013) found that the analytic sensitivity of the Taqman qPCR was highest when reactions used 5 μl compared to 3 μl of template DNA. The Taqman qPCR method appears to have a similar analytic sensitivity to a conventional PCR method (Garland, Wood & Skerratt 2011). However, the Nested PCR has previously been reported to have a higher analytic sensitivity than the Taqman qPCR and a conventional PCR (Goka et al. 2009). The preliminary analysis indicates that the Qiagen qPCR method was more reliable for detecting small concentrations of zoospores in samples than the Nested PCR and may also be more reliable than the Taqman qPCR methods.

PCR analysis has revolutionized our ability to detect disease in wildlife populations. However, determining disease impact on populations and options to mitigate the impact rely on accurate diagnoses of disease (or infection) state in individuals (Skerratt et al. 2011). Diagnostic PCR methods are not perfect (OIE 2012), although when the risk of misclassification of samples is considered, researchers can compensate for the uncertainty when interpreting results and informing management decisions.
Quantifying the demographic impact of disease upon sympatric threatened and non-threatened frogs
Chapter 4. Quantifying the demographic impact of disease upon sympatric threatened and non-threatened frogs

4.1 Introduction

Disease is an important influence and regulator of wildlife populations (Anderson & May 1979; May & Anderson 1979). Emerging infectious diseases pose a significant risk to wildlife as they can drive host population decline and even extinction (McCallum & Dobson 1995; Alford, Dixon & Pechmann 2001; de Castro & Bolker 2005; McCallum 2007; McCallum 2012). The risks posed by infectious diseases are increasingly recognized as a major threat to global biodiversity (Daszak, Cunningham & Hyatt 2000; Fisher et al. 2012; Tompkins et al. 2015). But determining the role of disease in decline is difficult as population fluctuations can also be driven by natural processes and other threats (Pechmann et al. 1991; Daszak, Cunningham & Hyatt 2003; McCallum 2012). This is important because 1) the impact of disease on populations could be under- or over-estimated, and 2) the success of management strategies for declining species depends on identifying the processes that drive decline (OIE 2010). Research is, therefore, required to quantify the demographic impact of disease when it is suspected to influence population dynamics.

Chytridiomycosis, caused by the fungal pathogen Batrachochytrium dendrobatidis (Chytrid fungus, hereafter chytrid), is a lethal disease for many amphibian species and has been linked to rapid population declines (e.g., Berger et al. 1998; Scherer et al. 2005; Lips et al. 2006; Schloegel et al. 2006; Skerratt et al. 2007; Ryan, Lips & Eichholz 2008). The pathogen is an identified threat for almost one quarter of IUCN Red List amphibians, but its actual demographic impact upon most species has not been quantified (Heard, Smith & Ripp 2011; McCaffery & Lips 2013; Newell, Goldingay & Brooks 2013). In a few cases, epidemic chytrid infections are clearly linked to decline because adequate monitoring occurred prior to the pathogen arriving (e.g. Lips 1999; Gillespie et al. 2014). Yet insufficient monitoring prior to observed declines often makes the timing of arrival and impact of chytrid uncertain (Phillips et al. 2012).
Chytrid is now endemic in many populations. When endemic, chytrid can substantially reduce the survival of susceptible species including the Cascade Tree Frog (*Litoria pearsoniana*) (Murray *et al.* 2009), Common Mist Frog (*Litoria rheocola*) (Phillott *et al.* 2013), Growling Grass Frog (*Litoria ranifromis*) (Heard *et al.* 2014), Mountain Yellow-Legged Frog (*Rana mucosa*) and Sierra Nevada Yellow-Legged Frog (*Rana sierrae*) (Briggs, Knapp & Vredenburg 2010). The impact of chytrid upon these species is influenced by host density (Briggs, Knapp & Vredenburg 2010), fluctuations in ambient temperature and rainfall (Murray *et al.* 2009; Phillott *et al.* 2013) or variation in microclimate and microhabitat (Heard *et al.* 2014). These and other factors (e.g. climate, pathogen virulence, host defences, sympatric species) are now known to influence the response of populations infected with chytrid (James *et al.* 2015). Some populations with endemic chytrid infections can decline more slowly than populations experiencing epidemic infections (Pilliod *et al.* 2010). Other endemically-infected populations may even recover or reach new stable states (Briggs, Knapp & Vredenburg 2010; Newell, Goldingay & Brooks 2013; Phillott *et al.* 2013).

Understanding how species persist in the presence of threats like chytrid is crucial when directing conservation efforts. Host-pathogen-environment relationships are important for disease outcomes (reviewed in James *et al.* 2015). Mounting evidence suggests that species can persistent if recruitment compensates for low adult survival when chytrid is endemic (Muths, Scherer & Pilliod 2011; Tobler, Borgula & Schmidt 2012; Phillott *et al.* 2013; Scheele *et al.* 2015). Recruitment of Boreal Toads (*Anaxyrus boreas*) can be higher in populations with low adult survival in the presence of chytrid compared to populations that have high adult survival in the assumed absence of chytrid (Muths, Scherer & Pilliod 2011). The population growth rates of Midwife Toads (*Alytes obstetricans*) can be the same at sites that are infected with chytrid and at sites which chytrid has not been detected (Tobler, Borgula & Schmidt 2012). Common Mist Frog (*Litoria rheocola*) populations have persisted despite chytrid infection for 15 years, as high recruitment compensated for annual fluctuations in adult mortality when chytrid prevalence was high (Phillott *et al.* 2013). Populations of Alpine Tree Frogs (*L. verreauxii alpina*) infected with chytrid can persist if adults breed prior to suffering high mortality and if infection prevalence in the tadpole and juvenile stages is low and does not dramatically limit recruitment (Scheele *et al.* 2015).
The demographic impact of disease and its role in decline can be evaluated by examining the fate of individuals in infected populations (McCallum 1994; Cooch et al. 2012; McCallum 2012) utilizing mark-recapture methods (Lebreton et al. 1992; Lebreton et al. 2009). Key demographic parameters including the probabilities of individuals becoming infected, or recovering from an infection, and probabilities of survival in either an infected or uninfected disease state can be estimated with multistate mark-recapture methods (Cooch et al. 2012).

I undertook a mark-recapture study at four sites to quantify and compare the demographic responses of amphibians to chytrid infection. Two species were selected that can co-occur and were assumed to have different responses to chytrid. Spotted Tree Frogs (*Litoria spenceri*) are Critically Endangered (Hero et al. 2004), with at least one localized extinction following an epidemic chytrid infection (Gillespie et al. 2014). Lesueur’s Frogs (*Litoria lesueurii*) are considered non-threatened and suspected to be a potential pathogen host reservoir (Chapter 2). *Litoria lesueurii* was encountered frequently at some sites and monitored over four years but fewer *L. spenceri* were encountered during this time period, so the species was monitored over six years to obtain additional data. The disease state of each individual was diagnosed at each capture by swabbing frogs and subsequent PCR analysis. A Bayesian multi-state model of the observation history and infection history of individuals was developed to estimate probabilities of infection, recovery and survival.

Finally, I examined the importance of compensating for four potential sources of bias in parameter estimates that could occur if: 1) the disease state is misclassified; 2) disease affects the probability of detection; 3) the marking method affects the probability of survival or recapture; and, 4) an incorrect assumption is made about the recovery of individuals. The disease state of individuals can be misclassified if diagnostic (PCR) results differ from an individual’s true disease state. This can occur if test diagnostic sensitivity and specificity (measures of the rates of true positive and negative samples) is imperfect (Chapter 3; Skerratt et al. 2011). The probability of detecting individuals can differ if their disease state influences their behaviour (Jennelle et al. 2007; Conn, Cooch & Caley 2012; Cooch et al. 2012). Chytrid can influence detection of amphibians (e.g. Growling Grass Frogs, *L. raniformis*) (Heard et al. 2014), but most studies have not identified an effect of disease on amphibian detection (Retallick, McCallum & Speare 2004; Kriger & Hero 2006; Murray et al. 2009; Pilliod et al. 2010; Phillott et al. 2013). Key assumptions of mark-recapture studies
are that marks are not lost and the method of marking animals does not affect the return rate (capture and survival) of individuals. Yet marking methods may reduce amphibian survival (Scherer et al. 2005) and return rates (Parris & McCarthy 2001; McCarthy & Parris 2004). The effect of marking should be assessed to avoid violating statistical assumptions, but also to ensure that benefits of marking outweigh any adverse impacts on the welfare or conservation of species (e.g., Parris & McCarthy 2001; May 2004; McCarthy & Parris 2008; Parris et al. 2010).

4.2 Methods

4.2.1 Study Species

*Litoria spenceri* is restricted to fast flowing mountain streams in north-eastern Victoria and south-eastern New South Wales, Australia (Gillespie & Hollis 1996). Most populations are now infected with chytrid (West et al in prep). In 1996, an abundant population of *L. spenceri* rapidly declined and dead frogs found at the site were diagnosed with chytridiomycosis (Gillespie & Marantelli 2000). Chytrid is believed to have caused the localized extinction of the species at the site as no other threats were identified (Gillespie et al. 2014). Other *L. spenceri* populations are also considered to have suffered declines (Gillespie & Hollis 1996), but the role of chytrid in these declines has not yet been determined. *Litoria spenceri* populations infected with chytrid may be at greater risk of decline as they are exposed to other threats including habitat disturbance and introduced fish (Gillespie & Hollis 1996; Gillespie & Robertson 1998; Gillespie & Marantelli 2000; Gillespie 2001b; Gillespie 2002).

In contrast, chytrid is not considered to have the same effect on *L. lesueurii*. This species is abundant and widely distributed in south-eastern Australia and sympatric with *L. spenceri* at some sites. *Litoria lesueurii* is one of several phylogenetically-related and ecologically similar stream-breeding species along the eastern sea-board of Australia (Donnellan & Mahony 2004). Chytrid is not known to have caused obvious population decline in any of the species within this species group despite suffering chytrid infections (Kriger & Hero 2006; Kriger, Pereoglou & Hero 2007; Chapter 2). Species within the *L. lesueurii* complex have been hypothesized to act as a chytrid reservoir and maintain pathogen transmission to
more susceptible species (Retallick, McCallum & Speare 2004; Kriger & Hero 2006; Chapter 2).

4.2.2 Mark-recapture study design

A mark-recapture study was conducted on the two frog species at four sites along mountain streams in Victoria, Australia. These sites were selected due to similarities in elevation (350 -450m asl), habitat characteristics and fish species composition (Gillespie and Hollis 1996). *Litoria spenceri* were detected at Sites 1, 2 and 3 and *L. lesueurii* were detected at all sites. Transects 1 km in length were established at Sites 1 and 2 and 700m in length at Sites 3 and 4. Sites 3 and 4 were surveyed over a four year period between November 2009 and March 2013 and sites 1 and 2 which had the largest populations of *L. spenceri* were surveyed for an additional two years (November 2009 – March 2015) to ensure samples sizes were sufficient to evaluate chytrid impact.

Each transect was sampled 5 - 8 times each year between November and March when the frogs are most active. Two observers searched each site during 21-39 nocturnal frog surveys. All frogs observed were hand-captured, and the species, age class, sex and snout-urostyle length were recorded. The feet, thighs, hands, forearms and left, right and central dorsal surfaces of each frog were swabbed with a sterile swab. Swabs were kept on ice and later transported to a laboratory and kept at 4°C prior to PCR analysis. Each frog was inspected for existing marks to determine if it had been previously captured. All frogs caught on the first occasion were given a unique mark by toe-clipping following Gillespie (2011b). I aimed to minimize the number toes clipped. Frogs were marked by clipping one (0.4%), two (39.4%), three toes (57.7%) or four toes (0.9%). Thumbs were not clipped. In 1.5% of cases, no toes were clipped as frogs could be identified by an existing unique mark or injury.

4.2.3 PCR Analysis

All swabs were extracted using a Chelex/ proteinase K method and analysed for chytrid in triplicate using a Qiagen quantitative PCR method described in Chapter 3. The Qiagen qPCR method differs from the published Taqman qPCR method (Boyle et al. 2004) by incorporating a Qiagen master mix instead of a Taqman master mix. The Qiagen qPCR has an equal or higher diagnostic sensitivity and equal diagnostic specificity to the Taqman
method (Chapter 3). The PCR method can be used to quantify the concentration of chytrid zoospores in a sample but I simplified the results to either positive or negative. I used this approach to avoid bias that may be encountered if the concentration of zoospores in a sample is not correlated with the intensity of infection. Zoospore counts can be inaccurate and influenced by variation in swabbing techniques (Skerratt et al. 2008), distribution of the chytrid infection on a frog’s skin (Berger, Speare & Skerratt 2005), fungus life-cycle stage or failure of zoospores to be released at time of swabbing (Clare et al. 2016), variation in ITS copies between chytrid strains (Longo et al. 2013) and PCR errors (Chapter 3). The actual presence and concentration of the analyte (chytrid zoospores) in wild samples was unknown. PCR results were classified as positive if any of the replicate tests were positive, otherwise results were negative. Previous research supports the classification of equivocal results (<3 positive replicates) as positive (Chapter 3). I modelled both the possibility that PCR results perfectly classified or misclassified the two possible disease states: uninfected or infected.

4.2.4 Multistate Mark-recapture Analysis

My multi-state mark-recapture analysis assessed the influence of disease on survival and capture probabilities. I assumed that individuals existed in either an uninfected or infected disease state. If an individual survived between surveys they could either remain in their current disease state or transition to the other disease state.

Importantly, some marked individuals are unobserved if they die or are not detected during each survey (Lebreton et al 1992). Multi-state models can deal with non-detection data by estimating the probability that an individual is detected when alive, and if alive the probability that the individual is infected (or uninfected). Some undetected individuals may emigrate from the site and so this analysis estimated individual’s apparent annual survival.

I compared models that accounted for four potential sources of bias: 1) that the PCR test results might be imperfect and an individual’s disease state could be misclassified; 2) that disease state may influence an individual’s probability of detection; 3) that the marking method might influence an individual’s return rate (probabilities of capture and survival); and, 4) that individuals may not be able to recover from infections. I evaluated the influence
of these potential sources of bias upon estimates of the probabilities of annual survival, detection, infection and recovery. To achieve this, models were constructed that explicitly accounted for one or more potential sources of bias (listed in Table 4.1).

Table 4.1 Assumptions of the candidate multistate mark-recapture models to account for four potential sources of bias when estimating the demographic impact of disease on frogs. These models addressed the research aims and so other models were not assessed. An assumption of no recovery is only possible when the disease state is assumed to be misclassified.

<table>
<thead>
<tr>
<th>Model</th>
<th>Disease state</th>
<th>Influence of disease upon Pr(Detection)</th>
<th>Influence of marking upon return rate</th>
<th>Recovery possible</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Misclassification (tpos = 0.95)</td>
<td>Equal</td>
<td>None</td>
<td>Yes</td>
</tr>
<tr>
<td>B</td>
<td>Misclassification (tpos = 0.95)</td>
<td>May vary</td>
<td>None</td>
<td>No</td>
</tr>
<tr>
<td>C</td>
<td>Perfect</td>
<td>Equal</td>
<td>None</td>
<td>Yes</td>
</tr>
<tr>
<td>D</td>
<td>Misclassification (tpos = 0.95)</td>
<td>May vary</td>
<td>Possible</td>
<td>No</td>
</tr>
<tr>
<td>E</td>
<td>Misclassification (tpos = 0.95)</td>
<td>May vary</td>
<td>None</td>
<td>Yes</td>
</tr>
<tr>
<td>F</td>
<td>Misclassification (tpos = 0.95)</td>
<td>Equal</td>
<td>Possible</td>
<td>Yes</td>
</tr>
<tr>
<td>G</td>
<td>Perfect</td>
<td>Equal</td>
<td>Possible</td>
<td>Yes</td>
</tr>
<tr>
<td>H</td>
<td>Misclassification (tpos = 0.90)</td>
<td>Equal</td>
<td>None</td>
<td>Yes</td>
</tr>
<tr>
<td>I</td>
<td>Perfect</td>
<td>May vary</td>
<td>None</td>
<td>Yes</td>
</tr>
</tbody>
</table>

tpos = probability that sample is truly positive

Models for each species were analysed by pooling data for captured adult frogs across all sites. I assumed that marked individuals were correctly identified when captured. The observation history data \((Y)\) of each individual was modelled to estimate the probability of capture from a Bernoulli distribution as follows:
where the probability of capture was assumed to depend on 1) the individual \(i\) being alive (where \(Z_{i,t} = 1\)) during the survey \((t)\); 2) the probability of detecting \(d\) the individual in either an uninfected \((B_{i,t} = 0)\) or infected \((B_{i,t} = 1)\) disease state; and 3) a potential effect of marking \(c\) upon the probability of capture, that was proportional to the number of toes clipped \((T_i)\). Note, toe clipping reduces detection with each toe removed when \(c < 1\) and increases detection when \(c > 1\).

All individuals were classified as alive at first capture \((Z_{i,First} = 1)\). If the true alive or dead state \((Z)\) of each individual was not known during subsequent surveys (where \(t = First + 1, \ldots n\) surveys), because they had not been observed, then the individual’s survival was modelled as a draw from a Bernoulli distribution:

\[
Z_{i,t} \sim Bernoulli\left[\left(1 - (1 - s_{i,t}) \times m^T \times Z_{i,t-1}\right)\right] \tag{Eqn. 4.2}
\]

where the probability that each individual \(i\) was alive was a function of the individual’s apparent annual probability of survival \((s_{i,t})\) of individual \(i\) during the previous period (from time \(t-1\) to time \(t\)) and the per toe effect of marking \((m)\). Survival to time \(t\) depends on the individual being alive at the previous time \((Z_{i,t-1} = 1)\). Note, toe clipping reduces mortality \((1 - s_{i,t})\) with each toe \((T_i)\) removed when \(m < 1\) and increases mortality when \(m > 1\).

The probability of apparent annual survival \((s_{i,t})\) was modelled as a power function of the daily survival \((s_{daily})\) given the individual’s current disease state \((B_{i,t} = 0,1)\) and was proportional to the number of days \((n)\) between successive survey periods at each site:

\[
s_{i,t} = s_{daily}^{n_{i,t}}_{B_{i,t}} \tag{Eqn. 4.3}
\]

In order to account for the potential misclassification of an individual’s disease state, each individual’s \(i\) true (latent) disease state \((B_{i,t} = 0,1)\) at each time step \((t)\) was modelled as a latent variable drawn from a Bernoulli distribution:
where the probability of actually being infected was a function of the probability that an individual with a negative PCR test \((q_{i,t} = 0)\) result may have been incorrectly diagnosed and was actually infected \((\text{tpos} \times (1 - q_{i,t}))\); and the probability that an individual with a positive test result \((q_{i,t} = 1)\) was actually uninfected \((\text{fpos} \times B_{i,t})\). I compensated for the potential misclassification of disease state by assuming the PCR method had a true positive probability \((\text{tpos})\) of 0.95 and a false positive probability \((\text{fpos})\) of 0.05. These probabilities were point estimates that approximated the PCR methods mean diagnostic sensitivity and 1 - the mean diagnostic specificity (Chapter 3). Candidate models were also constructed to investigate lower true positive and higher false positive probabilities (e.g. Model H) but were not supported (higher DIC values).

An individual’s PCR test result \((q_{i,t})\) in each time step was modelled as a Bernoulli distribution:

\[
q_{i,t} \sim \text{Bernoulli}\left(\left[I_{A,t} \times (1 - B_{i,t-1})\right] + \left((1 - R_{A,t}) \times B_{i,t-1}\right)\right)
\]

where an individual’s current probability of being infected was assumed to be a function of the individual’s true (latent) disease state \((B_{i,t-1})\) in the previous time step and either the probability of recovery \((R)\) if infected or the probability of infection \((I)\) if uninfected.

When I modelled perfect disease classification the PCR test result was assumed to be equal to the true disease state:

\[
q_{i,t} = B_{i,t}
\]

The probabilities of infection \((I)\) and recovery \((R)\) were estimated for two periods \((A)\): the frogs active period (November – March), when surveys were conducted and the frogs non-active period (April – October) during which ambient temperatures are generally low and surveys were not conducted.
The multi-state mark recapture model was analysed in a Bayesian framework in R (Version 3.2.1) (R Core Team 2013) and performed with JAGS (Version 3.4.0) using packages R2jags (Version 0.5-6) and rjags (Version 3-15). JAGS is a program for Bayesian analysis which combines prior knowledge and data to obtain samples from a posterior distribution using a Markov Chain Monte Carlo (MCMC) algorithm. The relationship between the prior knowledge and the data is defined by the model. Uninformative priors drawn from a uniform distribution on the interval [0,1] were assumed for all estimated parameters except c and m which were drawn from a uniform distribution on the interval [0,2]. The package jagstools (Version 1.3) was used to summarize and plot the results.

All models were performed with three replicate Markov chains that were each run for 200,000 MCMC iterations. The Markov chains were checked for convergence ensuring that the potential scale reduction factor (Rhat) values were less than 1.1. Parameters were estimated from the posterior distribution after discarding the first 100,000 iterations.

### 4.2.5 Model evaluation

I performed simulations to evaluate performance of models. To do this I assumed parameter values equalled the actual mean estimated values and then simulated mark-recapture data with a similar sampling intensity to my actual observation and PCR results data. The parameters were estimated from these simulated data confirming that they were consistent with assumed parameter values that had been used to generate the data.

### 4.2.6 Model selection

Deviance information criterion (DIC) were calculated for all candidate models during the JAGS analysis using a method described by Spiegelhalter et al. (2002). The model with the lowest DIC value is suggested as the one that best fits the data.
4.3 Results

4.3.1 Sample data and observations

I captured and marked a total of 850 individual adult *L. lesueurii* in the first four years and 295 individual adult *L. spenceri* over the six year period. For *L. spenceri*, 183, 101 and 11 adults were captured at Sites 1, 2 and 3 respectively. *L. spenceri* were not observed at Site 4 and this site was considered to be allopatric for *L. lesueurii*. For *L. lesueurii*, 2, 25, 331 and 494 adults were captured at Sites 1, 2, 3 and 4. Although, *L. lesueurii* did not breed at Site 1 and so the 2 individuals detected on only a single occasion each were considered transient and excluded from further analysis. Site 1 was deemed to be allopatric for *L. spenceri*. Across all sites 57.3% of *L. spenceri* (n=169) and 36.9% of *L. lesueurii* (n=313) were recaptured on at least one occasion. Recapture rates varied from 54.5 - 58.4% for *L. spenceri* and 29 - 44% for *L. lesueurii* among sites.

Individual *L. spenceri* and *L. lesueurii* were captured and tested for chytrid on 505 and 517 successive occasions respectively (Table 4.2). *Litoria spenceri* were diagnosed to remain negative during a greater proportion of successive capture occasions than *L. lesueurii* (Table 4.2). *Litoria spenceri* had lower proportion of shifts in both negative – positive and positive – negative PCR results than *L. lesueurii*. *Litoria spenceri* also remained positive during successive assessments less often than *L. lesueurii*. In total 47.1% (n=139) of *L. spenceri* and 70.2% (n=597) of *L. lesueurii* tested positive on at least one occasion. The final PCR result was determined to be positive for 29.8% (n=88) of *L. spenceri* and 60.6% (n=514) of *L. lesueurii*. 
Table 4.2 Chytrid PCR results of *L. spenceri* and *L. lesueurii* during successive sampling (capture) occasions over a 4 – 6 year mark-recapture study. The proportion of occasions that frogs either had the same or different successive PCR results for chytrid is shown. The number of subsequent PCR testing occasions is given in parenthesis.

<table>
<thead>
<tr>
<th>PCR results during successive testing</th>
<th>Species:</th>
<th><em>L. spenceri</em></th>
<th><em>L. lesueurii</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Duration</td>
<td>4yrs</td>
<td>6yrs</td>
</tr>
<tr>
<td></td>
<td>Study period</td>
<td>November 2009 and March 2013</td>
<td>November 2009 and March 2015</td>
</tr>
<tr>
<td>Negative - negative</td>
<td></td>
<td>0.398 (141)</td>
<td>0.517 (261)</td>
</tr>
<tr>
<td>Negative - positive</td>
<td></td>
<td>0.229 (81)</td>
<td>0.184 (93)</td>
</tr>
<tr>
<td>Positive – negative</td>
<td></td>
<td>0.144 (51)</td>
<td>0.133 (67)</td>
</tr>
<tr>
<td>Positive - positive</td>
<td></td>
<td>0.229 (81)</td>
<td>0.166 (84)</td>
</tr>
<tr>
<td>Total observations</td>
<td></td>
<td>354</td>
<td>505</td>
</tr>
</tbody>
</table>
Table 4.3 Demographic parameter estimates for *L. spenceri* and *L. lesueurii* as determined by the four candidate Bayesian multi-state mark-recapture models (A-D) with the lowest DIC values. Mean values are provided with 95% credible interval shown in parentheses. Median values are shown for estimates of infection and recovery probabilities. Model A assumes disease misclassification, equal detection, no impact of marking and potential recovery from infection. Other models differ from A as follows: B assumes varying detection and no recovery, C assumes perfect disease classification, D assumes toe clipping could influence survival and capture probabilities, varying detection and no recovery.

<table>
<thead>
<tr>
<th>L. lesueurii Models</th>
<th>DIC</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pr(initial infection)</strong></td>
<td>5193.2</td>
<td>0.614 (0.577 - 0.650)</td>
<td>0.585 (0.547 - 0.623)</td>
<td>0.602 (0.570 - 0.633)</td>
<td>0.579 (0.538 - 0.617)</td>
</tr>
<tr>
<td><strong>Pr(infection survey period)</strong></td>
<td>5552.3</td>
<td>0.653 (0.546 - 0.761)</td>
<td>0.209 (0.146 - 0.278)</td>
<td>0.679 (0.587 - 0.764)</td>
<td>0.151 (0.102 - 0.210)</td>
</tr>
<tr>
<td><strong>Pr(infection non-survey period)</strong></td>
<td>5448.3</td>
<td>0.018 (0.001 - 0.076)</td>
<td>0.004 (0.001 - 0.018)</td>
<td>0.018 (0.001 - 0.077)</td>
<td>0.003 (0- 0.015)</td>
</tr>
<tr>
<td><strong>Pr(recovery survey period)</strong></td>
<td>5870.3</td>
<td>0.343 (0.247 - 0.451)</td>
<td>0</td>
<td>0.388 (0.308 - 0.473)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Pr(recovery non-survey period)</strong></td>
<td>0.119 (0.032 - 0.305)</td>
<td>0.102 (0.029 - 0.211)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pr(detection when uninfected)</strong></td>
<td>0.104 (0.092 - 0.116)</td>
<td>0.099 (0.075 - 0.126)</td>
<td>0.102 (0.091 - 0.114)</td>
<td>0.138 (0.083 - 0.212)</td>
<td></td>
</tr>
<tr>
<td><strong>Pr(detection when infected)</strong></td>
<td>0.104 (0.092 - 0.116)</td>
<td>0.099 (0.084 - 0.117)</td>
<td>0.102 (0.091 - 0.114)</td>
<td>0.248 (0.156 - 0.366)</td>
<td></td>
</tr>
<tr>
<td><strong>Pr(annual survival when uninfected)</strong></td>
<td>0.843 (0.442 - 0.995)</td>
<td>0.791 (0.592 - 0.977)</td>
<td>0.757 (0.237 - 0.991)</td>
<td>0.701 (0.406 - 0.967)</td>
<td></td>
</tr>
<tr>
<td><strong>Pr(annual survival when infected)</strong></td>
<td>0.151 (0.077 - 0.227)</td>
<td>0.225 (0.165 - 0.288)</td>
<td>0.178 (0.089 - 0.39)</td>
<td>0.001 (0 - 0.005)</td>
<td></td>
</tr>
<tr>
<td><strong>Difference in Pr(detection) infected – uninfected</strong></td>
<td>0</td>
<td>-0.036 (-0.035)</td>
<td>0</td>
<td>0.109 (0.030 - 0.203)</td>
<td></td>
</tr>
<tr>
<td><strong>Pr(annual survival when uninfected)</strong></td>
<td>0.843 (0.442 - 0.995)</td>
<td>0.791 (0.592 - 0.977)</td>
<td>0.757 (0.237 - 0.991)</td>
<td>0.701 (0.406 - 0.967)</td>
<td></td>
</tr>
<tr>
<td><strong>Pr(annual survival when infected)</strong></td>
<td>0.151 (0.077 - 0.227)</td>
<td>0.225 (0.165 - 0.288)</td>
<td>0.178 (0.089 - 0.39)</td>
<td>0.001 (0 - 0.005)</td>
<td></td>
</tr>
<tr>
<td><strong>Difference in Pr(survival) infected – uninfected</strong></td>
<td>-0.692 (-0.898 - -0.175)</td>
<td>-0.567 (-0.771 - -0.348)</td>
<td>-0.579 (-0.878 - 0.153)</td>
<td>-0.700 (-0.966 - 0.405)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>L. spenceri Models</th>
<th>DIC</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pr(initial infection)</strong></td>
<td>3813.9</td>
<td>0.224 (0.173 - 0.279)</td>
<td>0.163 (0.117 - 0.214)</td>
<td>0.249 (0.202 - 0.301)</td>
<td>0.162 (0.118 - 0.211)</td>
</tr>
<tr>
<td><strong>Pr(infection survey period)</strong></td>
<td>3986.4</td>
<td>0.129 (0.082 - 0.191)</td>
<td>0.041 (0.021 - 0.069)</td>
<td>0.216 (0.161 - 0.278)</td>
<td>0.038 (0.019 - 0.062)</td>
</tr>
<tr>
<td><strong>Pr(infection non-survey period)</strong></td>
<td>3831.5</td>
<td>0.059 (0.036 - 0.090)</td>
<td>0.016 (0.008 - 0.027)</td>
<td>0.066 (0.04 - 0.1)</td>
<td>0.016 (0.008 - 0.027)</td>
</tr>
<tr>
<td><strong>Pr(recovery survey period)</strong></td>
<td>3995.9</td>
<td>0.212 (0.112 - 0.34)</td>
<td>0</td>
<td>0.373 (0.276 - 0.486)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Pr(recovery non-survey period)</strong></td>
<td>0.229 (0.109 - 0.441)</td>
<td>0.171 (0.082 - 0.299)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pr(detection when uninfected)</strong></td>
<td>0.217 (0.198 - 0.236)</td>
<td>0.212 (0.191 - 0.234)</td>
<td>0.217 (0.197 - 0.236)</td>
<td>0.177 (0.116 - 0.257)</td>
<td></td>
</tr>
<tr>
<td><strong>Pr(detection when infected)</strong></td>
<td>0.217 (0.198 - 0.236)</td>
<td>0.240 (0.177 - 0.309)</td>
<td>0.217 (0.197 - 0.236)</td>
<td>0.215 (0.134 - 0.319)</td>
<td></td>
</tr>
<tr>
<td><strong>Pr(annual survival when uninfected)</strong></td>
<td>0.642 (0.535 - 0.749)</td>
<td>0.752 (0.671 - 0.839)</td>
<td>0.639 (0.533 - 0.751)</td>
<td>0.766 (0.627 - 0.874)</td>
<td></td>
</tr>
<tr>
<td><strong>Pr(annual survival when infected)</strong></td>
<td>0.421 (0.277 - 0.581)</td>
<td>0.150 (0.066 - 0.255)</td>
<td>0.435 (0.297 - 0.594)</td>
<td>0.221 (0.025 - 0.490)</td>
<td></td>
</tr>
<tr>
<td><strong>Difference in Pr(survival) infected – uninfected</strong></td>
<td>-0.221 (-0.448 - 0.018)</td>
<td>-0.602 (-0.730 - -0.459)</td>
<td>-0.204 (-0.429 - 0.039)</td>
<td>-0.545 (-0.702 - -0.354)</td>
<td></td>
</tr>
</tbody>
</table>
4.3.2 Multistate Mark-recapture Model Analysis

4.3.2.1 Model selection

Model A was determined to have the lowest DIC value of the nine candidate models that I examined (Models A – I) for both species (Table 4.3). The other candidate models had much higher DIC values. This indicated strong support for the assumptions underpinning Model A that included disease state misclassification, equal detection of uninfected and infected individuals, no effect of marking on survival and capture probabilities and allowed recovery from infection.

Model C had the second lowest DIC value and differed from Model A by assuming perfect classification rather than misclassification of an individual’s disease states. Despite this, both models produced very similar parameter estimates (Table 4.3), although Model C estimates were slightly more uncertain. The results support an equal detection probability of infected and uninfected individuals, as assumed by both Model A and C.

4.3.2.2 Effect of marking frogs on capture and survival probabilities

The mean effect of toe-clipping upon the probability of capture of *L. lesueurii* was 0.801 (0.692 - 0.928) and the mean effect upon the probability of mortality was 0.767 (0.709 – 0.826) (for Model D). This suggests that toe-clipping increased recapture and reduced mortality, which is biologically unlikely and may be an artefact of the model. The impact of marking upon *L. lesueurii* is therefore likely to be small. The effect of toe-clipping upon *L. spenceri* was uncertain and could indicate a positive, negative or no effect upon both the probability of capture and survival. For *L. spenceri*, the mean effect of toe-clipping upon the probability of capture was 1.078 (0.926 - 1.252) and the mean effect upon the probability of mortality was 1.052 (0.872 – 1.287) (for Model D). The estimated effect of toe-clipping upon both species probability of capture and mortality was consistent regardless of the other model assumptions. But models that assumed a potential effect of marking (e.g. Model D *L. spenceri* DIC = 3996, *L. lesueurii* DIC = 5870) ranked lower than models that assumed no effect of marking (e.g. highest ranked model (Model A) *L. spenceri* DIC = 3814, *L. lesueurii* DIC = 5193).
4.3.2.3 Monthly probability of acquiring an infection

The monthly probabilities of infection of both species were found to be higher during the survey period than during the non-survey period (Figure 4.1). *Litoria lesueurii* was consistently estimated to have a higher probability of infection than *L. spenceri* during the survey period. Probabilities of infection during the non-survey period were similarly low for both species. The differences in the probability of infection between the two periods were greater for *L. lesueurii* than for *L. spenceri*, regardless of the candidate model assumptions. Models A and C estimates of the monthly probabilities of infection were almost identical for each species, although estimates for other models either differed or were more uncertain.

![Figure 4.1 Estimated monthly probabilities of uninfected *Litoria spenceri* and *L. lesueurii* becoming infected during the survey (Nov – March) and non-survey (April-Oct) periods under varying model assumptions (A – D). Points indicate the median estimate and lines indicate the 95% CI.](image)

Figure 4.1 Estimated monthly probabilities of uninfected *Litoria spenceri* and *L. lesueurii* becoming infected during the survey (Nov – March) and non-survey (April-Oct) periods under varying model assumptions (A – D). Points indicate the median estimate and lines indicate the 95% CI.
4.3.2.4 Monthly probability of recovery from an infection

There was no clear difference in monthly probability of recovery for *L. spenceri* during the survey and non-survey periods under the assumptions of Model A (Figure 4.2). The results of Model A indicated that *L. lesueurii* was likely to have a higher monthly probability of recovery during the survey period compared to the non-survey period, although the differences in recovery were uncertain. The two species were estimated to have similar probabilities of recovery, although estimates were sensitive to the other assumptions and so varied between candidate models.

![Figure 4.2: Comparison of estimated monthly probabilities of recovery of infected *L. spenceri* and *L. lesueurii* during the survey (Nov – March) and non-survey (April-Oct) periods under varying model assumptions (A – D). Points indicate the median estimate and lines indicate the 95% CI.](image)

Figure 4.2 Comparison of estimated monthly probabilities of recovery of infected *L. spenceri* and *L. lesueurii* during the survey (Nov – March) and non-survey (April-Oct) periods under varying model assumptions (A – D). Points indicate the median estimate and lines indicate the 95% CI.
4.3.2.5 Impact of chytrid on probability of detection

The analysis supported the assumption that infected and uninfected individuals have an equal probability of detection (Figure 4.3). The two models with the lowest DIC values (Model A and C) both assumed equal detection. Even the model that ranked third in terms of its DIC value (Model B) estimated that the probability of detection of infected and uninfected frogs was very similar despite assuming detection may be unequal. Some other models, with higher DIC values (e.g. Model I) estimated that infected frogs had a lower probability of detection than uninfected frogs.

![Figure 4.3](image)

**Figure 4.3** Comparison of estimated probabilities of detection of infected (+ve) and uninfected (-ve) *L. spenceri* and *L. lesueurii* under varying model assumptions (A – D). Points indicate the mean estimate and lines indicate the 95% CI.
4.3.2.6 Probability of apparent annual survival

*Litoria lesueurii* clearly had a lower annual probability of survival when infected compared to when uninfected (Model A mean difference: -0.692, 95%CI: -0.898 - -0.175) (Figure 4.4). The analysis also suggests that infected *L. spenceri* had a lower probability of survival than uninfected frogs (Model A mean difference: -0.221, 95% CI: -0.448 - 0.018), but the difference was more uncertain than for *L. lesueurii*. Model A indicated that annual probability of survival of infected *L. lesueurii* was lower than estimated survival of infected *L. spenceri*, but the differences in the probability of survival of uninfected frogs for each species was uncertain. Models B, C and D, for *L. spenceri*, all suggested that infected frogs had a lower probability of survival than uninfected frogs, although the differences were unclear for other candidate models (E - I).

![Figure 4.4 Comparison of estimated probabilities of apparent annual survival of infected (+ve) and uninfected (-ve) L. spenceri and L. lesueurii under varying model assumptions (A – D). Points indicate the mean estimate and lines indicate the 95% CI.](image-url)
4.4 Discussion

My results suggest chytrid infection reduced the apparent annual survival of the non-threatened frog species more than the threatened species. I had expected a negative effect of chytrid upon *L. spenceri*, given that one population had previously gone extinct subsequent to epidemic chytrid infection (Gillespie *et al.* 2014). Although, my results for *L. lesueurii* were unexpected given that declines have not been reported and that *L. lesueurii* occurred at a relative high abundance compared to *L. spenceri*. This finding indicates that even though chytrid can reduce the survival of both threatened and non-threatened adult frogs, the consequence of chytrid for the species at a population level appears to be different.

These findings support the hypothesis that species can persist if recruitment can compensate for reduced adult survival caused by chytrid (Muths, Scherer & Pilliod 2011; Tobler, Borgula & Schmidt 2012; Phillott *et al.* 2013; Scheele *et al.* 2015). *Litoria spenceri* is known to produce fewer eggs (mean eggs/female: 527.9) than *L. lesueurii* (mean eggs/female: 1510.2) (Gillespie 2011a). Furthermore, *L. lesueurii* can mature more quickly and breed at an earlier age than *L. spenceri* (*L. lesueurii* 2 - 4 years vs. *L. spenceri* 3 -5 years) (Gillespie 2010; Gillespie 2011a). Additionally, *L. spenceri* recruitment is reduced by introduced trout (*Salmo trutta* and *Oncorhynchus mykiss*). Trout can significantly reduce the survival of *L. spenceri* tadpoles but appear to have little effect on the survival of *L. lesueurii* tadpoles (Gillespie 2001). These recruitment differences suggest that *L. spenceri* has a lower capacity to compensate for chytrid induced adult mortality than *L. lesueurii*. My findings therefore support a general hypothesis that differing population trajectories of sympatric species can be explained by their fecundity index whereby species with smaller ovarian clutch sizes have poorer population resilience (Hero, Williams & Magnusson 2005).

The total number or density of hosts can influence amphibian population responses to chytrid (Briggs *et al.* 2005; Rachowicz & Briggs 2007; Briggs, Knapp & Vredenburg 2010; McCallum 2012). The *L. spenceri* population that became extinct following an epidemic chytrid infection occurred at a high density (Gillespie *et al.* 2014). However some *L. spenceri* populations have continued to persist at lower densities for long periods (at least 17 years) despite the presence of chytrid (and trout). Interactions with native sympatric species (like *L. lesueurii*) could increase pressure upon more susceptible species (like *L. spenceri*)
through competition or increased disease transmission. Mesocosom experiments with *L. spenceri* tadpoles suggest intraspecific density-dependant competition can influence their growth and development (Gillespie 2001a). But it is not clear if interspecific competitive relationships occur with *L. lesueurii* in wild stream systems. My observations support a hypothesis that *L. lesueurii* are a pathogen reservoir, because they occurred at a relative high abundance despite suffering chytrid-mediated mortality and could therefore maintain pathogen transmission to *L. spenceri* even as the threatened frog declines. Notably, *L. lesueurii* infection was highest during the active period when the species aggregates at breeding sites and could be indicative of density- or frequency-dependent disease transmission (sensu Rachowicz & Briggs 2007; McCallum 2012). In my study, the number of *L. spenceri* and *L. lesueurii* encountered at sites were inversely related. More broadly, *L. spenceri* population declines across all historic sites are also correlated with the presence of both chytrid and *L. lesueurii* (Chapter 2). These observations are important as pathogen reservoirs could drive local extinction of species that have less ability to cope with chytrid impacts (McCallum & Dobson 1995; McCallum 2012).

The different responses of *L. spenceri* and *L. lesueurii* to chytrid could be influenced by a range of abiotic and biotic interactions. Other amphibian population responses to chytrid vary due to host, pathogen or environment differences (including host defences or behaviour, pathogen strain and microclimate) (James *et al.* 2015). Microclimatic conditions can influence both host and pathogen survival and host infection and recovery (e.g. Piotrowski, Annis & Longcore 2004; Rowley & Alford 2013; Daskin *et al.* 2014; Heard *et al.* 2014). In my study, the differences in infection and recovery between species could be influenced by their microhabitat use and associated microclimatic differences. *Litoria spenceri* remain close to streams throughout their lives and can be observed basking during the day (Gillespie 2011b). In contrast, *L. lesueurii* mostly occur under vegetation or other substratum during the day and disperse away from streams following breeding (Anstis *et al.* 1998; Gillespie 2011a; Langkilde & Alford 2002; Rowley & Alford 2007b). At my study sites, diurnal temperatures during the survey period (November –March) peak above temperatures at which pathogen mortality occurs (≥ 30°C) (i.e. Woodhams, Alford & Marantelli 2003; Piotrowski, Annis & Longcore 2004; Rowley & Alford 2013) and at which other assessed species host defences are optimal (Daskin *et al.* 2014). However,
temperatures are rarely high enough to eradicate chytrid during April – October which may help explain why \textit{L. lesueurii} recovery may be lowest during the species’ non-active period.

I found that estimates of pathogen impact on individuals can be biased unless key assumptions are evaluated when modelling disease and population dynamics. The potential risk of disease state misclassification when assessing the impact of pathogens upon species will depend upon the accuracy of diagnostic tests. Here I used a PCR method with a high diagnostic sensitivity and specificity (Chapter 3) and so the misclassification risk was probably small. In fact candidate models that assumed perfect classification produced almost identical estimates to models that assumed a true positive rate of 0.95 and false negative rate of 0.05. Furthermore models that assumed lower true positive rates were not supported. Credible intervals of survival estimates will tend to be tighter when assuming perfect classification but they may be falsely precise. Misclassification can bias estimates of disease prevalence and undermine assessments of infection dynamics (McClintock \textit{et al.} 2010; Miller \textit{et al.} 2012). Clearly, disease impact studies should account for the risk of misclassifying disease state.

My analysis indicates uninfected and infected individuals of both species are likely to have an equal probability of detection. This was surprising since chytrid can affect the behaviour of amphibians. Infected individuals are known to display clinical symptoms that include lethargy and inappetance as disease progresses (Berger, Spear & Hyatt 1999; Lips 1999) which could mean infected individuals either hide or are less responsive and more conspicuous. Nevertheless, studies have mostly found equal detection of uninfected and infected individuals (Retallick, McCallum & Speare 2004; Kriger & Hero 2006; Murray \textit{et al.} 2009; Pilliod \textit{et al.} 2010; Phillott \textit{et al.} 2013) but not always (Heard \textit{et al.} 2014). Detection may be influenced by the intensity of an individual’s infection. Highly infected individuals have been detected more frequently than individuals with lower infection loads (Heard \textit{et al.} 2014). This may be important, although additional work is required to demonstrate that PCR results are reasonable approximations of infection intensity as quantitative errors in assessed infection load might bias analyses.

In my study, \textit{L. spenceri} was not detected at Site 4 despite historic records of occurrence and 21 surveys over the 4 year study period. I previously found that the mean probability of
detecting the species during a survey is 0.845 (95% CI: 0.822 – 0.867) (Chapter 2). Assuming independence of encounters, the probability of not seeing the species after the 21 visits is \(~9.9\times10^{-18}\) (based on an equation by McArdle (1990)) indicating that \(L. spenceri\) is now likely to be extinct at Site 4. \(L. lesueurii\) was abundant and persisted at this site throughout the study despite suffering high chytrid-induced mortality. \(Litoria lesueurii\) may have influenced \(L. spenceri\) extinction at this site by maintaining a chytrid reservoir and sustaining pathogen transmission even as \(L. spenceri\) declined.

I found the marking method used did not reduce the probability of survival or capture of \(L. lesueurii\). But the effect of marking on \(L. spenceri\) was uncertain and no larger than 28.7% and 25.2%, per toe clipped, on survival and capture probabilities respectively. My results for \(L. lesueurii\) differ from previous studies that indicate toe-clipping can reduce the return rate of amphibians (Parris & McCarthy 2001; McCarthy & Parris 2004; McCarthy, Weller & Parris 2009). Previous assessments have not be able to separate whether reductions in return rates are driven by increases in mortality or reductions in recapture rates (McCarthy, Weller & Parris 2009). My modelling approach could be used to disentangle the impact of marking on mortality and capture rates, although an effect of toe clipping on return rate (the product of survival and recapture probabilities) was very uncertain for \(L. spenceri\), and likely to be small for \(L. lesueurii\).

This study highlights the importance of considering species interactions and the role of multiple threats when assessing the impact of disease. In this case, species interactions could increase the risk of a chytrid-mediated decline of \(L. spenceri\) populations if introduced predatory trout reduce recruitment or \(L. lesueurii\) maintains pathogen transmission to \(L. spenceri\). The impact of chytrid at an individual level should not be discounted when species occur at a high density. Direct field assessments are required to ground truth assumed or predicted responses of species to chytrid (Tompkins et al. 2011; Riley, Berry & Roberts 2013). This study demonstrates that chytridiomycosis can reduce survival of both threatened and non-threatened frogs, and provides further evidence that species’ demographic susceptibility to chytrid can be offset by their capacity to compensate for reduced adult survival through recruitment.
Population-level responses of two frogs to multiple threats: chytrid fungus and introduced trout
Chapter 5. Population-level responses of two frogs to multiple threats: chytrid fungus and introduced trout

5.1 Introduction

Understanding the principle threatening processes driving population declines is critical to developing effective conservation management responses (Caughley 1994). Multiple threats are linked to most declines. But the combined impact of threats upon species is often unclear (e.g. Smith, Sax & Lafferty 2006; Heard, Smith & Ripp 2011) and difficult to disentangle, as interactions between threats can be complex (Blaustein et al. 2011), and natural processes can also influence population fluctuations (Pechmann et al. 1991; Alford & Richards 1999; Daszak, Cunningham & Hyatt 2003; McCallum 2012). Globally, 42% of amphibian species are considered to be in decline and 32% are listed as threatened or extinct by the International Union for Conservation of Nature (IUCN 2008), making amphibians one of the most threatened taxonomic groups (Hof et al. 2011). Habitat loss, climate change, pollution, disease and invasive species are all major threats to amphibians with combinations of these threats probably impacting most species (Hof et al. 2011). Some threats may not be feasible to manage and misdirected management intervention can hamper conservation efforts (e.g. Rayner et al. 2007). Effective mitigation strategies to conserve declining species depend on understanding the relative contribution of each threat to population change (Brook, Sodhi & Bradshaw 2008; Bolten et al. 2010).

Chytrid fungus (Batrachochytrium dendrobatidis) is thought to threaten almost one quarter of threatened/extinct IUCN listed amphibians (Heard, Smith & Ripp 2011) and causes a disease known as chytridiomycosis in susceptible individuals (Berger et al. 1998). Although the response of individuals and populations to chytrid can vary both between and within species (Blaustein et al. 2005; Briggs et al. 2005; Tobler & Schmidt 2010). Chytrid can cause rapid population declines of some species at some sites, (e.g. Lips et al. 2006; Gillespie et al. 2014) but other species can experience slower rates of population decline or may persist despite chytrid presence (Phillott et al. 2013; Scheele et al. 2015).
Understanding why species and populations have differing responses to chytrid could lead to new conservation solutions (Collins 2010; James et al. 2015).

Variation in disease outcomes at the population level is influenced by variance in site-specific environment, host and pathogen interactions at the individual-level (James et al. 2015). For instance, microclimatic or microhabitat conditions, immune defences and pathogen virulence can influence an individual’s risk of infection and ability to survive or recover from infection (Tobler & Schmidt 2010; Gahl, Longcore & Houlanahan 2012; Doddington et al. 2013; Daskin et al. 2014; Gervasi et al. 2014; Heard et al. 2014). The same site-specific microclimatic conditions (particularly temperature) can influence pathogen growth and survival (Piotrowski, Annis & Longcore 2004) and amphibian demographic rates including age to maturation, clutch size and longevity (Morrison, Hero & Browning 2004). However, populations may persist despite chytrid infection as long as sufficient adult frogs survive and successfully reproduce (Briggs et al. 2005). Several authors have recently hypothesized that an infected population’s ability to compensate for disease induced mortality through recruitment could determine its risk of decline (Muths, Scherer & Pilliod 2011; Tobler, Borgula & Schmidt 2012; Phillott et al. 2013; Scheele et al. 2015).

Chytrid infected populations may be at greater risk of decline if impacted by other threats (Phillott et al. 2013) and most species are probably influenced by additional threats (Bielby et al. 2008; Heard, Smith & Ripp 2011). However, the actual risk to most species and the need for management intervention is unclear as the impacts of multiple threats can be complex (Blaustein & Kiesecker 2002) and could be additive, synergistic or antagonistic (Brook, Sodhi & Bradshaw 2008; Darling & Côté 2008). Furthermore, the population-level impacts may vary depending on how the dominance of threat changes in space or time. For example, the impacts of chytrid could either become more or less severe under climate change if host-pathogen dynamics are altered by shifts in ambient temperatures and precipitation (reviewed in Blaustein et al. 2010). Similarly, chemical contaminants (e.g. herbicides) can independently kill amphibians (Mann, Bidwell & Tyler 2003), although in some instances may reduce chytrid impacts by negatively affecting the pathogen more than the host (Gahl, Pauli & Houlanahan 2011). The implications for species are most serious if threats combine to have additive or synergistic effects (Brook, Sodhi & Bradshaw 2008;
Wake 2012). In some cases, pesticides may reduce host skin peptide defences against chytrid and increase individual’s disease risk (Davidson et al. 2007). Invasive and native amphibians that are tolerant of chytrid and maintain a pathogen-reservoir can increase disease risk for native amphibians that are intolerant of the pathogen (Fisher & Garner 2007; Reeder, Pessier & Vredenburg 2012).

Introduced fish (such as trout species) can severely effect amphibians and are implicated in species declines (Gillespie 2001b; Matthews et al. 2001; Vredenburg 2004; Knapp 2005). The impacts of introduced fish could be particularly severe for species that are also influenced by chytrid, as the threats may simultaneously effect different life stages. Introduced fish can reduce amphibian larval survival (Gillespie 2001b; Hunter et al. 2011), whereas the impacts of chytrid upon survival may be most severe following metamorphosis (Berger, Spear & Hyatt 1999). The threats could therefore combine to accelerate species decline. Alternatively, the threats may have opposing (antagonistic) effects; chytrid impacts may be reduced if disease dynamics are influenced by population density, and fish predation may limit some species’ population density. An understanding of these effects and interactions is therefore crucial to developing sound conservation strategies for amphibians where multiple threats are operating and potentially interacting.

Here, I compare the population-level responses of a threatened and a non-threatened frog species to two threats, chytrid and introduced trout. The Spotted Tree Frog (Litoria spenceri) is Critically Endangered (Hero et al. 2004) and breeds in mountain streams between 300-1110 m elevation in south-eastern Australia (Gillespie & Hollis 1996). Broad-scale monitoring over a 55 year period indicates that L. spenceri currently occupies around 50% of known historic sites and faces continuing decline without management intervention (Chapter 2). Multiple threats have been implicated in the species’ decline, although ongoing population pressure is believed to be primarily driven by chytrid and introduced trout (Gillespie 2001b; Gillespie et al. 2014). One abundant high-elevation population suffered rapid extinction following epidemic chytrid infection (Gillespie et al. 2014). However, the population-level impact of chytrid upon other L. spenceri populations has not been assessed. Introduced trout (Salmo trutta and Oncorhynchus mykiss) can significantly reduce the survival of L. spenceri tadpoles but appear to have little effect on the survival of Lesueur’s Frogs (Litoria lesueurii) tadpoles (Gillespie 2001), a frog species that occurs sympatrically
with *L. spenceri* at some sites. *Litoria lesueurii* are considered non-threatened and are suspected to be a potential chytrid-host-reservoir (Chapter 2). Recent multistate mark-recapture analysis indicates that chytrid can reduce the apparent annual survival of both species at an individual-level (Chapter 3). However population-level responses to the pathogen are suspected to differ among the species, perhaps due to different impacts of trout or demographic variation between sites correlated with temperature and elevation. I examine conditions that influence population viability and threat management of each species.

### 5.2 Methods

#### 5.2.1 Case Study

*Litoria spenceri* and *L. lesueurii* both breed annually in mountain streams during late spring and summer (November – January) in south-eastern Australia. Eggs hatch and tadpoles metamorph into frogs within a single season, prior to April when both species become inactive, due to onset of cooler temperatures. Activity generally resumes in mid-late October. *Litoria spenceri* matures less quickly and breeds at a later age than *L. lesueurii* (minimum maturity ages: *L. spenceri*: 3-5 years versus *L. lesueurii*: 2-4 years) (Gillespie 2010). Age to maturity for both species is expected to vary with site-specific climatic conditions and skeletochronology indicates both species reach maturity earlier at low elevations compared to higher elevations (Table 5.1) (Gillespie 2010; G.Gillespie unpublished data). Skeletochronology also indicates that *L. spenceri* can live longer than *L. lesueurii*, (Gillespie 2010; G.Gillespie unpublished data). Longevity estimates for both species (Gillespie 2010; G.Gillespie unpublished data) and the general observed pattern in other amphibians (Morrison & Hero 2003) suggests that mean survivorship of *L. spenceri* and *L. lesueurii* is longer at cooler high elevations compared to warmer low elevations.

Chytrid is known to occur at all but two *L. spenceri* sites, where the pathogens presence is uncertain (Chapter 2, G Gillespie and M West unpublished data). When chytrid is present, I assumed that frogs exist in one of two disease states: uninfected or infected. For each species, the disease dynamics for individuals at low elevation sites can be approximated as shown in Figure 5.1, assuming an annual time step and a pre-birth census. At mid and high elevation sites, both species spend longer periods in a subadult state (Appendix 3).
Figure 5.1 Multi-state life-cycle graphs and corresponding population matrices for *L. spenceri* and *L. lesueurii* at low elevation sites when chytrid is present. Frogs occur in either an uninfected (U) or infected (I) disease state and can either remain in that state or transition to the alternative disease state. $P$ represents the probability of survival and probability of transition between states, and $F$ represents fecundity.
Table 5.1 Initial annual demographic parameters of *L. spenceri* and *L. lesueurii* at low, mid and high elevation sites. Parameter estimates are generally based on mean published data derived during mark-recapture studies (Chapter 4; Todd *et al.* in prep.), counts of egg clutches (Gillespie 2011a) or skeletochronology (Gillespie 2010). Some parameters are based on expert opinion or unpublished data*.

<table>
<thead>
<tr>
<th>symbol</th>
<th>parameter</th>
<th><em>L. spenceri</em></th>
<th><em>L. lesueurii</em></th>
<th>Refs.</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>low</td>
<td>mid</td>
<td>high</td>
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<td>0.5</td>
<td>0.5</td>
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<td>b</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>f</td>
<td>fecundity (mean clutch size)</td>
<td>736*</td>
<td>528</td>
<td>482*</td>
</tr>
<tr>
<td>S_e</td>
<td>probability survival of eggs-year1</td>
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<td>0.011</td>
<td>0.011</td>
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<tr>
<td>M_1</td>
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<td>0.391</td>
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<tr>
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<td>0.270</td>
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<tr>
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<td>0.306</td>
<td>0.268</td>
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<td>0.272</td>
<td>0.310</td>
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<tr>
<td>a_A</td>
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<td>0.221</td>
<td>0.272</td>
<td>0.310</td>
</tr>
<tr>
<td>p_I</td>
<td>probability of apparent infection</td>
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</tr>
<tr>
<td>p_R</td>
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<td></td>
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<td></td>
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<td>coefficient to manipulate disease impact on all age classes</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>J</td>
<td>coefficient to manipulate M_sa1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* Gillespie (2010); ** Gillespie (2011a); *** Gillespie (2011b); ^ Todd *et al.* (in prep.); ^ Chapter 4; * G. Gillespie unpublished data.
A series of intensive mark-recapture and life history studies provided demographic parameter estimates for *L. spenceri* (Table 5.1). Stage-specific apparent annual survival probabilities and population growth rates were estimated at two low elevation sites ( ~330m) following a 21 – 23-year mark-recapture study (Todd *et al.* in prep.). Mean estimates of stage-specific survival, using a Cormack-Jolly-Seber model, were very similar at both sites. Chytrid and introduced trout were present throughout the mark-recapture study, but their relative influence upon survival was not distinguished. To address this, disease state-specific apparent annual survival probabilities of adult *L. spenceri* were estimated during a 6-year multi-state mark-recapture study (Chapter 4). Estimates for probabilities of apparent annual infection of uninfected frogs and apparent annual recovery of infected frogs were also derived using a Bayesian multi-state mark-recapture analysis (Chapter 4). A comparison of the species life history at low (~330m) versus high elevation (~1110m) sites indicates an equal sex ratio in terrestrial life stages and relative high mortality in juvenile compared to adult age-classes (Gillespie 2010). Approximately half of females reach maturity a year earlier than females within the same cohort (Gillespie 2010; Gillespie 2011b; Gillespie 2011a). Whilst clutch size does not appear to vary with age, it can vary with body size (Gillespie 2011a).

*Litoria lesueurii* produce larger clutches (mean eggs/female: 1510.2) than *L. spenceri* (mean eggs/female: 527.9) (Gillespie 2011a), but the clutch sizes of both species can vary and are larger at lower elevation sites (Table 1; G. Gillespie unpublished data). Disease state-specific apparent annual survival probabilities of adults, and probabilities of apparent annual infection and recovery have been estimated for *L. lesueurii* during a 4-year multi-state mark-recapture study (Chapter 4). Stage-specific survival estimates were not available for earlier *L. lesueurii* life stages, so survival of subadults in each year was assumed to equal that for *L. spenceri*.

Introduced brown trout (*Salmo trutta*) can significantly reduce the survival of *L. spenceri* tadpoles (Gillespie 2001b). An assessment of the *L. spenceri* population age structure suggests survival of eggs, tadpoles and metamorph frogs to year 1 (eggs-year 1) is approximately 4.75–times lower at sites when trout are present compared to when trout are absent (Gillespie 2010). The survival of *L. lesueurii* eggs-year 1 is not known but was
assumed to equal the survival of *L. spenceri* in the absence of trout, given that trout appear to have little effect on the survival of *L. lesueurii* tadpoles (Gillespie 2001b).

Probabilities of survival that had been derived at low elevation sites (Todd *et al.* in prep.; Chapter 4) were scaled to medium and high elevation sites. Since mean longevity is approximately the reciprocal of species mean mortality rate (for all ages) (McCarthy 2007), survival at other elevations was calculated relative to the ratio of maximum longevity at low elevation sites ($\text{long}_{\text{low}}$) compared to the maximum longevity at either the mid or high elevation sites ($\text{long}_{\text{elev}}$):

\[
\text{Su}_{y,elev} = \text{Su}_{y,\text{low}} \times \frac{\text{long}_{\text{low}}}{\text{long}_{\text{elev}}} \quad \text{Eqn. 5.1}
\]

\[
\text{Si}_{y,elev} = \text{Si}_{y,\text{low}} \times \frac{\text{long}_{\text{low}}}{\text{long}_{\text{elev}}} \quad \text{Eqn. 5.2}
\]

where $\text{Su}_{y,elev}$ and $\text{Si}_{y,elev}$ respectively represent the annual survival probability of subadults in age-class ($y$ : year 1, 2, or 3) when uninfected or infected with chytrid at either a mid or high elevation; $\text{Su}_{y,\text{low}}$ and $\text{Si}_{y,\text{low}}$ represent annual survival probability of uninfected and infected subadults in age-class 1, 2, or 3 and at a low elevation.

The same calculations were made to convert probabilities of survival of adults in uninfected ($\text{Su}_{A,\text{low}}$) or infected ($\text{Si}_{A,\text{low}}$) disease states derived at low elevation sites to state-specific survival estimates at mid and high elevations.

\[
\text{Su}_{A,elev} = \text{Su}_{A,\text{low}} \times \frac{\text{long}_{\text{low}}}{\text{long}_{\text{elev}}} \quad \text{Eqn. 5.3}
\]

\[
\text{Si}_{A,elev} = \text{Si}_{A,\text{low}} \times \frac{\text{long}_{\text{low}}}{\text{long}_{\text{elev}}} \quad \text{Eqn. 5.4}
\]

I assumed that the survival of frogs following metamorphosis was influenced by their chytrid infection status. Disease state-specific estimates of survival for each species have only been derived for adult frogs (Chapter 4). The relative influence of disease upon survival of individuals in other age-classes was assumed to be proportional to the difference in disease state-specific adult survival. Probabilities of survival of subadults when either uninfected ($\text{Su}_{y,elev}$) or infected ($\text{Si}_{y,elev}$) with chytrid were as assumed to be:
\[ S_{y, \text{elev}} = \frac{S_{y, \text{elev}}}{S_{A, \text{elev}}} \times S_{uA, \text{elev}} \quad \text{Eqn. 5.5} \]

\[ S_{i,y, \text{elev}} = \frac{S_{y, \text{elev}}}{S_{iA, \text{elev}}} \times S_{iA, \text{elev}} \quad \text{Eqn. 5.6} \]

where subadult survival in each disease state was calculated as the ratio of subadult survival \((S_{y, \text{elev}})\) to adult survival \((S_{A, \text{elev}})\) ignoring disease state and proportional to either the probability of survival of uninfected adults \((S_{uA, \text{elev}})\) or infected adults \((S_{iA, \text{elev}})\). Disease state-specific survival was calculated in the same manner for each age-class \((y: \text{years 1, 2, or 3})\) and at each elevation \((\text{elev})\). I assumed that the survival of \(L. \text{lesueurii}\) in each subadult age-class was equal to the survival of \(L. \text{spenceri}\) in each subadult age-class when disease state was unknown. Disease state specific estimates of subadult survival were then calculated relative to those of adults (Chapter 4). Age-specific baseline mortality probabilities for subadults in each age class \((M_{y, \text{elev}})\) and adults \((M_{A, \text{elev}})\) for each elevation were:

\[ M_{y, \text{elev}} = 1 - S_{u,y, \text{elev}} \quad \text{Eqn. 5.7} \]

\[ M_{A, \text{elev}} = 1 - S_{uA, \text{elev}} \quad \text{Eqn. 5.8} \]

The added mortality probabilities due to chytridiomycosis for subadults in each age class \((a_{y, \text{elev}})\) and for adults \((a_{A, \text{elev}})\) at each elevation were:

\[ a_{y, \text{elev}} = S_{u,y, \text{elev}} - S_{i,y, \text{elev}} \quad \text{Eqn. 5.9} \]

\[ a_{A, \text{elev}} = S_{uA, \text{elev}} - S_{iA, \text{elev}} \quad \text{Eqn. 5.10} \]

### 5.2.2 Population Models

Three multistate age-structured population projection matrices were constructed for each frog species to represent their population dynamics at a low, medium and high elevation site (Table 1). Each projection matrix \(A\) was composed of four submatrices:

\[
A = \begin{bmatrix}
A_1 & A_3 \\
A_2 & A_4
\end{bmatrix} \quad \text{Eqn. 5.11}
\]
where submatrices described the survival and disease transition probabilities and reproduction of individuals that either remain uninfected \((A_1)\) or infected \((A_4)\), or transition from an uninfected to infected disease state \((A_2)\), or from an infected to uninfected disease state \((A_4)\) in each time step.

Submatrices for each species were constructed using lower level demographic parameters. The reproduction elements \((F; \text{ in Figure 5.1})\) were calculated as a function of the sex ratio \((sr)\), probability of breeding in first \((b_1)\) or subsequent adult year \((b)\), clutch size \((f; \text{ fecundity})\) and survival probability of eggs-year 1 \((S_e)\). The survival and transition elements \((P; \text{ in Figure 1})\) were calculated for each age class as a function of the baseline mortality, added mortality due to chytrid, and disease impact coefficient \((D)\). Reproduction and survival elements were also a function of \(T_i\), the probability of an individual remaining in a current disease state or transitioning to another disease state.

The matrix construction for each species at other elevations was modified so the dimensions of each submatrix represented the number of age classes prior to female maturation and timing of first breeding (see Appendix 3).

The population growth rate was the dominant eigenvalue for each population projection matrix \(A\) using the popbio package in program R (R Core Team 2013). A sensitivity analysis was performed using the popbio package to evaluate the sensitivity of growth rates to perturbations in the species’ demographic parameters/vital rates. The sensitivity analysis also examined the change in growth rate with respect to a change in key demographic parameters across a biologically plausible range. Mortality was manipulated using coefficients; changes in mortality of subadults in year 1-year 2 \((M_1)\) were examined by varying coefficient \(J\), and the added mortality due to chytrid across all life stages was manipulated by varying a disease coefficient \(D\).

Changes in the population growth rate were compared to potential changes in key demographic parameters that could be influenced by management. Growth rates were estimated after modifying demographic parameter values that were likely to be effected under different management scenarios, whilst holding all other parameter values constant. Management activities to mitigate the impacts of trout are likely to influence the probability
of survival of eggs-year 1. Management activities to mitigate the impacts of chytrid are likely to influence the probability of added mortality due to chytrid fungus and/or the probabilities of infection and recovery. If chytrid management activities affected frog survival, the added mortality due to chytrid across all life stages was manipulated by varying a disease coefficient ($D$).
For *L. spenceri* at a low elevation site, submatrices $\mathbf{A}_1$ and $\mathbf{A}_2$ were:

$$
\mathbf{A}_i =
\begin{bmatrix}
0 & 0 & sr \times b \times 1 \times f \times S_e \times T_i & sr \times b \times f \times S_e \times T_i \\
(1 - (M_1 \times J)) \times T_i & 0 & 0 & 0 \\
0 & (1 - M_2) \times T_i & 0 & 0 \\
0 & 0 & (1 - M_3) \times T_i & (1 - M_A) \times T_i
\end{bmatrix}
$$

Eqn. 5.12

for $i=1,2$ where $T_1 = 1 - pl$ is the probability of an uninfected individual remaining uninfected and $T_2 = pl$ is the probability of an uninfected individual becoming infected. *Litoria spenceri* submatrices $\mathbf{A}_3$ and $\mathbf{A}_4$ at low elevations were:

$$
\mathbf{A}_i =
\begin{bmatrix}
0 & 0 & sr \times b \times 1 \times f \times S_e \times T_i & sr \times b \times f \times S_e \times T_i \\
(1 - (M_1 \times J + a_3 \times D))) \times T_i & 0 & 0 & 0 \\
0 & (1 - (M_2 + a_2 \times D)) \times T_i & 0 & 0 \\
0 & 0 & (1 - (M_3 + a_3 \times D)) \times T_i & (1 - (M_A + a_A \times D)) \times T_i
\end{bmatrix}
$$

Eqn. 5.13

for $i=3,4$ where $T_3 = pR$ is the probability of an individual recovering from an infection and $T_4 = 1 - pR$ is the probability of an individual remaining infected.
For *L. lesueurii* at a low elevation site, submatrices $A_1$ and $A_2$ were:

$$A_i = \begin{bmatrix}
0 & sr \times b \times f \times S_e \times T_i & sr \times b \times f \times S_e \times T_i & sr \times b \times f \times S_e \times T_i \\
(1 - (M_1 \times f)) \times T_i & 0 & 0 & 0 \\
0 & (1 - M_2) \times T_i & 0 & 0 \\
0 & 0 & (1 - M_3) \times T_i & (1 - M_4) \times T_i \\
\end{bmatrix}$$  \hspace{1cm} \text{Eqn. 5.14}

for $i=1,2$ where $T_i = 1 - pI$ is the probability of an uninfected individual remaining uninfected and $T_2 = pI$ is the probability of an uninfected individual becoming infected. *Litoria lesueurii* submatrices $A_3$ and $A_4$ at low elevations were:

$$A_i = \begin{bmatrix}
0 & sr \times b \times f \times S_e \times T_i & sr \times b \times f \times S_e \times T_i & sr \times b \times f \times S_e \times T_i \\
(1 - (M_1 \times f + a_3 \times D)) \times T_i & 0 & 0 & 0 \\
0 & (1 - (M_2 + a_2 \times D)) \times T_i & 0 & 0 \\
0 & 0 & (1 - (M_3 + a_3 \times D)) \times T_i & (1 - (M_4 + a_A \times D)) \times T_i \\
\end{bmatrix}$$  \hspace{1cm} \text{Eqn. 5.15}

for $i=3,4$ where $T_3 = pR$ is the probability of an individual recovering from an infection and $T_4 = 1 - pR$ is the probability of an individual remaining infected.
5.3 Results

Under all scenarios examined (Table 5.2), population growth rates ($\lambda$) of both species decreased with elevation despite increased longevity.

Under current conditions, *L. spenceri* populations at high ($\lambda_1 = 0.88$) and mid ($\lambda_2 = 0.93$) elevation sites and *L. lesueurii* at high elevations sites ($\lambda = 0.97$) are considered to be nonviable when impacted by both trout and chytrid fungus. In contrast, populations of *L. spenceri* at low elevation sites ($\lambda_3 = 1.07$) should be considered viable under current conditions (in the absence of stochasticity), while *L. lesueurii* populations have a greater capacity to persist at both mid ($\lambda_4 = 1.38$) and low ($\lambda_5 = 2.39$) elevation sites.

Variation in the survival of eggs-year 1 ($S_e$) had the greatest impact on estimated growth rate of *L. spenceri*, which was relatively insensitive to changes in other parameters (Figures 5.2 and 5.3). The growth rate of *L. lesueurii* was sensitive to changes in the survival of eggs-year 1 age-class ($S_e$), the baseline mortality of year 1 subadults ($M_1$), the added mortality of year 1 subadults due to chytrid ($a_1$), the added mortality due to disease ($D$) of all age classes, and the probability of infection ($p_I$) (Figures 5.2 and 5.3).

Notably, if the *L. lesueurii* eggs-year 1 survival was equal to the current estimate for *L. spenceri* ($S_e = 0.011$) then its growth rate could drop below 1 at mid elevations ($\lambda = 0.89$). However, the growth rate of *L. lesueurii* could remain above 1 at low elevation sites ($\lambda = 1.30$), even if eggs-year 1 survival was reduced to the same current level as *L. spenceri*.

If management intervention were able to completely mitigate the impacts of both threats, the growth rate of *L. spenceri* could increase by around 56 to 69% (Table 5.2). Whilst complete mitigation of both threats was estimated to have the greatest benefit, management actions that reduce the impacts of either threat could also improve the growth rate of *L. spenceri*. If management targeted only one threat, a reduction of trout impacts upon the eggs-year 1 age-class is expected to most improve the growth rate of *L. spenceri* at low elevation sites, even if the risk of becoming infected by chytrid increases (Table 5.2: Trout Management A and B). However, the potential benefit to *L. spenceri* by managing trout at other elevations depended on no change in the impact of chytrid. Under chytrid-only management scenarios,
the greatest benefit to *L. spenceri* could be achieved by eliminating the probability of infection \((pI)\) (i.e. preventing disease transmission) or avoiding the added mortality due to chytridiomycosis \((D)\). Actions that only increase the probability of recovery of infected frogs were unlikely to improve population growth rate (Table 5.2).

Population growth rates of *L. lesueurii* were predicted to increase under all chytrid management scenarios. Complete mitigation of chytrid impacts was predicted to increase the growth rate of this species by between 105 and 113% from current estimates, depending on the sites’ elevation (Table 5.2: Chytrid Management B and C). Actions to mitigate chytrid impacts could have the greatest benefit to high elevation *L. lesueurii* populations. As with *L. spenceri*, actions that only increase the probability of recovery of infected frogs were unlikely to improve population growth rate of *L. lesueurii* (Table 5.2). This analysis identified that *L. lesueurii* populations might decline \((\lambda<1)\) at mid elevations if the probability of infection increased and the probability of recovery was to drop to zero (Table 5.2: Trout Management B). However, *L. lesueurii* growth rates at mid elevations were predicted to be at or greater than 1 as long as the probability of recovery was at least 0.3 (Figure 5.3).

The population growth rate of *L. spenceri* at low elevation sites could fall below 1 if female age of maturity were delayed or females produced fewer eggs. For example, if females reached maturity in year 4 instead of year 3, then \(\lambda = 0.99\) at low elevations. Similarly, if females produced 528 eggs (mean size at mid elevations) instead of 736 eggs, then \(\lambda = 0.99\) at low elevations. Any delay in age of maturity or any reduction in egg production could, therefore, also accelerate decline of *L. spenceri* at other elevations. Conversely, if *L. spenceri* were able to produce more eggs, for example the same number produced by *L. lesueurii*, then population growth rates could increase from current estimates (Table 5.2) to 1.38, 1.12 and 1.03 respectively at low, mid and high elevation sites. But if *L. lesueurii* produced the same number of eggs as *L. spenceri*, then population growth rates could decrease from current estimates (Table 5.2) to 1.57, 1.02 and 0.79 respectively at low, mid and high elevation sites.
Table 5.2 Estimated deterministic population growth rates of *L. spenceri* and *L. lesueurii* at low, mid and high elevation sites, under current conditions or varying hypothetical management scenarios to mitigate the impacts of introduced trout or chytrid fungus. Management was assumed to influence *L. spenceri*’s probability of survival of eggs-year1 (*S_e*), species probability of infection (*pI*) or recovery (*pR*), or the added mortality across all terrestrial life stages due to disease (*D*). Mean vital rates are assumed unless listed. Percentage change from current conditions is shown in parenthesis.

<table>
<thead>
<tr>
<th>Management Scenario</th>
<th>Assumptions</th>
<th>Estimated Population Growth Rates</th>
<th>L. spenceri</th>
<th>L. lesueurii</th>
<th>L. spenceri</th>
<th>L. lesueurii</th>
<th>L. spenceri</th>
<th>L. lesueurii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current (no management)</td>
<td>Mean vital rates</td>
<td>1.07</td>
<td>0.93</td>
<td>0.88</td>
<td>2.39</td>
<td>1.38</td>
<td>0.97</td>
<td></td>
</tr>
</tbody>
</table>
| Trout management A (no change in chytrid impacts) | *L. spenceri S_e = 0.052;*  
*L. lesueurii S_e = no change* | 1.57                             | 1.24                       | 1.11        | 2.39        | 1.38        | 0.97        |
|                                           |                                                                             | (46.7%)                         | (33.3%)     | (26.1%)     | (0%)        | (0%)        | (0%)        |             |
| Trout management B (but increase in chytrid impacts) | *L. spenceri S_e = 0.052;*  
*L. lesueurii S_e = no change;*  
Both species *pI* = 1, *pR* = 0. | 1.33                             | 0.98                       | 0.84        | 1.98        | 0.78        | 0.53        |
|                                           |                                                                             | (24.2%)                         | (0.5%)      | (-4.5%)     | (-16.9%)    | (-42.9%)    | (-45.3%)    |             |
| Chytrid management A (no change in trout impacts) | Both species *pI* = mean, *pR* = 1  
Both species *pI* = mean, *pR* = 1  | 1.07                             | 0.93                       | 0.89        | 2.40        | 1.39        | 0.98        |
|                                           |                                                                             | (0%)                            | (0.3%)      | (1.1%)      | (0.7%)      | (1.1%)      | (1.7%)      |             |
| Chytrid management B (no change in trout impacts) | Both species *pI* = 0, *pR* = 1  
Both species *pI* = 0, *pR* = 1 | 1.24                             | 1.12                       | 1.09        | 5.08        | 2.83        | 2.2         |
|                                           |                                                                             | (15.7%)                         | (20.4%)     | (23.8%)     | (112.8%)    | (105.1%)    | (127.6%)    |             |
| Chytrid management C (no change in trout impacts) | Both species 50% reduction in added mortality due to disease (*D* = 0.5) | 1.16                             | 1.03                       | 1.00        | 4.01        | 2.26        | 1.72        |
|                                           |                                                                             | (8.2%)                          | (10.7%)     | (13.6%)     | (67.7%)     | (63.9%)     | (77.2%)     |             |
| Chytrid management D (no change in trout impacts) | Both species 100% reduction in added mortality due to disease (*D* = 0) | 1.24                             | 1.12                       | 1.09        | 5.08        | 2.83        | 2.21        |
|                                           |                                                                             | (15.7%)                         | (20.4%)     | (23.8%)     | (112.8%)    | (105.1%)    | (127.6%)    |             |
| Trout and chytrid management (complete threat mitigation) | *L. spenceri S_e = 0.052;*  
*L. lesueurii S_e = no change;*  
Both species *pI* = 0, *pR* = 1, *D* = 0 | 1.81                             | 1.49                       | 1.37        | 5.08        | 2.83        | 2.21        |
|                                           |                                                                             | (69.1%)                         | (60.2%)     | (55.7%)     | (112.8%)    | (105.1%)    | (127.6%)    |             |
Figure 5.2 Sensitivity of population growth rate to each of the vital rates of *L. spenceri* and *L. lesueurii* for multistate age-structured models constructed to represent populations at low, mid or high elevation sites.
$L.\ spenceri$

$L.\ lesueurii$
Figure 5.3 The potential change in deterministic population growth rates (lambda) of *L. spenceri* and *L. lesueurii* if vital rates vary from current mean parameter estimates (shown at grey vertical dashed line). Vital rates examined: survival of eggs-year1 (*S*); proportional change in baseline mortality of year1 subadults (*M*); proportional change in added mortality due to chytridiomycosis (*D*) on all life stages; apparent probability of infection (*pI*) of uninfected frogs; apparent probability of recovery (*pR*) of infected frogs. Population growth rates were estimated for populations at low (blue dotted line), mid (black solid line) or high (red dashed line) elevation sites.
5.4 Discussion

My findings support the hypothesis that species can persist if recruitment can compensate for chytrid-induced mortality (Muths, Scherer & Pilioid 2011; Tobler, Borgula & Schmidt 2012; Phillott et al. 2013; Scheele et al. 2015). My models suggest that interspecific differences in the age at maturity, clutch size and survival of individuals between egg-year 1 account for the differences in the capacity of *L. spenceri* and *L. lesueurii* to compensate for chytrid-induced adult mortality. *Litoria lesueurii* populations cope with chytrid infection better at low and mid elevations despite individuals having a lower probability of survival when infected and a higher probability of infection than *L. spenceri*.

Results also indicate that the impact of threats upon species can vary spatially if species’ vital rates vary between sites. *Litoria spenceri* populations were estimated to be non-viable ($\lambda<1$) at mid and high elevation sites. However, an earlier age of female maturity (year 3 versus year 4 or 5) and a larger clutch size (736 eggs versus 528 or 482 eggs) may reduce the overall population-level impacts of chytrid and trout on *L. spenceri* at low elevation sites ($\lambda=1.07$) in contrast to higher elevation sites. The earlier maturation age and increased number of eggs produced by *L. lesueurii* compared with *L. spenceri* also appears to buffer *L. lesueurii* against the impact of chytrid at low ($\lambda=2.39$) and mid ($\lambda=1.38$) elevation sites. Although the later age of maturity and smaller number of eggs produced mean that *L. lesueurii* was nonviable at high ($\lambda=0.97$) elevation sites. Elevation, in this study, reflects a gradient of a longer frog active season and warmer conditions at low elevations versus a shorter active season and cooler conditions at higher elevations (Gillespie 2011b). Intraspecific variation in amphibian demography (particularly age to maturity, longevity and clutch size) is generally correlated with climatic differences between sites often across an elevation gradient (e.g. Miaud, Guyetant & Faber 2000; Morrison & Hero 2003; Morrison, Hero & Browning 2004; Leskovar et al. 2006; Cvetković et al. 2009; Gillespie 2011b). I found that age of maturity, clutch size and egg-year 1 survival were more important for species persistence than longevity. In turn this supports the hypothesis that high-elevation, range-restricted, aquatic amphibian species with low fecundities are generally at greatest risk of chytrid associated declines (Bielby et al. 2008).
Predation of eggs and tadpoles by introduced trout is likely to severely restrict the ability of *L. spenceri* to compensate for chytrid-induced mortality of adults. The predatory impact of introduced trout may be particularly severe as *L. spenceri* tadpoles are not consumed by native fish species (Gillespie 2001b). Introduced trout species now occur at all extant *L. spenceri* sites (Gillespie & Hollis 1996; Gillespie 2001b). In contrast, *L. lesueurii* has a competitive advantage over *L. spenceri* as trout do not consume *L. lesueurii* tadpoles (Gillespie 2001b). My models suggest that *L. lesueurii* would be non-viable at all elevations if tadpoles were impacted by trout to the same extent as *L. spenceri* tadpoles.

In the absence of effective strategies to directly mitigate chytrid an alternative strategy for conserving *L. spenceri* may be to increase recruitment by managing introduced trout. I acknowledge that the benefits of trout management to *L. spenceri* populations are currently uncertain, particularly at mid and high elevation sites. This is because, potentially positive responses of *L. spenceri* populations to trout management could be reduced if chytrid transmission rates vary. Chytrid transmission may be influenced by host density or disease prevalence (density- or frequency-dependent transmission) (Briggs *et al.* 2005; Rachowicz & Briggs 2007), amongst other factors such as seasonal climatic variation (Phillott *et al.* 2013) and host behaviour (Rowley & Alford 2007a). High population density may have contributed to the rapid decline and extirpation of *L. spenceri* at a high elevation site during an epidemic chytrid infection (Gillespie *et al.* 2014). The population-level consequences of an increase in disease transmission may vary between sites and could depend upon the populations’ ability to compensate for an increase in adult mortality through recruitment. *Litoria spenceri* occur at lower densities at all remaining sites (Gillespie & Hollis 1996). My analysis suggests that the risk of *L. spenceri* extinction might be reduced if trout were managed at low elevation sites even if infection probability increased and recovery probability decreased (assuming the survival probability of infected frogs remains constant). Experimental fish management is technically feasible and has benefited other frog populations known to be threatened by introduced trout (Vredenburg 2004; Knapp, Boiano & Vredenburg 2007). Management trials are warranted to evaluate the benefits and consequences of mitigating introduced fish predation on *L. spenceri* populations at all elevations. But trout management is expected to be most beneficial and least risky for *L. spenceri* populations at low elevation sites.
The success of any mitigation strategies could be influenced by other species interactions. For instance, extinction risk of *L. spenceri* may increase when both *L. lesueurii* and chytrid are present at sites. Pathogen host reservoirs can form when species have different responses to disease (Fisher, Garner & Walker 2009). The ability of *L. lesueurii* populations to persist at sites despite the impacts of chytrid could increase chytrid transmission rates through *L. spenceri* populations when the species are sympatric. Previously research found that the presence of both chytrid and *L. lesueurii* are correlated with a decline in occupancy of *L. spenceri* (Chapter 2). In contrast, *L. lesueurii* may now occupy more *L. spenceri* sites despite chytrid (Chapter 2). These observations suggest that *L. lesueurii* may be an important pathogen host reservoir for *L. spenceri*.

The deterministic discrete-time model approach used in this study provides simple but important insights into the potential population-level impacts of threats upon the two frog species with contrasting population dynamics. This type of information is generally lacking for most amphibian species (Biek et al. 2002; Gillespie 2011b). Further research on predicting population viability could also consider additional factors, such as density or frequency–dependent effects and impacts of environmental and demographic stochasticity. For example, whilst the population growth rate suggests *L. spenceri* can persist at low elevations despite the presence of both threats. Both species are vulnerable to stochastic events that reduce recruitment (i.e. flood or bushfire (Gillespie & West 2012)) or age of maturity (i.e. a series of cool summers (*sensu* Morrison & Hero 2003)). However, the risk of extinction when exposed to unfavourable stochastic events is higher for *L. spenceri* than *L. lesueurii* as it has a more restricted distribution, smaller population sizes and a lower growth rate and capacity to recover. Furthermore, disease dynamics may need to be modelled on a continuous scale or shorter discrete time step, rather than the annual dynamics examined in my study. My results are sensitive to the selected parameter values, particularly estimates of eggs-year 1 survival, although the parameter estimates are broadly consistent with those derived for other species. For instance, survival from hatching to metamorphosis in other species is considered generally less than 10% (Wells 2010). Additional research to evaluate the components of the egg-year 1 period most sensitive to mortality could provide further insight into the mechanisms of decline. Similarly additional research is required to clarify age-specific survival of *L. lesueurii* which was assumed to be equivalent to *L. spenceri* in this study.
Whilst variation in environmental factors, host susceptibility and pathogen virulence can lead to varying epidemiological outcomes (James et al. 2015), interactions with co-occurring species can also influence population-level responses to disease. Threat impacts at the population-level can vary spatially for both threatened and non-threatened species. This case study provides clear evidence that an amphibian population’s capacity to cope with chytrid is influenced by environmental processes and other threats that can restrict recruitment.

Currently options to mitigate chytrid impacts upon wild infected populations are limited (Scheele et al. 2014b), particularly for lotic systems. However, strategies that increase recruitment to offset mortality caused by chytrid have been proposed (e.g. Scheele et al. 2015), and this could be achieved for L. spenceri by managing trout. In contrast to chytrid, direct trout mitigation options are technically feasible (i.e. piscicide use, removal via electrofishing). At warmer low elevation sites, fish management may be the optimal approach, but strategies to manage chytrid or both threats concurrently may be required at cooler, higher elevation sites. The challenge is to identify a feasible management approach before L. spenceri becomes extinct. Due to the current uncertainty in the species responses to intervention, management options should be examined in an experimental framework to avoid exacerbating the impacts of either threat. The results indicate that spatially-explicit knowledge of species demography and population-level impacts of interacting threats is important when designing effective management strategies for threatened species.
Synthesis and General Discussion
Chapter 6. Synthesis and General Discussion

6.1 Species decline driven by multiple interacting threats

Understanding threats to species is difficult. Researchers, like detectives, must search for evidence (by conducting targeted research) and piece together snippets of information (using statistical approaches) to assemble clues into a coherent picture (Schnute 1987 cited in Hilborn and Mangel (1997)).

Species’ population decline and the underlying causes are often uncertain, particularly as little information exists prior to declines and multiple factors can be involved. Data about decline can be challenging to acquire, and managers are often under pressure to undertake conservation actions without understanding the underlying processes influencing species (Rayner et al. 2007). Failing to collect and thoroughly consider the evidence of decline can bias assessments of population viability, waste or misdirect investment, and lead to ineffective or undesirable conservation outcomes (Rayner et al. 2007; Sabo 2008; Chadès, Curtis & Martin 2012). In this thesis, I provide clear evidence of a species’ decline and disentangle causal factors for the Critically Endangered Spotted Tree Frog, Litoria spenceri in south-eastern Australia.

In Chapter 2, using 55 years of data and a two-species dynamic occupancy model, I uncovered an approximate 50% reduction in L. spenceri site occupancy coinciding with increasing L. lesueurii site occupancy and chytrid presence. The analysis confirmed that L. spenceri has declined substantially from its historical distribution, as first suspected during the late 1980’s (Watson et al. 1991). At the same time a global decline of amphibians was beginning to be realised (Barinaga 1990; Blaustein & Wake 1990). As with other species at the time (Pechmann et al. 1991) evidence regarding L. spenceri decline was mostly anecdotal. The analysis in Chapter 2 expands previous assessments of L. spenceri decline (Watson et al. 1991; Gillespie & Hollis 1996) by piecing together historic fragmented data with more intensively collected post-decline data, and by using contemporary occupancy modelling techniques (MacKenzie et al. 2002; Royle & Kery
2007) to specifically account for imperfect detection of species at sites. A process-based model underpinned the occupancy model, evaluating extinction risk and generating hypotheses about the factors that caused decline. While the role of introduced trout could not be assessed using this approach (given the near ubiquity of trout among the focal sites), the analysis did suggest that *L. spenceri* decline could have been driven by either *L. lesueurii* or chytrid, or both factors acting in combination. *Litoria spenceri* and *L. lesueurii* were predicted to have different responses to chytrid infection, and I hypothesized that *L. spenceri* has declined most severely at sites when the presence of less-susceptible host reservoir species (or total host density) facilitated chytrid persistence and maintained chytrid transmission.

To verify the suspected differing responses of *L. spenceri* and *L. lesueurii* to chytrid, I tracked individual frogs and their infection status for between 4 and 6 years at 4 sites using mark-recapture (Chapter 4). First however, I sought to validate a modified diagnostic PCR method to accurately diagnose infected individuals and to minimise cost (Chapter 3). By assessing deliberate chytrid-spiked swabs, I found that the PCR method had a high diagnostic sensitivity and specificity, but that misclassification of the individual’s infection state was possible (Chapter 3). The misclassification of an individual’s disease state could bias disease impact assessments unless acknowledged by service providers and considered by practitioners. Other types of bias were also possible when assessing pathogen impacts on species, including: potential imperfect detection of individuals relative to their disease state; potential reduction in probability of capture or survival due to the marking method, and; uncertainty about the actual ability of individuals to recover from an infection.

In Chapter 4, I used modern multi-infection-state mark-recapture modelling to quantify the impact of chytrid on both *L. spenceri* and *L. lesueurii* while accounting for each of the potential sources of bias (discussed above). The modelling indicated that some misclassification of individual infection states occurred and that individuals could recover from infections if they survived. Both infected and uninfected individuals had similar probabilities of detection. Marking did not appear to affect *L. lesueurii* capture or survival rates and the effect on *L. spenceri* was uncertain. Models with the most support (lowest DIC values) did not include an effect of marking. Individuals of both species had a lower survival probability when infected with chytrid compared to uninfected individuals. Infections during
the inactive period and recovery probabilities were similar for both species, but *L. lesueurii* infection probability was higher during the active season than that of *L. spenceri*. Importantly, the results indicated that chytrid infections reduced the survival of both species with the impact being more severe for *L. lesueurii*. This ran counter to the hypothesised differential effect of chytrid on survival for these species, and instead indicated that species’ population-level responses to the pathogen were influenced by recruitment rather than survival (e.g. Muths, Scherer & Pilliod 2011; Tobler, Borgula & Schmidt 2012; Phillott *et al.* 2013; Scheele *et al.* 2015), with *L. lesueurii* being able to offset increased mortality through recruitment and *L. spenceri* having less capacity to do so. As such, the data did not support the possibility that *L. spenceri* and *L. lesueurii* have different inherent defences against the pathogen, such as differences in mucosal peptides and microbiota (see: Woodhams *et al.* 2006; Woodhams *et al.* 2007; Searle *et al.* 2011; Gahl, Longcore & Houlanah 2012).

To clarify why the focal species had different population-level responses despite similar individual-level responses to chytrid, I constructed multi-state deterministic population models (Chapter 5) for each species at low, mid and high elevation sites. Models were constructed at these elevations as egg production and maturation rates are negatively correlated with elevation for both species, whereas longevity is positively correlated with elevation (Gillespie 2010; Gillespie 2011b; Gillespie 2011a; G. Gillespie unpublished data). Species demographic rates are influenced by site-specific climatic conditions which vary with elevation (e.g. Morrison & Hero 2003). The analysis revealed intraspecific and interspecific differences in species population-level responses to chytrid, confirming that their ability to compensate for chytrid-induced mortality is mediated by recruitment. *Litoria spenceri* produces fewer eggs and matures later than *L. lesueurii*. As such, *L. spenceri* has a lower capacity to cope with increased mortality resulting from chytrid infections. The population models developed in Chapter 5 also revealed that chytrid impacts are also influenced by species interactions with the environment and introduced trout. For both species, the capacity to cope demographically with chytrid infection was highest at warmer, low elevation sites, where egg production is higher and sexual maturation occurs relatively quickly (growth rates for *L. spenceri* were 1.07, 0.93, 0.88 and for *L. lesueurii* were 2.39, 1.38, 0.97 at low, medium and high elevation sites respectively). Lastly, the population modelling completed in Chapter 5 clarifies the impact of introduced trout on *L. spenceri*, and
its interactive effect with chytrid. Tadpole predation by trout further restricts *L. spenceri* recruitment and exacerbates the impact of chytrid-induced adult mortality at all elevations whereas trout do not eat *L. lesueurii* tadpoles (Gillespie 2001b). The interactive impacts of chytrid and trout predation threaten *L. spenceri* populations, particularly at high and mid elevation sites. In contrast, *L. lesueurii* is able to thrive at both low and mid elevations and is likely to be less sensitive than *L. spenceri* to stochastic processes.

This thesis also provides evidence that *L. lesueurii* could be a reservoir host for chytrid as the species is more tolerant of the pathogen than *L. spenceri* (Chapter 5), despite suffering high mortality (Chapter 4). *Litoria lesueurii* could, therefore, further exacerbate chytrid impacts by maintaining pathogen transmission when the frog species are sympatric, even as *L. spenceri* declines. This is supported by the finding in Chapter 2 indicating *L. spenceri* extinction risk was highest when both chytrid and *L. lesueurii* were present at sites. The duration of most species’ association with chytrid is largely unresolved (Phillips *et al.* 2012). However, as demonstrated in Chapter 2, the negative association of *L. spenceri* with both *L. lesueurii* and chytrid held true regardless of when I assumed chytrid had arrived at the study sites. This finding supports the role of *L. lesueurii* as a reservoir host, although other potential negative interactions such as competition between the two frog species could also exist. For instance *L. lesueurii* tadpoles could be a superior competitor to those of *L. spenceri* (Gillespie 2001a). Conversely, the expansion of *L. lesueurii* at sites could occur if the loss of *L. spenceri* at sites released competitive pressure upon *L. lesueurii* (e.g. Rodriguez 2006).

### 6.2 Implications for Spotted Tree Frogs: future research priorities and recommendations

*Litoria spenceri* is predicted to continue to decline without management intervention, and could become extinct at all sites by as early as 2035 (Chapter 2). The complex site-specific interactions will influence the rate of future decline at each of *L. spenceri*’s remaining extant sites (Chapters 2 and 5). The population assessment and evaluation of causal factors presented in this thesis, building on the work of others (Watson *et al.* 1991; Gillespie & Hollis 1996; Gillespie 2001b; Gillespie 2001a; Gillespie 2002; Gillespie 2011a; Gillespie *et
al. 2014), now provides a clear evidence base on which to take further action. I advocate that we must adopt an adaptive management strategy if we are to prevent *L. spenceri* extinction in the decades ahead.

Adaptive management is a systematic approach for improving management by learning from management outcomes (Walters & Hilborn 1978; Walters & Holling 1990; Westgate, Likens & Lindenmayer 2013). In this context, managers can take action to assist threatened species, whilst resolving uncertainty in the optimal management solution and remaining responsive to new information as it is acquired (Walters & Hilborn 1978; Walters & Holling 1990; McDonald-Madden *et al.* 2010; Runge 2011). The process involves: management goal setting; identifying management options; creating a conceptual model of how the system might respond to management; designing and implementing statistically robust management experiments; monitoring of the system response to experimental management intervention, and; refining management experiments in response to results from monitoring (Westgate, Likens & Lindenmayer 2013).

The current overarching management goal for *L. spenceri* is to minimise its probability of extinction in the wild and to increase the probability of populations becoming self-sustaining in the long term (Gillespie & Clemann in review). My thesis highlights several management options that could be structured as adaptive management experiments to achieve this goal:

1. **Trout mitigation:** Actions to mitigate trout are expected to increase *L. spenceri* recruitment and therefore increase the frog’s ability to compensate for chytrid induced mortality (Chapter 5). Trout management could increase *L. spenceri* population growth rates by between 26% and 47%, and could be a useful approach for all sites (Chapter 5). Trout mitigation has benefited other threatened frog populations (Vredenburg 2004; Knapp, Boiano & Vredenburg 2007) which can rapidly increase to a similar abundance as other populations that are trout free (Vredenburg 2004). However, the effectiveness of this option for *L. spenceri* is currently uncertain. Rotenone (a chemical piscicide) is likely to be the most effective method to eradicate trout (Jackson *et al.* 2004) but political sensitivities around the use of rotenone and eradication of trout have prevented an earlier attempt to control invasive fish (S. Smith, Dept. Environment Land Water and
The extent of fish suppression required to increase *L. spenceri* recruitment without rotenone using other control options (such as fishing) is unknown. Importantly, chytrid infection rates might increase if disease transmission increases with increased population density (Rachowicz & Briggs 2007; Bielby et al. 2015). Despite the risk of an increase in chytrid impacts, trout management could still increase *L. spenceri* growth rates at low elevation sites by around 24%, although in this scenario trout management may not be effective at mid and high elevation sites (Chapter 5). Trout management may also lead to increased colonization and occupancy of sites and further buffer *L. spenceri* against extinction processes.

Trout management is becoming a more feasible option. A trout barrier has already been constructed on one stream, below one of the mark-recapture sites monitored as part of this thesis. Some of the political sensitivities that previously obstructed introduced fish management may be softening and trials are currently underway in south-eastern Australia to enhance protections for native Australian fish (T. Raadic Arthur Rylah Institute (ARI) DELWP Victoria pers. comm.). The multi-state mark-recapture model developed in Chapter 4 and extensive knowledge of the species’ demography, including the current impacts of chytrid at the mark-recapture sites, now provides a clear opportunity for experimental trout management and an ability to measure outcomes including changes in chytrid impacts.

2. **Translocation**: Translocation may help to reduce *L. spenceri* extinction risk at sites by increasing recruitment through assisted colonisation. The potential increase in the growth rate of *L. spenceri* could be similar to the increase expected under trout management. My dynamic-occupancy model (Chapter 2) predicted that extinction risk was lowest under an assumption of constant colonisation, and the method could be extended to evaluate the level of translocation required to ensure that key sites remain occupied.
Translocations can also re-establish a species following its local extirpation. Reintroduction of captive-bred *L. spenceri* stock has been attempted at two separate high elevations sites. However, the success of both translocations has been low, as chytrid has reduced the survival of individuals following release (D. Hunter, New South Wales Office of Environment and Heritage pers. com.; M. West unpublished report). In retrospect, this outcome is not surprising as modelling in Chapter 5 indicated that the probability of persistence at high elevation sites will be low unless chytrid is managed. However, my models suggest that population persistence may be more likely at all elevations if chytrid can be managed. The recent translocations highlight that chytrid management must be considered. In an attempt to do this, a trial has recently been initiated to introduce *L. spenceri* to a stream at which it has not previously been recorded, but where trout and other sympatric frogs (that may be chytrid reservoir hosts) are absent (Scheele *et al.* 2014b).

Conceptual models developed in this thesis (Chapters 2 and 5) could help clarify how populations may respond to future translocation attempts under various scenarios. For example, translocation scenarios should consider the potential increased risk associated with chytrid as population density increases, and that the survival of released frogs may differ from wild frogs even if chytrid can be managed. Translocations will need to use captive-bred stock, as *L. spenceri* abundance at all remaining sites is considered low and chytrid-infected individuals might be transferred if sourced at other wild sites (unless a disease free wild site can be established). Captive *L. spenceri* colonies are currently maintained at two quarantine facilities. Individuals have been produced and released from one site (Amphibian Research Centre), but captive production at the other site (Healesville Sanctuary) has so far been low. These captive populations may be vital for future translocation efforts, or could be used to develop insurance colonies for populations in immediate risk of extinction. The population model developed in Chapter 5 could be extended by including stochasticity or continuous time to evaluate the benefits of varying translocation scenarios.
3. **Reducing the environmental suitability of sites for site pathogen:** Management options that reduce *L. spenceri* infection risk or increase survival or recovery when infected by chytrid could increase the species’ population growth rate by around 16-24% (Chapter 5). This may be achieved by manipulating environmental conditions that support pathogen persistence. Chytrid survival and reproduction can be reduced by increasing temperature, salinity and pH (Piotrowski, Annis & Longcore 2004; Stockwell, Clulow & Mahony 2015). Manipulation of water chemistry (to change pH or salinity) is proposed for lentic (still water) systems (Heard *et al.* 2014; Stockwell, Clulow & Mahony 2015), but may be impractical, or impossible to achieve, in lotic systems. Alternatively, canopy reduction has been proposed as an option to increase ambient or water temperatures and opportunities for basking (Rowley & Alford 2013; Heard *et al.* 2014). Canopy reduction might be a potentially feasible management option at a small scale for *L. spenceri*. Equally, sites that have recently been affected by bush fire could offer a potential natural experiment to investigate changes in chytrid impacts. In a similar respect, the changes in canopy cover following a cyclone altered microclimatic conditions and reduced infection risk for a frog population in north-eastern Australia (Roznik *et al.* 2015).

4. **Pathogen host reservoir control:** My results indicate that *L. lesueurii* may act as a pathogen host reservoir and that *L. spenceri* extinction risk could be reduced if *L. lesueurii* is absent at sites (Chapter 2) or by reducing the probability of infection of *L. spenceri* (Chapter 5). The exclusion of pathogen reservoir hosts has been hypothesised as a general management strategy to conserve susceptible species (Woodhams *et al.* 2011; McCallum 2012) and may be a crucial conservation measure for some species like the Southern Corroboree Frog (*Psuedophryne corroboree*) (Scheele *et al.* 2014b). Whilst pathogen reservoir hosts exclusion might be effective at a small scale for lentic breeding species (e.g. by excluding reservoir species from breeding sites), their exclusion might be more difficult in complex lotic systems where the movement of frogs is more difficult to restrict and where risk of re-infection from upstream sources is high. Alternatively, *L. spenceri* infection risk may be reduced via reservoir host
suppression, as opposed to complete exclusion, although the degree of suppression required to reduce infection risk is currently unknown.

The optimal management solution will likely involve a combination of the above discussed management strategies that increase recruitment and either reduce the infection rate or increase recovery and survival rates. In fact, efforts to manage both trout and chytrid could increase the growth rate of *L. spenceri* by between 56% and 69% (Chapter 5). Long-term solutions to manage chytrid are currently unclear (Scheele *et al.* 2014b) and all of the above discussed management options will require constant intervention (i.e to remove trout, suppress sympatric hosts, reduce canopy cover or maintain colonization through translocation). Given that the success of the potential management options remains uncertain, they would be most appropriately addressed in an adaptive management framework. Adaptive management strategies must be considered a medium – long term undertaking, as short duration projects have rarely succeeded (Westgate, Likens & Lindenmayer 2013).

A potential threat not currently addressed in this thesis is climate change. Climate change is predicted to be an important threat for amphibians, although many of the impacts are uncertain (Blaustein *et al.* 2010). Population modelling in Chapter 5 indicates that *L. spenceri* could be particularly sensitive if a changing climate impacts the species’ rate of maturation, production of eggs or survival of eggs, tadpoles or metamorph frogs. Conversely, climate changes could benefit frogs if the pathogen, rather than the host, is adversely affected (Blaustein *et al.* 2010). Environmental conditions can influence the population growth rate of *L. lesueurii* (Chapter 5) and expansion in *L. lesueurii* could also have implications for *L. spenceri* extinction risk as demonstrated in Chapter 2. Population modelling could be expanded to evaluate stochasticity or continuous time, and be integrated with biophysical models to evaluate future risk associated with climate change. This approach may also help to define refugia at which some of the current threats to *L. spenceri* (particularly chytrid and trout) may be reduced under future climate change.

The time is right to instigate adaptive management and to harness the current knowledge, existing monitoring and research momentum. A failure to do so could undermine recent efforts and limit the ability to distinguish the response of populations to experimental
intervention or result in further expense to re-establish knowledge of the system’s current state. Current and future predicted site occupancy estimates (Chapter 2) could now be used to help prioritize sites for management action. Monitoring data have been crucial but must now be integrated within a management plan and the reasons for future monitoring should be explicit (Lindenmayer, Piggott & Wintle 2013). I recommend that we should now define: 1) an optimal monitoring strategy to identify future population changes at sites, 2) triggers for intervention, and; 3) establish a clear plan for management intervention that can be implemented if triggers are reached (sensu Lindenmayer, Piggott & Wintle 2013). In addition to the management options discussed, other strategies such as establishing captive insurance populations may be required for emergency intervention at some sites. When undertaking experimental interventions, managers should be alert to the potential for other factors that could influence these species. Site prioritization, setting of goals, defining of triggers and planning for intervention should be undertaken collaboratively with other stakeholders including land managers and the Spotted Tree Frog Recovery Team.

6.3 Broader implications for addressing species declines

Understanding species population trajectories and causal factors in decline is crucial for most conservation efforts, to both prioritize action and ensure effective outcomes. This challenge extends beyond my case study, and amphibians. Globally, threatened species lists of other assessed taxon groups are expanding and few improvements in species conservation status have been achieved (Butchart et al. 2010; Hoffmann et al. 2010). Species recovery is most straightforward and successful when a clear threat affecting species can be managed in a discrete location (e.g. removal of predators from islands, prohibition of hunting, protection of nesting sites) (Hoffmann et al. 2010; Woinarski, Burbidge & Harrison 2015). But as highlighted in this thesis, most species are threatened by more complex interactions involving multiple factors (Brook, Sodhi & Bradshaw 2008). The challenge of how to conserve species in the face of multiple threats is recognized for taxa including freshwater crayfish (Richman et al. 2015), birds (Gurevitch & Padilla 2004; Ockendon et al. 2014), reptiles (Bohm et al. 2013), Australian mammals (McKenzie et al. 2007; Woinarski, Burbidge & Harrison 2015) and plants (Gurevitch & Padilla 2004; Enright et al. 2015).
Management intervention without understanding the interacting causes of decline can impede conservation gains or even accelerate declines (Chadès, Curtis & Martin 2012). For example, an introduced Domestic Cat (*Felis catus*) eradication program on a New Zealand island caused increased predation upon Cook’s Petrel (*Pterodroma cookii*) eggs and chicks by an invasive mesopredator, the Pacific Rat (*Rattus exulans*) (Rayner *et al.* 2007). The rat accelerated the petrel’s decline and prevented recovery until it was also removed (Rayner *et al.* 2007). In Australia, the Woylie (*Bettongia penicillata*) initially recovered and its threat status was down-listed following the control of an introduced predator (Red Fox, *Vulpes vulpes*), only to subsequently decline and the species threat status to be upgraded (Groom 2010). The Woylie’s second decline could have been driven by multiple other threats (Wayne *et al.* 2013). In particular, introduced cat (*F. catus*) predation on Woylies increased dramatically following fox (*V. vulpes*) management, highlighting the importance of simultaneously managing multiple threats to stimulate recovery (Marlow *et al.* 2015). The consequence of inaction in the face of decline is clear (Lindenmayer, Piggott & Wintle 2013), but the consequences of action without understanding can be unexpected (Ruscoe *et al.* 2011) and costly (Bergstrom *et al.* 2009). Expanding threatened species lists, continued declines, protracted recovery times and wasted resources are symptomatic of a failure to understand and adequately address the complex problems facing threatened species (Chadès, Curtis & Martin 2012). Understanding the potential interactive impacts of threats upon species is therefore crucial to prioritize management actions (Rayner *et al.* 2007; Evans, Possingham & Wilson 2011; Bode, Baker & Plein 2015).

Chytrid clearly is a problem for amphibians. However, as demonstrated in my thesis, other threatening processes and species interactions can have important implications for mitigating pathogens impacts (e.g. Shoemaker *et al.* 2014). There are currently no long term solutions to eradicate chytrid, particularly in complex systems or at a broad scale (Scheele *et al.* 2014b). However, studies such as mine suggest several levers that could be pulled to mitigate chytrid impacts at a smaller scale (e.g. Heard *et al.* 2014; Scheele *et al.* 2014b; Scheele *et al.* 2015). Diseases are increasingly recognized to be important threats to wildlife populations (e.g. Fisher *et al.* 2012). Whilst some diseases are capable of independently driving species extinction through frequency-dependent transmission (McCallum *et al.* 2009; McCallum 2012), they rarely cause extinction in isolation (Smith, Sax & Lafferty 2006; Fisher *et al.* 2012; McCallum 2012). As for chytridiomycosis, other wildlife disease
impacts are most severe when reservoir host species and other interacting threats are present (Smith, Sax & Lafferty 2006; McCallum 2012). In some cases, other threats may even influence population trajectories more than disease (Gardner, Barlow & Peres 2007; Heard, Smith & Ripp 2011). An approach that systematically assesses population declines and the underlying causes similar to that described in my thesis may be crucial when managing other wildlife diseases (e.g. white-nosed syndrome for bats (Blehert et al. 2009)), particularly when multiple factors could be involved (Blaustein et al. 2011).

In this thesis, I have strategically evaluated and quantified a species’ decline and some causal factors by direct field assessments and contemporary analytical methods (particularly occupancy, mark-recapture and population modelling). There are trade-offs that need to be considered between the amount of time and cost associated with assessing species versus the imperative to take action in the face of uncertainty when time and resources for action are limited (Runge, Converse & Lyons 2011; Canessa et al. 2015). However, the steps in this thesis, and similar approaches, are crucial to ensure that conservation intervention is necessary, appropriate and ultimately effective (Dreitz 2006; Muths & Dreitz 2008; Chadès, Curtis & Martin 2012; Lindenmayer, Piggott & Wintle 2013; Wayne et al. 2013).

6.4 Conclusion

Throughout this thesis I have responded to three major challenges in conservation ecology - the need for: 1) a more rigorous assessment of amphibian decline and the underlying causes (Pechmann et al. 1991; Alford & Richards 1999); 2) disentangling interacting threats when multiple causes are suspected (Blaustein & Kiesecker 2002), and; 3) a greater understanding of varied responses of amphibians to chytrid (Wells 2010). I used field assessments, occupancy, mark-recapture and population modelling to compare the population responses of two ecologically similar sympatric species in south-eastern Australia to chytrid and other threatening processes.

I provide clear evidence that the Critically Endangered Spotted Tree Frog *Litoria spenceri* has declined in occupancy at historic sites by approximately 50%. Meanwhile, site occupancy of the non-threatened Lesueur’s Frog *L. lesueurii* increased. Variation in recruitment between and within species, and site-specific threats drove different population-
level outcomes, despite severe individual-level pathogen impacts in both species. Chytrid is a key threat for all extant *L. spenceri* populations (Chapter 2, 4 and 5), but the pathogen’s impacts are exacerbated by site-specific environmental limits to recruitment (Chapter 5), the presence of *L. lesueurii* which may be a pathogen reservoir host (Chapter 2), and predation of tadpoles by introduced trout (Chapter 5).

I advocate that adaptive management strategies should now be initiated to prevent *L. spenceri* extinction. Experimental interventions, like trout eradication, could reduce chytrid impacts by increasing *L. spenceri* recruitment. However, increasing population density might exacerbate chytrid infection. Chytrid impacts should be monitored and concurrent action may be required to increase survival and recovery of infected frogs or reduce infection rates (potentially via habitat manipulation or reservoir host management).

The impact of threats can be difficult to predict when multiple processes are involved and can be severe for both threatened and non-threatened species. Understanding the causes and magnitude of a species decline is crucial to ensure that conservation intervention is necessary, appropriate and ultimately effective.
Appendix 1 Other occupancy model results

Figure A1.1 Estimated historic (1958-2012) and predicted future (2013-2057) change in proportion of sites occupied by *Litoria spenceri* and *L. lesueurii* assuming that species extinction was influenced by the presence of chytrid. Three different models of *L. spenceri* colonization assumptions were compared: by A) no colonization; B) colonization is linked to mean probability of occupancy of sites within a major stream catchment that are spatially-linked; C) constant probability of colonization. Colonization by *L. lesueurii* was always assumed to be constant. Black lines and green shading indicate mean and 95% CI estimated occupancy changes for *L. spenceri*. Blue lines and shading indicate mean and 95% CI estimated occupancy changes for *L. lesueurii*.
Figure A1.2 Estimated historic (1958-2012) and predicted future (2013-2057) change in proportion of sites occupied by *Litoria spenceri* and *L. lesueurii* assuming that species extinction was influenced by the presence of the other (sympatric) frog species. “Symp”: sympatric frog. Three different models of *L. spenceri* colonization assumptions were compared: by A) no colonization; B) colonization is linked to mean probability of occupancy of sites within a major stream catchment that are spatially-linked; C) constant probability of colonization. Colonization by *L. lesueurii* was always assumed to be constant. The presence of chytrid was ignored. This model is equivalent to an assumption that chytrid was always present at sites. Black lines and green shading indicate mean and 95% CI estimated occupancy changes for *L. spenceri*. Blue lines and shading indicate mean and 95% CI estimated occupancy changes for *L. lesueurii*. 
A1.1 Evaluating the influence of ambient temperature:

Dynamic occupancy models were constructed to evaluate the impact of ambient temperature and the presence of absence of chytrid at sites. To do this the probability of annual extinction \(E(s)_{i,t}\) of each frog species \((s)\) was drawn from a uninformative uniform prior distribution:

\[
E(s)_{i,t} = \hat{E}(s)_{i,t} \times [B_{i,t-4}, \text{temp}_i]
\]

(Alternative to Eqn. 2.4)

Extinction \((\hat{E}(s)_{i,t})\) was indexed by the presence chytrid \((B_{i,t-4})\), assuming a 4 year time lag, and the sites ambient temperature category \((\text{temp}_i; \text{values 1:4})\). The proportion of days that maximum temperature reached or exceeded \(30^\circ\text{C}\) between October and April was used to classify sites into four ambient temperature categories: cool sites (proportion of days \(\geq 30^\circ\text{C}\): 0.02 - 0.08), mild sites (proportion of days \(\geq 30^\circ\text{C}\): 0.08 - 0.14), warm sites (proportion of days \(\geq 30^\circ\text{C}\): 0.14 - 0.22) or hot sites (proportion of days \(\geq 30^\circ\text{C}\): 0.22 - 0.28).

**Ambient temperature data:** Interpolated maximum daily temperature was obtained from the Australian Water Availability Project Climate database (resolution 0.05° x 0.05°) (Raupach et al. 2009) for each year from 1958 to 2012 for each of the 49 known historic sites. The data was clipped to only include information when the frogs were known to be active from October to April of each survey year. The maximum daily temperature at each site in each season was corrected by comparing the difference in elevation at each of the sites to the average elevation within each 0.05° x 0.05° grid cells at which the sites occurred and multiplying the difference in elevation by a adiabatic lapse rate of 0.00645°C m⁻¹ change in elevation (Kearney et al. 2014). The data was then summarized to determine the proportion of days at each site and during each survey season where maximum daily temperature was at or above \(30^\circ\text{C}\). This cut off temperature was selected as chytrid mortality occurs at temperatures above \(30^\circ\text{C}\) (Piotrowski, Annis & Longcore 2004). The mean proportion of days at or above \(30^\circ\text{C}\) was estimated for the period between 1996 and 2012 during which chytrid was known to occur at sites.
Figure A1.3 Comparing *Litoria spenceri* and *L. lesueurii* extinction probabilities relative to the ambient temperature at sites and the presence (+) or absence (-) of “Bd”: chytrid fungus (*Batrachochytrium dendrobatidis*). Sites were classified into four ambient temperature categories depending upon the proportion of days that maximum temperature reached or exceeded 30°C between October and April: cool sites (0.02 - 0.08 days above 30°C), mild sites (0.08 - 0.14 days above 30°C), warm sites (0.14 - 0.22 above 30°C) or hot sites (0.22 - 0.28 above 30°C). Points and lines represent the mean and 95% credible interval estimates. Results are shown for models that assume: no *L. spenceri* colonization (black solid lines); spatially-linked *L. spenceri* colonization (grey dashed lines); constant colonization (grey solid lines).
Litoria spenceri extinction probability tended to be highest at sites classified as cool or warm compared to mild or hot (Figure A1.3). However the differences between sites relative to ambient temperature were uncertain. The analysis supported other models that indicated an increase in L. spenceri extinction probability when chytrid was present compared to when it was absent, particularly at cool – warm sites. This response was less clear at hot sites. Interactions between chytrid and the ambient temperature at sites may influence L. spenceri but additional factors may be involved. The impact of chytrid and ambient temperature upon L. lesueurii was uncertain.
Figure A1.4 Estimated historic (1958-2012) and predicted future (2013-2057) change in proportion of sites occupied by *Litoria spenceri* and *L. lesueurii* assuming that species extinction was influenced by the presence of chytrid (*Bd*: *Batrachochytrium dendrobatidis*) and an interaction with the proportion of days when ambient temperature (Temp) was at or above 30°C. Three different models of *L. spenceri* colonization assumptions were compared: by A) no colonization; B) colonization is linked to mean probability of occupancy of sites within a major stream catchment that are spatially-linked; C) constant probability of colonization. Colonization by *L. lesueurii* was always assumed to be constant. Black lines and green shading indicate mean and 95% CI estimated occupancy changes for *L. spenceri*. Blue lines and shading indicate mean and 95% CI estimated occupancy changes for *L. lesueurii*.
Appendix 2 Results of the preliminary assessment to compare the diagnostic sensitivity and specificity of four PCR methods

A2.1 Preliminary Experiment A

Preliminary assessments of the diagnostic sensitivity and specificity of four different PCR methods found that all methods had a high sensitivity (means of between 0.752 - 0.917) when the concentration of zoospores in the sample was high (~400 or 4000 zoospores/sample), regardless of how equivocal samples were defined (Figure A2.1). When equivocal samples were defined as negative, the estimated diagnostic sensitivity of all PCR methods tended to be low for samples containing a low number of zoospores (~4 or 10 zoospores/sample). The diagnostic sensitivity was clearly lowest for the two Taqman qPCR methods and the Nested PCR method when samples contained 4 zoospores compared to higher concentrations of zoospores (~400 – 4000/sample) (Figures A2.1 b –d and j - l). Both the Taqman qPCR using a 1:15 extraction dilution and the Nested PCR failed to identify any of the 10 samples at the lowest concentration (~4 zoospore/sample) as positive for all threes replicates. In comparison the Taqman PCR at the 1:10 dilution identified two samples and the Qiagen qPCR identified five samples at the lowest concentration as positive for all three replicates. The diagnostic sensitivity of the Nested PCR method (95% CI: 0.296-0.691) also appeared to be less than the sensitivity of the other methodologies (95% CI minimum: 0.601) for samples containing ~10 zoospores (Figure A2.1 d Vs a-c).

When equivocal samples are defined as positive all PCR methods correctly identified all samples as positive when they contained between 10 – 4000 zoospores/ sample (Figures A2.1 a - d Vs e - h). At the lowest concentration (~4 zoospores/sample), however, the number of positive samples (n = 10) for the Qiagen, Taqman 1:15, Taqman 1:10 and Nested PCR methods increased from 5, 0, 2 and 0 samples, when equivocal samples were negative, to 10, 6, 8 and 2 samples respectively when equivocal results were defined as positive. The diagnostic sensitivity of the Qiagen qPCR method was then equally high (95% CI: 0.713 –
0.998; mean 0.917) for all concentrations of zoospores evaluated (Figure A2.1 e) when equivocal results were defined as positive. The diagnostic sensitivity for the lowest zoospore samples of the other three PCR methods were generally less than for higher concentrations of zoospores (Figures A2.1 f - h).

The assessment indicated that the diagnostic specificity of all PCR methods was similarly high when equivocal samples were defined as negative (Figure A2.2). The Qiagen, Taqman 1:10 and Nested PCR were determined to have a mean diagnostic specificity of 0.916 (95% CI 0.720 -0.998) and the Taqman 1:15 method falsely identified one true negative sample as positive which resulted in an estimated mean diagnostic specificity of 0.829 (95% CI 0.569 - 0.976). When equivocal samples were defined as positive there was a slight reduction in the diagnostic specificity (95% CI 0.569 - 0.976, mean 0.829) of the Qiagen qPCR as one true negative sample test result was equivocal (one of the three replicates was positive).

### A2.2 Pilot Experiment B

The addition of contaminants to samples did not generally affect the estimated diagnostic sensitivity of the PCR methods when compared to samples that did not contain contaminants (Figures A2.11 a-h Vs Figures A2.1 i – p). The only exception was an increase in the diagnostic sensitivity of the Qiagen qPCR method when equivocal results were classified as negative and samples contained both a low number of spiked zoospores (~4) and contaminants (95% CI: 0.720 - 0.998; mean: 0.916) compared to samples without the contamination (95% CI: 0.243 - 0.756; mean: 0.499) (Figure A2.1 a Vs i). The difference in diagnostic sensitivity was likely to be because the wild frog contaminant mix contained some zoospores. However this did not significantly alter the diagnostic sensitivity of the other PCR methods despite all evaluating the same extracted samples. There was no clear evidence that the contaminants caused inhibition.
Test 1
Qiagen PCR

Test 2
Taqman PCR (1:15)

Test 3
Taqman PCR (1:10)

Test 4
Nested PCR

a.  
b.  
c.  
d.  
e.  
f.  
g.  
h.  
i.  
j.  
k.  
l.  
m.  
n.  
o.  
p.  

Sensitivity
Figure A2.1 A comparison of the estimated diagnostic sensitivity of four PCR tests used to detect varying concentrations of chytrid zoospores that had been added to either sterile (Figure 2 a – h) or deliberately contaminated (Figures 2 i – p) samples. PCR methods were a Qiagen qPCR; Taqman 1:10 qPCR, Taqman 1:15 qPCR; and a Nested PCR. To estimate diagnostic sensitivity four groups of ten swab samples were either spiked with approximately 4, 10, 400 or 4000 zoospores (shown above each line on each plot). Points represent the estimated median sensitivity and lines represent the 95% credible interval as determined using a Bayesian binomial model. Figures with red lines show the estimated diagnostic sensitivity when equivocal results were defined as negative, Figures with blue lines show the estimated diagnostic sensitivity when equivocal results were defined as positive.
Figure A2.2 A comparison of the estimated diagnostic specificity of four PCR tests used to detect chytrid when equivocal samples are either interpreted as negative (red lines; Figures 3 a - d) or positive (blue lines; Figures 3 e - h). PCR methods were a Qiagen qPCR, Taqman 1:10 qPCR, Taqman 1:15 qPCR, and a Nested PCR. Figures (a - d) with red lines show the estimated diagnostic specificity when equivocal results were defined as negative, Figures (e - h) with blue lines show the estimated diagnostic specificity when equivocal results were defined as positive.
Appendix 3 Life graphs for each species at mid and high elevation sites.

*L. spenceri* at mid elevation sites

*L. spenceri* at high elevation sites
*L. lesueurii* at mid elevation sites and corresponding matrix model

*L. lesueurii* at high elevation sites
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Author/s:
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Title:
Contrasting population responses of ecologically-similar sympatric species to multiple threatening processes

Date:
2015

Persistent Link:
http://hdl.handle.net/11343/115191

File Description:
Contrasting population responses of ecologically-similar sympatric species to multiple threatening processes