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# **Emerging amphibian diseases in Queensland and host immune response to disease**

Thesis submitted by

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BVSc (Hons) Uni Syd, BSc (Vet) (Hons) Uni Syd, MVS (Zoo & Wildlife Medicine) Uni Melb

in August 2012

for the degree of Doctor of Philosophy

in the School of Public Health, Tropical Medicine and Rehabilitation Sciences

James Cook University

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### DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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## DECLARATION ON ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Statement on Ethical Conduct in Research Involving Humans (1999)*, the *Joint National Health and Medical Research Council/Australian Vice Chancellors' Committee Statement and Guidelines on Research Practice (1997)*, the *James Cook University Policy on Experimentation Ethics Standard Practices and Guidelines (2001)*, the *James Cook University Statement and Guidelines on Research Practice (2001)* and the *Australian Code for the Responsible Conduct of Research (2007)*.

The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee:

Approval Number:     A1085

Signature:

Date:                     14 August 2012

## STATEMENT ON THE CONTRIBUTION OF OTHERS

My research was funded primarily by the Australian Government Department of the Environment, Water, Heritage and the Arts. I was also awarded a James Cook University Postgraduate Research Scholarship. Some of this research required multidisciplinary skills and involved collaboration and consultation with colleagues at James Cook University and other institutions within Australia and overseas. My supervisors Rick Speare, Lee Berger and Lee Skerratt gave advice throughout on research methodology, interpretation of results and manuscript and thesis preparation. The specific scientific and intellectual contributions of others to each chapter are summarised below.

### CHAPTER 3

**Publication:** Young, S., Speare, R., Berger, L., Skerratt, L. F. & Mendez, D. (2010). Emerging Amphibian Diseases and Disease Surveillance in Queensland – Stage 2 (February 2007 – April 2010). Final report for the project Emerging Amphibian Diseases and Disease Surveillance in Queensland – Stage 2 (February 2007 – April 2010) to the Australian Government Department of the Environment, Water, Heritage and the Arts.

This published funding body report was my original writing with minor editorial contributions from Rick Speare, Lee Berger, Lee Skerratt and Diana Mendez. I performed the majority of the surveillance work and specimen collection, and all of the clinical examinations, physical data collection, post mortem examinations and diagnostic sample collection. Rick Speare, Lee Berger and Lee Skerratt gave advice on study design and interpretation of results throughout the research. Rick Speare, Lee Berger and Diana Mendez assisted with histological analysis. Harry Hines assisted with field collection of specimens. Rebecca Webb processed the tissue samples for histology and Stephen Garland ran the chytrid PCR analyses. Ron Slocombe performed histological analysis and provided images for cases 054 and 085. Liliana Tatarczuch performed transmission electron microscopy and provided images for these two cases, and Ron Slocombe assisted with electron microscopic image interpretation. Microsporidial PCR analysis was performed by Louis Weiss (case 054), poxvirus PCR analysis was performed by Jianning Wang (case 085), and papillomavirus PCR was performed by Annika Antonnson (case 085). Louis Weiss and Ann Cali assisted with microsporidial identification.

**Publication:** Young, S., Skerratt, L. F., Mendez, D., Speare, R., Berger, L. & Steele, M. (2012a). Using community surveillance data to differentiate between emerging and endemic amphibian diseases. *Diseases of Aquatic Organisms* 98: 1-10.

This published peer-reviewed paper represents my original data organisation, classification, analysis and writing. Rick Speare, Lee Skerratt and Lee Berger assisted with data interpretation. Diana Mendez performed the histological analysis. Mike Steele performed the original statistical analysis and I performed additional revised analyses with advice from Lee Skerratt. Lee Berger, Lee Skerratt, Diana Mendez and Rick Speare provided substantial editorial input, Diana Mendez wrote the histology results section, and Mike Steele wrote the majority of the statistical methods section.

**Publication:** Phillot, A. D. & Young, S. (2009). Occurrence of cloacal prolapse in wild hylids in the Wet Tropics, Australia. *Diseases of Aquatic Organisms* 86: 77-80.

This published peer-reviewed paper represents original research led by Andrea Phillott, the primary investigator. My role in the paper included provision of veterinary technical expertise for results interpretation and substantial written contributions to the introduction and discussion sections.

#### **CHAPTER 4**

**Publication:** Young, S., Warner, J., Speare, R., Berger, L., Skerratt, L. F. & Muller, R. Hematologic and plasma biochemical reference intervals for health monitoring of wild Australian tree frogs. *Veterinary Clinical Pathology* (In press).

This paper represents my original research and writing and has been accepted for publication in the peer-reviewed journal *Veterinary Clinical Pathology* (scheduled for print in December 2012). I received study design advice from Rick Speare, Lee Berger and Lee Skerratt, technical methodology advice and cell identification assistance from Jeff Warner, and editorial contributions from Jeff Warner, Lee Berger and Lee Skerratt. Reinhold Muller performed all of the statistical analyses. Helen Martin and Deb Buckett at Gribbles Veterinary Pathology Laboratory performed the serum protein electrophoresis.

#### **CHAPTER 5**

**Publication:** Young, S., Whitehorn, P., Berger, L., Skerratt, L. F., Speare, R., Garland, S. & Webb, R. *Batrachochytrium dendrobatidis* suppresses immune function in tree frogs. *Diseases of Aquatic Organisms* (submitted).

This manuscript represents my original research and writing and has been submitted for publication to the journal *Diseases of Aquatic Organisms*. I received study design advice from

Rick Speare, Lee Berger and Lee Skerratt. Paul Whitehorn assisted with field collection of frogs, along with all husbandry and some experimental procedures. Stephen Garland ran the PCR analyses, Rebecca Webb processed the tissue samples for histology and Ammar Aziz assisted with microtitre assays. Helen Martin and Deb Buckett at Gribbles Veterinary Pathology Laboratory performed the serum protein electrophoresis. I performed all of the statistical analyses with advice from Lee Skerratt. Lee Berger and Lee Skerratt gave substantial editorial input to the final written paper.

## **CHAPTER 6**

**Publication:** Young, S., Whitehorn, P., Garland, S., Speare, R., Skerratt, L. F., Berger, L. & Voyles, J. Chytridiomycosis makes frogs more susceptible to reinfection. *Research in Veterinary Science* (submitted).

This manuscript represents my original research and writing and has been submitted for publication to the peer-reviewed journal *Research in Veterinary Science*. I received study design advice from Rick Speare, Lee Berger and Lee Skerratt. Paul Whitehorn assisted with all husbandry and the majority of experimental procedures. Paul Whitehorn and Jamie Voyles assisted with field collection of frogs. Stephen Garland ran the PCR analyses. I performed all of the statistical analyses with advice from Lee Skerratt. Rick Speare, Jamie Voyles, Lee Skerratt and Lee Berger assisted with interpretation of results. Lee Berger and Lee Skerratt gave substantial editorial input to the final written paper.

## **CHAPTER 7**

**Publication:** Voyles, J., Berger, L., Young, S., Speare, R., Webb, R., Warner, J., Rudd, D., Campbell, R. & Skerratt, L. F. (2007). Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis. *Diseases of Aquatic Organisms* 77: 113-118.

This published peer-reviewed paper represents original research led by Jamie Voyles and Lee Berger, the primary investigators. My role in the paper included provision of veterinary technical expertise for methodology and results interpretation, and substantial intellectual and editorial contributions to the methods, results and discussion sections.

**Publication:** Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W. F., Dinudom, A., Cook, D., Webb, R., Alford, R. A., Skerratt, L. F. & Speare, R. (2009). Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science* 326: 582-585.



This published peer-reviewed paper represents original research led by Jamie Voyles, the primary investigator. My role in the paper included: surgical implantation of the cardiac bio-transmitters in experimental frogs, blood biochemistry and urine sediment sample processing, provision of veterinary technical expertise for methodology and results interpretation, and substantial intellectual and editorial contributions to the methods, results and discussion sections.

## **CHAPTER 8**

**Publication:** Young, S., Berger, L. & Speare, R. (2007). Amphibian chytridiomycosis: strategies for captive management and conservation. *International Zoo Yearbook* 41: 85-95.

I drafted the original management and conservation review for this published peer-reviewed paper, and subsequently received significant editorial contributions from Lee Berger and Rick Speare, along with specific management advice from Rob Puschendorf.

**Publication:** Young, S., Speare, R., Berger, L. & Skerratt, L. F. (2012b). Chloramphenicol with fluid and electrolyte therapy cures terminally ill green tree frogs (*Litoria caerulea*) with chytridiomycosis. *Journal of Zoo and Wildlife Medicine* 43: 330-337.

This published peer-reviewed paper represents my original research and writing, with substantial study design advice from Rick Speare and editorial contributions from Lee Berger and Lee Skerratt.

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A huge thank you to Jamie Voyles who helped me collect frogs, shared ideas late into the night and gave me wonderful support and friendship throughout. A big thank you also to Rob Puschendorf for his frog collecting, support and friendship, and to Diana Mendez for helping me jump over some big hurdles at the start and for her continued support.

Thanks to Rebecca Webb for preparing a huge number of histology slides and to Stephen Garland for analysing the hundreds of swabs I collected. Their contributions were crucial to my work and I imagine they are very relieved that I have finally completed this.

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I gratefully acknowledge James Cook University and the Australian Government Department of the Environment, Water, Heritage and the Arts for providing financial support.

Finally, Paul Whitehorn, thank you for everything. I could not have done this or so very many other things without you.

## ABSTRACT

Emerging infectious diseases are a significant issue for amphibian conservation and the global spread of chytridiomycosis has decimated many frog populations. As well as developing effective disease management strategies, it is crucial to improve surveillance and health monitoring for the early detection and prevention of other emerging diseases. The aims of this research were to investigate emerging and endemic amphibian diseases in Queensland, to evaluate amphibian disease surveillance techniques, and to study the effect of chytridiomycosis on the host immune system.

Significant diseases, including two new anuran pathogens, were discovered through the passive disease surveillance network I established at James Cook University in Cairns. Over 160 sick or dead amphibian specimens were received from various sources. Papovavirus particles were identified with electron microscopy in a dermal squamous papilloma from a white-lipped tree frog (*Litoria infrafrenata*). Subsequent PCR testing to further classify the virus was unsuccessful. This is the first known report describing a viral aetiology for dermal papillomas in anurans. Systemic microsporidiosis was diagnosed histologically in a common green tree frog (*L. caerulea*), and electron microscopic analysis identified the organism as belonging to the genus *Anncaliia*. This is the first known report of *Anncaliia* species infection, and only the third published report of a pathogenic microsporidial infection, in amphibians.

A new endemic disease in *L. infrafrenata*, manifesting as irreversible emaciation, was detected by retrospective analysis of syndromic submission data from a community frog group. I identified the cestode *Spirometra erinacei* as a likely primary aetiologic agent of this fatal wasting syndrome. Over a six-year period, 877 *L. infrafrenata* submitted to the community group were classified according to origin, season and presenting category. Irreversible emaciation accounted for 9 % of submissions, but the lack of significant spatial or temporal patterns in case presentation suggests that this is not a currently emerging disease. I also found a high overall prevalence (27 %) of *S. erinacei* infection in *L. infrafrenata* populations in northern Queensland. This is the first known report of *S. erinacei* infection in this species. While community wildlife groups can play a valuable role in urban disease monitoring, passive syndromic surveillance has a number of limitations. All wildlife disease investigations must involve professionals to establish syndromic case definitions, and for diagnostic pathology, complementary active disease surveillance, and data analysis and interpretation.

To improve diagnostic capabilities for amphibian disease investigations, I established blood

reference intervals for two species of Australian tree frogs, described their leucocyte morphology, and analysed the effects of season, year and parasite status on blood values. This is currently the most comprehensive study that defines haematologic and biochemical reference intervals for amphibians. Blood was collected from reference sample populations of wild adult tree frogs (*L. caerulea*,  $n = 80$ , and *L. infrafrenata*,  $n = 66$ ) for manual haematologic, automated plasma biochemical, and serum protein electrophoretic analysis. Intra-erythrocytic haemogregarine infections were found in 19 % of *L. infrafrenata*, and multiple haematologic and biochemical parameters varied in infected frogs. Wide inter-species and seasonal variations highlight the need to establish species- and season-specific reference intervals in amphibians.

I also investigated host immune response to chytridiomycosis. Global spread of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) has caused mass mortality leading to population declines and extinctions in many frog species. Wide variation in susceptibility to chytridiomycosis exists between species, populations and individuals, but the mechanisms of host immunity appear complex and much remains unknown.

My first experimental infection trial is the first of its kind to show that *B. dendrobatidis* reduces systemic adaptive immune function in frogs. I used a range of haematologic and protein electrophoresis biomarkers, along with various functional tests, to assess immune competence in *L. caerulea* experimentally infected with *B. dendrobatidis*, and in experimentally exposed *L. infrafrenata*. Stimulation of infected *L. caerulea* resulted in a minimal immune system response. Compared with uninfected frogs, *B. dendrobatidis* infection reduced splenic, white blood cell, acute-phase protein and immunoglobulin responses, indicating a significantly impaired ability of infected frogs to respond adequately to antigenic stimulation. Although *L. infrafrenata* failed to maintain infection after exposure, sub-clinical immunologic effects occurred in recovered compared with unexposed frogs. This host immune suppression is likely a key factor enabling chytridiomycosis to be a formidable disease with unprecedented effects on biodiversity.

In the second experimental infection trial, I found that prior infection with *B. dendrobatidis* had no significant effect on infection rate compared with naïve frogs, and also appeared to have a long-term adverse effect on host resistance. *Litoria caerulea* were experimentally infected with *B. dendrobatidis*, treated to clear infection, and then re-exposed to the pathogen. A greater proportion of exposed frogs (78%) became infected compared with naïve frogs (28%). Furthermore, infected re-exposed frogs had a higher infection intensity that increased at a greater rate compared with infected naïve frogs. A greater proportion of infected naïve frogs (59%) self-cured compared with the infected re-exposed group (0%), indicating a greater ability

of naïve frogs to control and clear infection. These results indicate that vaccination-based control programmes for chytridiomycosis are unlikely to be successful.

Collaborative research during my study identified fatal terminal pathophysiological changes in experimentally infected *L. caerulea*. While the virulence of *B. dendrobatidis* has been clearly demonstrated, the mechanism by which the pathogen kills its host has not been determined. We identified epidermal degeneration, inhibited epidermal electrolyte transport, systemic electrolyte disturbances and asystolic cardiac arrest as the pathogenic mechanisms of mortality in infected frogs. Amphibian skin exchanges respiratory gases, water and electrolytes to maintain homeostasis; this disruption to cutaneous function may be a key factor enabling the pathogen to be lethal to such a large range of host species, including phylogenetically distant amphibian taxa.

I validated effective antifungal treatments and discovered a clinical protocol for curing terminally ill amphibians during *B. dendrobatidis* infection trials. There are few reports of successful treatment of chytridiomycosis, and none that include curing amphibians with severe disease. Three terminally ill *L. caerulea* with heavy *B. dendrobatidis* infections were cured using a combination of continuous shallow immersion in chloramphenicol solution, parenteral isotonic electrolyte fluid therapy, and increased ambient temperature. All terminally ill frogs recovered rapidly within five days of commencing treatment. In contrast, five untreated terminally ill *L. caerulea* with heavy *B. dendrobatidis* infections died within 24 to 48 hours of becoming moribund. Sub-clinical infections in 15 experimentally infected *L. caerulea* were cured within 28 days by continuous shallow immersion in chloramphenicol solution without adverse effects. This is the first known report of a clinical treatment protocol for curing terminally ill *B. dendrobatidis*-infected frogs.

I identified key roles of zoological institutions to limit the impact of chytridiomycosis on wild amphibian populations during my review of captive disease management. Prevalence in the international amphibian trade is high and importation of infected frogs into zoos has caused disease epidemics in established amphibian collections. Control strategies for zoos to reduce the risk of pathogen spread must include strict quarantine, hygiene, disinfection and translocation protocols, increased public education, routine surveillance of captive and wild populations for rapid diagnosis of and response to outbreaks, and timely, well-planned captive-breeding and reintroduction programmes with extensive institutional collaboration worldwide.

Outcomes from this research fill critical knowledge gaps about systemic adaptive immune function and host resistance in frogs with chytridiomycosis, and about successful clinical

treatment of infected frogs. Validation of diagnostic tests and establishment of blood reference intervals, along with assessment of disease surveillance data and techniques, will contribute significantly to the future ability of researchers to detect and investigate emerging and endemic amphibian diseases. These are key results that contribute to global amphibian conservation efforts aimed firstly at managing chytridiomycosis, and secondly at improving disease surveillance and diagnosis.

Future investigations are needed to better characterise the irreversible emaciation syndrome in *L. infrafrenata*, the role of *S. erinacei* infection in this disease, and the clinical significance of haemogregarines in Australian *L. infrafrenata* populations. Further work to understand mechanisms of *B. dendrobatidis* immune suppression should aim to identify and isolate pathogen-specific immunosuppressive factors. Researchers need to confirm whether susceptible amphibians can mount protective adaptive immune responses against *B. dendrobatidis* and if not, the focus of future research needs to shift beyond this to alternative methods of host immune modulation. Broader research to determine pathophysiology for chytridiomycosis among amphibian species should incorporate field studies with naturally infected susceptible host species, tolerant reservoir species and naturally resistant species. In the short-term, global resources must be dedicated to captive institutions for threatened species breeding programmes and for emergency response to species and population declines. While research is underway to improve *in situ* management of chytridiomycosis in wild amphibian populations, habitat protection and restoration also remain priorities for amphibian conservation.

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# CHAPTER 1

## Scope of the thesis

### 1.1 Overview of the research

All of the procedures reported in this thesis received the prior approval of the James Cook University Animal Ethics Committee (approval number A1085) and the Queensland Parks and Wildlife Service (Scientific Purposes Permit number WISP03866106).

The initial broad objectives of my research were: 1) to investigate emerging and endemic amphibian diseases in the Wet Tropics of Queensland; 2) to evaluate amphibian disease surveillance techniques with integration of community surveillance data into the Australian Wildlife Health Network; and 3) to study the effect of disease on the amphibian immune response.

This research was driven largely by the intense shift in focus at the start of this century towards emerging infectious diseases in humans and the role of wildlife species in recent human disease pandemics and epidemics. At the same time, emerging infectious diseases in amphibians received significant attention due to discovery of the formidable disease chytridiomycosis. The impact of this disease on frog populations since its emergence was discovered in 1998 has been devastating.

The momentum gathered by researchers to investigate emerging infectious diseases was accompanied by heightened awareness of the need to dramatically improve national and international biosecurity measures in an effort to control and prevent the spread of these diseases. Australian biosecurity received particular scrutiny due to the wide stretches of largely uninhabited, accessible and unpatrolled coastline in the northern parts of the country. Northern Queensland was, and still is, considered to be of particularly high risk for incursion of emerging infectious diseases into Australia due to its close proximity to neighbouring Asian-Pacific islands, large isolated coastal fringes, the relatively large but isolated port city of Cairns, the Cairns international airport, and favourable climatic conditions.

Early emerging amphibian disease investigations indicated that significant species-specific differences in host response to pathogens existed and that validation of diagnostic tests in amphibians was in its infancy. Priorities for future research were identified to include the need

to validate a range of tests to improve diagnostic capabilities in amphibian disease investigations, and to greatly broaden the taxonomic focus of these investigations in an attempt to better understand aspects of aetiology, pathogenesis and epidemiology.

The Cairns Frog Hospital (CFH) is a small, non-profit community wildlife group that has been receiving injured and diseased amphibians from the public since 1998. Increased media exposure about the plight of amphibians that coincided with the emergence of chytridiomycosis prompted lobbying efforts by the CFH to gain financial support. Many attempts were made to secure government and non-government organisation funding to investigate what the CFH claimed to be novel emerging viral, fungal (but not chytridiomycosis) and bacterial diseases in submitted frogs, and media interest created some political pressure to respond. However, the lack of supporting diagnostic pathology and scientific data rendered the majority of these lobbying attempts unsuccessful. Researchers at James Cook University eventually agreed to collaborate in the preliminary investigation of a possible new disease syndrome manifesting as emaciation in white-lipped tree frogs (*Litoria infrafrenata*).

This collaboration led to one of the initial objectives of my research, which was to comprehensively investigate the aetiology and epidemiology of this syndrome in *L. infrafrenata*, along with other amphibian diseases identified during analysis of CFH submission data. However, limited specimen availability precluded real-time data collection and analyses, and this component necessarily became retrospective. Additionally, ongoing difficulties with real-time data collection from, and co-operative relationships with, the CFH made further data utilisation and investigations logistically impossible. Accordingly, other objectives of my research were prioritised due to their importance in global amphibian research and the greater likelihood of achieving tangible and practical outcomes.

At the start of the project I set up a passive amphibian disease surveillance network at James Cook University in Cairns. Many colleagues and members of the public, both within and external to the university, contributed to the success of this network and I received over 160 specimens for analysis. Numerous interesting pathological lesions were identified in preserved and fresh specimens, but ultimate aetiological identification was not always possible. Significant limitations associated with disease diagnosis in amphibians include rapid autolysis of fresh tissue samples and the few validated diagnostic testing protocols for this taxonomic group.

Outcomes from my experimental research to validate a range of diagnostic test protocols for amphibians were very successful, and the focus of my research shifted to include more detailed

investigations into amphibian immunity and chytridiomycosis. Significant discoveries were made about systemic adaptive immunity in *Batrachochytrium dendrobatidis*-infected frogs, and I developed a successful treatment protocol for terminally ill frogs with chytridiomycosis.

## **1.2 Significance of the research**

This research project enabled a comprehensive assessment of adaptive immunity in *L. infrafrenata* and *L. caerulea*. This in turn led to the development of valid immunological tests to assess the immune response to, and for the diagnosis of, specific endemic and emerging amphibian diseases. Investigating *B. dendrobatidis*-specific immunity led to significant advancements in our knowledge about amphibian immune responses to fungal pathogens and mechanisms of host resistance against this formidable infectious disease. Determining baseline values for a range of haematologic and biochemical variables, and assessing the effect of season on these values, has led to enhanced diagnostic capabilities for diseased individuals of these two anuran species.

Retrospective aetiological and epidemiological investigations into the emaciation syndrome in *L. infrafrenata* provided an opportunity to study a previously undocumented amphibian disease that may significantly impact *L. infrafrenata* populations in far northern Queensland. Evaluating disease surveillance techniques led to recommendations that will benefit a number of government organisations, community groups, amphibian ecologists, scientists and veterinarians. The outcomes from this part of the study will ultimately enhance both the capacity of community groups to deal with amphibian diseases, as well as the ability of national bodies to monitor and diagnose important and emerging diseases affecting these species.

Investigating new and emerging amphibian diseases in Queensland, with a particular focus in the region of the port city of Cairns, has improved our knowledge base regarding amphibian diseases and the risks they pose to amphibian populations globally. This research is significant to global amphibian conservation efforts and is an important contribution to the field of wildlife disease investigation and management. The formidable emerging infectious disease, chytridiomycosis, has caused an unprecedented loss of species and may be a current driving force in the evolution of amphibians. Australia has been at the forefront of research and management of amphibian diseases, and this research project has contributed to maintaining this position.

### **1.3 Contribution of others to this thesis**

Parts of my research involved collaboration and consultation with colleagues at James Cook University and other institutions within Australia and overseas. The public were instrumental in providing specimens to contribute to my disease surveillance work. Specialised diagnostic tests, such as PCR, electron microscopy and protein electrophoresis, were performed by collaborators and these are clearly identified in the relevant sections of the thesis. I was the primary investigator for all of the research described in this thesis, with the exception of one published paper in Chapter 3 reporting cloacal prolapse in wild hylids and two published papers in Chapter 7 describing the pathogenesis of chytridiomycosis. The contributions of others are clearly identified at the start of the thesis and in the relevant sections of each chapter.



## CHAPTER 2

### Background and justification of aims

#### 2.1 Scope of the chapter

This chapter is a review of the available literature at the start of the candidature and aims to establish context and rationale for the research and to identify gaps in knowledge. References to the literature reflect the situation at the time of formulating the objectives for the thesis in August 2006. Citations 'in press' in August 2006 have been updated accordingly.

#### 2.2 Declining amphibian populations

Global declines and extinctions of amphibians have been accelerating over the past three decades. Analysis of data collected by The World Conservation Union (IUCN) shows that 1856 amphibian species (32.5 %) are globally threatened, and at least 2468 species (43.2 %) are experiencing population declines (IUCN, 2001, 2004; Stuart et al., 2004). Since 1980, rapid population declines have been reported in over 400 amphibian species, currently listed as critically endangered, with just over half of these attributed to habitat degradation and overexploitation (IUCN, 2001; Stuart et al., 2004). Until recently, in at least 200 of these species declines had been enigmatic, predominantly affecting stream-associated frogs in forests and tropical montane habitats in the Neotropics and Australia (Stuart et al., 2004). Affected species have been greatly over-represented by four anuran families: Bufonidae, Leptodactylidae, Hylidae and Ranidae (Stuart et al., 2004). Hypotheses for causes of population declines in these species have included infectious disease, introduction of predators and/or competing species, increased solar ultraviolet B radiation, acid precipitation, environmental pollution and global climate change (Carey & Bryant, 1995; Berger et al., 1998; Mann & Bidwell, 1999; Starnes et al., 2000; Middleton et al., 2001).

Many of these enigmatic amphibian population declines have now been linked to the formidable emerging infectious disease chytridiomycosis (Berger et al., 1998, 1999; Bosch et al., 2001; Carey et al., 2003; Daszak et al., 2003; McDonald et al., 2005; Skerratt et al., 2007). The impact of this disease on frog populations is thought to represent the most spectacular loss of biodiversity resulting from disease in recorded history (Skerratt et al., 2007). However, preliminary investigations indicated that chytridiomycosis was not the cause of an anecdotal

increase in numbers of diseased frogs presenting to community wildlife care groups in the Cairns region in tropical northern Queensland, Australia (R. Speare, unpublished observations).

### **2.3 Overview of emerging amphibian diseases in Australia**

Two infectious diseases are currently recognised worldwide as important emerging amphibian diseases: chytridiomycosis and ranaviral infections. Both diseases have been causally associated with global mass mortalities, have the potential to adversely impact amphibian populations, and are present in Australia. Ranavirus infection can cause high mortality but epidemics only occur locally or in captivity; chytridiomycosis causes rapid and widespread population declines in susceptible species and populations (Drury et al., 1995; Berger et al., 1998, 1999; Bollinger et al., 1999; Carey et al., 1999; Daszak et al., 1999, 2003; Berger, 2001; Hyatt et al., 2001; Docherty et al., 2003).

#### **2.3.1 *Chytridiomycosis***

Chytridiomycosis, first described by Berger et al. (1998) and Nichols et al. (1998), is an emerging infectious amphibian disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Berger et al., 1998; Longcore et al., 1999). This virulent and highly transmissible pathogen has been linked to global declines of amphibian populations and its presence has been recorded in Australia, New Zealand, the Caribbean, Europe, Africa, and South, Central and North America (Berger et al., 1998, 1999; Daszak et al., 1999, 2003; Alpin & Kirkpatrick, 2000; Mutschmann et al., 2000; Berger, 2001; Bosch et al., 2001; Waldman et al., 2001; Carey et al., 2003; Muths et al., 2003; Burrowes et al., 2004; Hanselmann et al., 2004; Weldon et al., 2004; Garner et al., 2005; McDonald et al., 2005; Lips et al., 2006; Moyer & Weldon, 2006; Skerratt et al., 2007). In Australia, *B. dendrobatidis* has been associated with dramatic frog population declines particularly in the high altitude rainforest areas of Queensland (Berger et al., 1998, 1999; Berger, 2001).

Virulence of *B. dendrobatidis* varies experimentally with pathogen dose and strain, and environmental temperature, and there is wide inter-species variation in host susceptibility to infection (Ardipradja, 2001; Berger, 2001; Nichols et al., 2001; Woodhams et al., 2003; Berger et al., 2004, 2005a). Mortality rates of up to 100 % have been recorded during experimental transmission and natural outbreaks of *B. dendrobatidis* in susceptible captive anuran species (Berger et al., 1998; Longcore et al., 1999; Berger, 2001; Nichols et al., 2001).

Many native Australian frog species across 13 genera are susceptible to chytridiomycosis, including *Litoria caerulea*, *L. chloris*, *L. nannotis*, *L. rheocola*, *Mixophyes fasciolatus*, *M. fleayi*, *Nyctimystes dayi* and *Limnodynastes tasmaniensis* (Berger et al., 1999, 2005b; Ardipradja, 2001; Berger, 2001). Chytridiomycosis is thought to have caused extinction of the sharp-snouted day frog, *Taudactylus acutirostris* (Schloegel et al., 2006). Transmission studies have shown the common green tree frog, *L. caerulea*, to be a highly susceptible host compared with three other native extant species (Ardipradja, 2001; Berger et al., 2005b). Experimentally, the fungus has been shown to persist and survive in environmental samples, independent of its host, for one to seven weeks in water and up to 12 weeks in moist sand (Johnson & Speare, 2003, 2005). Factors implicated in the spread of *B. dendrobatidis* include movement through water bodies and via surface water during precipitation, the movement of individual infected amphibians, and translocation on fomites and vectors such as moist substrate and birds (Speare et al., 2001; Johnson & Speare, 2003, 2005).

Infection with *B. dendrobatidis* occurs through waterborne zoospores that invade the superficial keratinised epidermal layers of the host, causing hyperkeratosis, sloughing and erosions of the epidermis, and occasional ulcerations, in post-metamorphic frogs (Berger et al., 1998, 1999; Berger, 2001). Minimal skin inflammation occurs, which may be due to a lack of stimulation of the immune system. Proposed reasons for this include the superficial site of infection, insufficient epidermal damage or low inherent antigenicity of the fungus (Berger et al., 1999). The mechanisms by which the fungus causes death are unknown, but have been postulated to be due to toxin release or host osmoregulatory disruption (Berger et al., 1998; Daszak et al., 2001). *Batrachochytrium dendrobatidis* can be carried on the keratinised mouthparts of tadpoles, and many of these exhibit clinical signs of abnormal mouthpart structure and pigmentation (Berger et al., 1999; Nichols et al., 2001). Infected tadpoles may be smaller and take longer to reach metamorphosis (Parris & Beaudoin, 2004; Parris & Cornelius, 2004). Diagnosis of chytridiomycosis requires laboratory confirmation by routine histological examination of skin sections, direct examination of unstained skin smears, immunohistochemical staining of skin sections using specific *B. dendrobatidis* antibodies, or standard and real-time polymerase chain reaction (PCR) assay (Berger et al., 1998, 1999, 2000, 2002, 2005b; Pessier et al., 1999; Berger, 2001; Annis et al., 2004; Boyle et al., 2004; Speare et al., 2005).

### **2.3.2 *Ranavirus infection***

*Ranavirus* is one of five genera belonging to the *Iridoviridae* family, members of which are pathogenic to three classes of poikilothermic vertebrates: reptiles, amphibians and fish (Mao et al., 1997, 1999; Hyatt et al., 1998, 2001; Carey et al., 1999; Daszak et al., 1999). Although

ranavirus infection can cause high mortality rates in wild populations of tiger salamanders (*Ambystoma tigrinum*) and spotted salamanders (*A. maculatum*) in North America, and common frogs (*Rana temporaria*) in the United Kingdom, it has not been associated with the population declines or extinctions caused by chytridiomycosis (Cunningham et al., 1996; Jancovich et al., 1997, 2001; Bollinger et al., 1999; Cunningham, 2001; Docherty et al., 2003). Despite this, the potential for ranaviruses to negatively impact amphibian populations globally cannot be discounted, particularly because the unregulated international trade and movement of potentially infected animals may result in the introduction of the pathogen to naïve populations and species (Cunningham et al., 2003; Daszak et al., 2003).

Two closely related ranaviruses have been identified in Australia: Bohle iridovirus and epizootic haematopoietic necrosis virus, first described in the ornate burrowing frog, *Limnodynastes ornatus* (Speare & Smith, 1992; Hengstberger et al., 1993), and redfin perch, *Perca fluviatilis* (Langdon et al., 1986), respectively. Subsequent studies have demonstrated that Bohle iridovirus is pathogenic to other native Australian amphibian species, fish and reptiles (Moody & Owens, 1994; Cullen et al., 1995; Ariel, 1997; Cullen & Owens, 2002). However, outbreaks of mass mortality caused by ranavirus have not occurred in Australia (Berger et al., 2004). Various other iridoviruses have been identified in amphibians outside Australia, including frog virus-3 (a *Ranavirus* species closely related to Bohle iridovirus), tadpole edema virus, and erythrocytic iridoviruses (Wolf et al., 1968, 1969; Granoff 1989; Gruia-Grey et al., 1989; Speare et al., 1991; Gruia-Grey & Desser, 1992; Hengstberger et al., 1993; Goorha & Granoff, 1994).

*Ranavirus*-specific antibodies are present in populations of the introduced cane toad (*Bufo marinus*, synonym *Rhinella marina*) throughout most of its known range in Australia, and this range has been steadily expanding since it first arrived in 1935 (van Beurden, 1981; Easteal, 1983; Zupanovic et al., 1998; Hyatt & Robinson, 2004). The potential for *B. marinus* to act as a vector for disease transmission to native amphibians and other wildlife is of significant concern, particularly based on the pathogenicity and apparent lack of species specificity of the Australian ranaviruses (Speare & Smith, 1992; Cullen et al., 1995; Drury et al., 1995; Ahne et al., 1997; Zupanovic et al., 1998).

The host effect of ranavirus infection in amphibians appears to be largely dependent upon the life cycle stage of the individual. A study in native Australian anurans found that juveniles were highly susceptible to Bohle iridovirus, manifesting signs of acute disease with high mortality rates, while adults were generally relatively resistant (Cullen & Owens, 2002). Species evaluated in this study include *Litoria caerulea*, *L. rubella*, *L. alboguttata*,

*Limnodynastes terraereginae*, *Cyclorana brevipes*, *Pseudophryne coriacea* and *Taudactylus acutirostris*. An earlier study in *B. marinus* found that infected juveniles exhibited an acute, fatal disease, while the adults again were found to be relatively resistant (Cullen, 2000). Some amphibians exposed to Bohle iridiovirus survive infection and continue to harbour the virus subclinically, and may become important viral reservoirs facilitating spread of the disease (Cullen & Owens, 2002).

Clinical signs in ranavirus-infected amphibians are generally non-specific, ranging from emaciation and lethargy to sudden death with no visible external signs (Chinchar & Mao, 2000; Cullen & Owens, 2002). Diagnosis is primarily based on histopathological examination of tissues and PCR amplification of the virus (Cullen & Owens, 2002). Classic histopathological changes include focal cellular necrosis and degeneration of haemopoietic tissue in the kidney, spleen and liver, often with associated haemorrhage and melanin deposition, and sometimes with haemosiderosis (Cullen et al., 1995; Cullen & Owens, 2002; Greer et al., 2005).

## **2.4 Structure and function of the immune system**

Understanding normal immune function in a given species, and subsequent determination of the type and magnitude of the immune response to a specific disease syndrome, are important tools for predicting possible aetiology and epidemiology. Furthermore, this information can assist in the development of specific diagnostic tests, and treatment, prevention and management strategies for emerging, epidemic and endemic diseases.

### **2.4.1 Overview of mammalian immunity**

A number of complex defense mechanisms function synergistically to establish and maintain a state of immunity against infection within the body. The immune system is regularly challenged by, and responds to, a multitude of antigens, including bacteria, viruses, fungi, endo- and ectoparasites, and other foreign material (Day, 1999a; Roitt, 2006). The humoral immune component includes antibodies, complement and soluble lymphocyte products, and the cellular component consists of the various cell types of the reticuloendothelial system (Boyle & Borsos, 1983). Immunity is classically divided into two types, innate and adaptive, and these are intrinsically linked in the response to antigenic challenge (Roitt, 2006). Antibody, complement and neutrophils confer protection against most extracellular microbes, while T-lymphocytes, lymphokines, macrophages and natural killer cells function to combat intracellular infections (Escobar & Swenson, 1983; Yoshida & Suko, 1983; Roitt, 2006).

Specific organs, circulating blood cells, tissue cells and various plasma protein and enzyme systems are integral to the efficient functioning of the immune system. Organs involved in immune function include the bone marrow, thymus, spleen, liver and various lymphatic nodes and nodules throughout the body (Tortora & Grabowski, 2004; Roitt, 2006). All cells of the immune system are derived from a single population of pluripotential stem cells found in the liver in early embryonic life, that later populate the bone marrow. This same stem cell population also gives rise to the other cellular blood elements (Friedman et al., 1983).

Innate immunity consists of non-specific antimicrobial systems that are present from birth, the function of which is not enhanced with repeated exposure to microbes (Roitt, 2006).

Components of innate immunity include physical barriers, commensal flora and soluble chemical factors associated with the skin and mucous membranes, along with internal defenses involving cellular mechanisms, antimicrobial proteins and inflammation (Day, 1999a; Tortora & Grabowski, 2004).

Commensal bacteria suppress the growth of many potentially pathogenic organisms by competition for essential nutrients and/or production of inhibitory compounds (Roitt, 2006). Soluble bactericidal factors are released in response to tissue injury and include a range of enzymes such as lysozyme. These enzymes activate the clotting system and disrupt bacterial cell walls, thereby limiting the spread of infection (Roitt, 2006). Concentrations of acute phase proteins increase dramatically in response to tissue injury and specific microbial products. Bacterial endotoxins stimulate release of interleukins, and this in turn stimulates hepatic synthesis and secretion of C-reactive protein, an acute phase protein that binds to microbes and activates the 'classical' complement pathway (Roitt, 2006). Virus-infected cells secrete broad-spectrum anti-viral proteins (interferons) into the extracellular fluid; these factors bind to adjacent uninfected cells and enhance their ability to resist infection (Escobar & Swenson, 1983; Roitt, 2006).

The phagocytic cell system forms the first line of non-specific cellular defense against antigens (Tracey, 1983). Phagocytes consist primarily of two leucocyte types, neutrophils and macrophages, the functions of which are antigen recognition, adherence, engulfing and digestion (Roitt, 2006). The 'alternative' complement pathway involved in innate immunity includes a complex series of proteins that, along with the blood clotting process, form an important triggered enzyme system in blood plasma (Day, 1999a; Roitt, 2006). Activation of the complement system produces a highly amplified cascade response to the presence of microbes; this functions to promote microbial cell lysis and to enhance the efficacy of the phagocytic cell system by facilitating antigen adherence to these cells and stimulating their

antimicrobial activity (Tracey, 1983; Day, 1999a). Natural killer cells (morphologically distinct granular lymphocytes) and eosinophils effect extracellular killing of virus-infected host cells and parasites, respectively, as part of the non-specific immune response (Roitt, 2006).

Adaptive immunity is the development of disease resistance resulting from the production of specific cell types or antibodies directed against particular antigens, a strategy that has evolved in response to the ability of microbes to mutate and evade non-specific defenses (Roitt, 2006). Humoral adaptive pathways involve antibodies (immunoglobulins) produced by B-lymphocytes, and these have three intrinsic properties: 1) antigen binding, 2) phagocytic cell stimulation, and 3) 'classical' complement pathway activation (Boyle & Borsos, 1983; Nicholas, 1987; Roitt, 2006). Immunoglobulins are divided into classes based on the structure of their heavy chain constant regions; humans have five classes: immunoglobulin (Ig) G, A, M, D and E (Friedman et al., 1983; Nicholas, 1987). When antigen binds tightly to an Ig receptor, the B-lymphocyte is triggered to divide and differentiate into antibody-secreting plasma cells that produce antibodies identical to those of the original lymphocyte (Day, 1999a; Roitt, 2006). The antibody-antigen complex directly causes microbial agglutination, precipitation and cell lysis (Nicholas, 1987). The classical complement cascade is also activated by antibody-antigen binding, and this enhances the ability of the innate immune system to initiate acute inflammatory reactions, along with stimulating direct cytotoxicity (Müller-Ebhard, 1968; Boyle & Borsos, 1983).

Repeat exposure of the immune system to the same antigen results in a more rapid and abundant secondary antibody response, due to adaptive cellular memory (Day, 1999c). A small portion of the original antigen-reactive B-lymphocytes become non-dividing memory cells, as opposed to antibody-producing effector cells, making the adaptive secondary response more rapid and of greater magnitude (Day, 1999c). Adaptive immunity shows considerable specificity i.e. establishment of memory or immunity by one microbe does not confer protection against another unrelated microbe (Roitt, 2006). However, the specificity is not absolute and there is some cross-reactivity between specific antibodies and closely related antigens (Lefford, 1983). The specificity and memory of the adaptive immune response form the basis for vaccination (Roitt, 2006).

Cell-mediated adaptive pathways involve B- and T-lymphocytes (Nicholas, 1987; Day 1999a). Analogous to the B-lymphocyte system, T-lymphocytes are selected and activated by combination with antigen, expanded by clonal proliferation, and mature to produce T-helper and T-effector cells, together with an enlarged population of memory cells (Day, 1999b,c). T-lymphocytes are specialised to recognise antigen, through T-cell receptors (TCR), only when it

is presented on the surface of a host cell in combination with a specific host cell surface marker, the major histocompatibility complex (MHC) (Zinkernagel & Doherty, 1979; Apasov & Sitkovsky, 2005). This phenomenon is known as MHC-restricted T-cell recognition (Nicholas, 1987). The majority of T-lymphocytes express antigen-binding  $\alpha\beta$  chains in their TCR and are involved in the development of adaptive immune responses. These cells have a very diverse repertoire of antigen-recognition receptors and are subdivided into two separate functional lineages based on their major surface co-receptor molecules, CD4 and CD8 (Apasov & Sitkovsky, 2005; Roitt, 2006). A small percentage of T-lymphocytes express  $\gamma\delta$  chains in their TCR and are found in the skin and some mucosal surfaces; their role is not fully understood but they are considered to be a relatively primitive part of the innate T-lymphocyte response (Apasov & Sitkovsky, 2005).

All nucleated cells of the body express MHC Class 1 molecules that bind to cytosol-derived antigenic peptides for cell surface presentation to CD8<sup>+</sup> lymphocytes. Activated CD8<sup>+</sup> cells form effector cytotoxic T-lymphocytes that undergo dramatic clonal expansion, making them very efficient antigen-specific effector cells that destroy target cells by apoptosis (Apasov & Sitkovsky, 2005; Roitt, 2006). Only a few cell types, the 'professional' antigen presenting cells (macrophages, B-lymphocytes and dendritic cells), express MHC Class 2 molecules that bind intracellular proteins and pathogen-derived peptides for surface presentation and recognition by CD4<sup>+</sup> lymphocytes (Apasov & Sitkovsky, 2005). Activated CD4<sup>+</sup> cells produce cytokines as effector T-helper (Th) cells and are further divided based on their cytokine profile into Th1, Th2 and regulatory T-cell (Treg) subsets. Pro-inflammatory cell-mediated immunity is driven by Th1 cells, along with delayed-type hypersensitivity, specific IgG-isotype production by B-lymphocytes and specific protozoal responses. Non-inflammatory immediate immune responses are mediated by Th2 cells, along with B-lymphocyte production of IgG, IgA and IgE (Apasov & Sitkovsky, 2005). Regulatory T-lymphocytes participate in all cell-mediated immune responses to maintain peripheral tolerance, down-modulate immune response amplitude and prevent autoimmune diseases (Apasov & Sitkovsky, 2005; Roitt, 2006).

Both B- and T-lymphocytes provide specific immunity via a range of mechanisms that increase the efficacy of the innate immune response and confer protection against subsequent exposure to the same microbe (Roitt, 2006).

#### **2.4.2 Comparative amphibian immunity**

Both larval and post-metamorphic amphibians have fewer lymphoid organs than mammals, and those present differ in architecture and reticuloendothelial cell number to varying extents (Du



Pasquier et al., 1989). Metamorphosis is a critical transition period during which increased concentrations of thyroid and corticosteroid hormones drive the loss or reorganisation of many tissues and organ systems, including the immune system (Rollins-Smith, 1998). During metamorphosis, lymphocyte populations have been shown to undergo an apoptotic decline in the spleen, thymus and liver, potentially resulting in temporary immunosuppression (Du Pasquier & Weiss, 1973; Rollins-Smith et al., 1984; Rollins-Smith, 1998). The immune system is then reorganised and expanded post-metamorphosis to provide a more complete, mature immune system with enhanced B- and T-lymphocyte antigen recognition and antibody repertoires, along with immunological tolerance to a new range of antigens (Du Pasquier et al., 1979; Hsu & Du Pasquier, 1992; Rollins-Smith, 1998).

The South African clawed frog (*Xenopus laevis*) has the best characterised immune system of any amphibian to date, and provides a unique non-mammalian system to understand fundamental immunology and its evolution (Gantress et al., 2003). The post-metamorphic immune system of *X. laevis* is fundamentally similar to that of mammals and involves antibody production in response to immunisation (Du Pasquier et al., 2000), immunoglobulin isotype heterogeneity and leucocyte-derived cytokines (Watkins et al., 1987; Haynes & Cohen, 1993), cytotoxic and helper T-cell responses (Blomberg et al., 1980; Harding et al., 1994), complement activity (Sekizawa et al., 1984), and MHC Class I- and Class II-restricted T-cell recognition (Du Pasquier et al., 1989). In addition to antigenic challenge of the immune system, factors that may influence the amphibian immune response include age, body condition, season, ambient temperature and developmental stage of the immune system (Harris, 1972; Lin & Rowlands, 1973; Anvier & Pond, 1984; Zapata et al., 1992; Wright, 2001b; Grasman, 2002).

Amphibian plasma cells produce three different classes of membrane-bound immunoglobulin (Hadji-Azimi, 1971; Du Pasquier et al., 2000). Immunoglobulin M is similar to all known vertebrate IgM molecules; it is the most abundant isotype in amphibians and has the lowest non-glycosylated heavy chain constant region molecular weight (Hadji-Azimi, 1971; Schwager et al., 1988). Immunoglobulin Y is functionally homologous with mammalian IgG, but the heavy chain constant region is higher in molecular weight than the mammalian isotype; the IgY non-glycosylated heavy chain constant region has the highest molecular weight of the three amphibian isotypes (Hadji-Azimi, 1971; Amemiya et al., 1989; Mussmann et al., 1996). Immunoglobulin X is preferentially expressed in the amphibian gastrointestinal tract, and is not directly homologous with any specific mammalian Ig isotype (Hsu et al., 1985; Mussman et al., 1996).

In addition to a competent adaptive immune response capability, adult amphibians also have an effective innate immune response, although this has not been as well characterised (Rollins-Smith, 1998). One important distinct component of innate local immunity is the ability to synthesise antimicrobial peptides in the skin (Mor & Nicholas, 1994; Reilly et al., 1994). Natural killer cells are also present in post-metamorphic amphibians and mediate an immediate cytotoxic response against virus-infected or tumour target cells (Horton et al., 1998; Rollins-Smith, 1998).

The spleen of the post-metamorphic amphibian is the most highly organised and sophisticated immune organ and has delineated regions of red and white pulp, centres of haemopoiesis and lymphopoiesis respectively, similar to its mammalian counterpart (Manning & Horton, 1982; Turner, 1988). This organ is the main site of B-lymphocyte differentiation in amphibians and also contains interleukin-producing cells, and cytotoxic and helper T-lymphocytes (Du Pasquier et al., 2000). Seasonal variation in splenic size has been documented in adult anurans (Wright, 2001b).

The larval amphibian thymus contains proliferating T-lymphocytes, IgM-producing plasma cells and macrophages. It involutes and translocates from the pharyngeal pouch region towards the tympanum during metamorphic climax, undergoes a second wave of histogenesis post-metamorphosis and then regresses at the onset of sexual maturity (Du Pasquier & Weiss, 1973; Rollins-Smith et al., 1984). Involution and atrophy of the thymus may also occur as a result of malnutrition, stress and hibernation (Wright, 2001b).

Post-metamorphic amphibians possess functional bone marrow, within which erythropoiesis, myelothrombocytopoiesis, and possibly lymphopoiesis, may occur depending on the species (Turner, 1988; Greenhalgh et al., 1993; Du Pasquier et al., 2000). Amphibians represent the first ancestral taxonomic group to have bone marrow (Campbell, 1970; Tanaka, 1976). Functional equivalents of bone marrow are also found in liver and kidney sites, and gut-associated lymphoid tissue is also present in some species (Manning & Horton, 1982; Wright, 2001b). There are no lymph nodes in anuran amphibians, although lymphoid accumulations are found near the heart in some species (Zapata & Amemiya, 2000).

Haemopoiesis is well described in mammals, but only a few amphibian species have been studied to characterise and localise the haemopoietic process, and reports are often conflicting. Sites of haemopoiesis have been shown to vary in those species studied (*X. laevis*, *Rana pipiens*, *R. catesbeiana* and *R. temporaria*) and include the spleen, kidney, liver, bone marrow, gut, lymphoid accumulations and lung (Foxon, 1964; Manning & Horton, 1982; Turner, 1988). The

spleen is reported to be the primary site of erythropoiesis in adult *R. catesbeiana* (Jordan & Speidel, 1923; Turner, 1988), but Maniatis & Ingram (1971) describe haemopoietic tissue in the larval spleen and maturing erythroid cells in the adult bone marrow in the same species. Erythropoiesis has also been described in the peripheral circulation in *X. laevis* with experimentally induced anaemia (Thomas & Maclean, 1974).

There are notable distinctions between mammalian and amphibian circulating blood cells. Amphibian erythrocytes are nucleated, with the exception of a subpopulation of anucleate forms described in lungless urodeles (Foxon, 1964; Cohen, 1982). Thrombocytes are also nucleated, usually with spindle-shaped cytoplasmic extensions at each pole, but these are not always evident with light microscopy and thrombocytes can be difficult to distinguish from small lymphocytes (Turner, 1988).

Amphibian lymphocytes form a complex cell population, but small lymphocytes are morphologically similar to those in other vertebrates and are common circulating blood cells (Turner, 1988). Amphibian monocytes are large mononuclear cells with a variable nucleus shape depending on the species, and cytoplasm that may contain small azurophilic granules (Cannon and Cannon, 1979; Turner, 1988). Amphibian granulocytes are generally classified into three groups based on morphology and staining characteristics, resembling the neutrophils, eosinophils and basophils of higher vertebrates (Hadji-Azimi et al., 1987; Turner, 1988). Neutrophils are abundant circulating blood cells with a distinctive multilobulated nucleus and variable cytoplasmic granulation. Eosinophils are generally less commonly seen in peripheral blood and have prominent round or oval cytoplasmic granules (Turner, 1988). Numbers of circulating basophils vary considerably according to species, from less than one percent to being the predominant granulocyte, and the cytoplasm usually contains large metachromatic granules (Jordan, 1938; Cannon & Cannon, 1979; Hadji-Azimi et al., 1987; Turner, 1988; Pfeiffer et al., 1990).

Some authors divide granulocytes with eosinophilic granules into two populations: eosinophils (large eosinophilic granule leucocytes) and heterophils (small eosinophilic granule leucocytes with smaller rod-shaped granules); these two cell types have been shown to have different histochemical properties and immune functions (Cannon & Cannon, 1979; Turner, 1988; Wright, 2001a). Eosinophils are poorly phagocytic and appear to play little or no role in combating bacterial and fungal infections (Wright, 2001a). Heterophils are phagocytic and circulating numbers often increase in acute bacterial infections, thereafter decreasing as they are depleted (Wright, 2001a). Several authors consider neutrophils and heterophils to be synonymous (Hadji-Azimi et al., 1987; Turner, 1988; Cathers et al., 1997; Romanova &

Egorikhina, 2006), while others consider neutrophils to be a distinct population of granulocytes (Surbis, 1978; Cannon & Cannon, 1979; Pfeiffer et al., 1990). Azurophilic granulocytes, with small azurophilic cytoplasmic granules, have also been described and are thought to be the same cell type as the monocyte (Hadji-Azimi et al., 1987; Cathers et al., 1997), although several authors consider the azurophil to be a separate granulocyte population (Montali, 1988; Hawkey & Dennett, 1989; Wright, 2001a). Further intra- and inter-species histochemical and comparative studies are required to determine whether these additional distinctions from mammalian cell types are warranted (Wright, 2001a).

### **2.4.3 Methods for assessing immune function**

Laboratory and field studies have been developed in various mammalian and avian species as measures of immune structure and function. Methods commonly applied across broad taxonomic groups to assess the structure of the immune system and to test specific immunologic functions include mass, cellularity and histology of immune organs, total and differential peripheral leucocyte counts, plasma/serum protein concentrations, *in vivo* phytohaemagglutinin (PHA) skin response test, *in vivo* anti-sheep red blood cell (SRBC) haemagglutination test, and several more complex *in vitro* assays and tissue culture techniques including enzyme-linked immunosorbent assays (ELISAs), cell stimulation assays and flow cytometry (Grasman, 2002; Roitt, 2006).

Immunisation with SRBCs to successfully evaluate humoral immunity via serum and splenic haemolytic antibody production has been reported in three species of anurans: *Rana pipiens*, *Bufo arenarum* and *Xenopus laevis* (Evans, 1963; Evans et al., 1965; Horton et al., 1976; Gearing et al., 1984; Zettergren et al., 1991; Rosenberg et al., 2002). Thymectomy of larval *X. laevis* resulted in an inability to mount an antibody response to SRBCs (Gearing et al., 1984), and splenectomised *X. laevis* showed an impaired antibody response to SRBC immunisation (Collie & Turner, 1975). A recent study assessed immune status over time in three *Rana* species in response to an anthropogenically transformed environment (Romanova & Egorikhina, 2006). T-lymphocyte activity was evaluated by rosette formation with chicken red blood cells, and B-lymphocyte activity was assessed by rosette formation with SRBCs sensitised by antibodies against frog immunoglobulins.

The use of PHA, a T-cell mitogen, has been quite widely reported in a number of *in vitro* studies evaluating splenocyte, thymocyte and lymphocyte proliferative responses in anurans, including *X. laevis* and *R. pipiens* (Rollins-Smith & Cohen, 1982; Rollins-Smith et al., 1984; Rollins-Smith & Blair, 1993; Kinney & Cohen, 2005). It has also been reported in several *in*

*vitro* studies designed to evaluate the immune system of reptiles (Work et al., 2001; Burnham et al., 2005). No reports have been found documenting the use of the PHA skin test as an *in vivo* indicator of immune function in amphibians. However, there is a report describing the successful use of the PHA skin test to assess immune activity in the Galapagos marine iguana, *Amblyrhynchus cristatus* (Berger et al., 2005).

The development of ELISAs using specific antisera against amphibian immunoglobulins to identify and assess adaptive immunity has been described in several anuran species, including *X. laevis* (Hsu & Du Pasquier, 1984a,b) and *B. marinus* (Whittington & Speare, 1996). These methods have since been adapted for the detection of antibodies against ranaviruses in the serum of *X. laevis* (Gantress et al., 2003) and *B. marinus* (Whittington et al., 1997; Zupanovic et al., 1998). Difficulties associated with developing and validating ELISAs for amphibians include the often relatively low antibody titres present, limited volume of serum available, marked inter-species variation in anti-immunoglobulin monoclonal antibody reactivity, lack of species-specific anti-immunoglobulins available to assess specificity, non-specific serum protein binding, and determination of optimal assay incubation temperature and length (Hsu & Du Pasquier, 1984a; Whittington & Speare, 1996; Whittington et al., 1997; Rosenberg et al., 2002).

Rapid progress has been made in identifying genes involved in innate and adaptive immunity in virtually all vertebrate taxa (Du Pasquier & Flajnik, 1999). However, little is known regarding the mechanisms of defense against viral and fungal pathogens that have been causally implicated in global amphibian population and species declines (Zupanovic et al., 1998; Berger et al., 1999; Carey et al., 1999; Daszak et al., 1999; Robert et al., 2005). Important basic knowledge about amphibian anti-viral immune defenses, using *Xenopus* as a model for studying the emerging ranavirus frog virus-3, has only very recently been documented (Robert et al., 2005). No reports have been found describing adaptive immunity in *Litoria* species.

Establishing baseline values for a range of haematologic and biochemical parameters can be valuable for assessing some aspects of immune function and for clinical interpretation of blood results in diseased individuals. Many of the published reports on amphibian haematology have little clinical relevance due to the wide range of reported normal values for healthy amphibians resulting from restricted sample size, variations in sampling techniques, sampling conditions, analytical techniques, physiological state, sex and season, and unrecognised pathologies (Wright, 2001a). The differential circulating leucocyte count has been shown to vary according to species, sex, reproductive stage, health status, season, temperature and environmental pollution, further complicating the establishment of blood reference values (Harris, 1972;

Cathers et al., 1997; Romanova & Egorikhina, 2006). Amphibian biochemistry values have received little attention in the literature, with the exception of plasma glucose concentrations which have been shown to vary widely in the northern leopard frog (*Rana pipiens*) as a result of geographic origin, season, time of day, handling, anaesthesia and assay method (Farrar & Frye, 1979). Sex-related differences in plasma protein, calcium and sodium values have been documented in the bullfrog, *Rana catesbeiana* (Cathers et al., 1997). Few clinical reports based on controlled studies of normal haematology and blood biochemistry values for anurans exist, and there have been no baseline or reference values published for white-lipped (*Litoria infrafrenata*) or common green (*L. caerulea*) tree frogs.

Reference values describe the dispersion of variables in healthy individuals and are usually reported as population-based reference intervals comprising 95 % of the healthy population (Grasbeck & Saris, 1969; Grasbeck, 1983; Solberg, 1987a,b). They are one of the most powerful tools in laboratory medicine to aid in the clinical decision-making process and for disease diagnosis (Grasbeck, 1983; Henny & Hytloft Petersen, 2004). Reference individuals are assumed to be clinically 'healthy' (Solberg, 1987a). Defining the health status of individuals based on strict inclusion and exclusion criteria, along with the use of quality-controlled analytic procedures, are critical steps in determining reference values and therefore reference intervals (PetitClerc & Solberg, 1987; Solberg & Stamm, 1991). International human recommendations state the preferred method as a priori non-parametric determination from at least 120 reference individuals selected according to predetermined criteria (Solberg, 1987b).

Establishing reference values in veterinary clinical pathology can be challenging due to a limited number of available reference individuals, difficulties associated with defining health status, lack of validated analytic tests, and the significant allocation of resources required. Identifying and recording the objective of the future use of the reference interval is critical, and helps to define population characteristics and to choose inclusion, exclusion and partitioning criteria for reference individual selection (PetitClerc & Solberg, 1987). Minimal exclusion criteria, based on a thorough physical examination and history, should include any clinical signs of disease or administration of medications, with the possible exception of anthelmintics (PetitClerc & Solberg, 1987; Solberg, 1987a). Other quantifiable exclusion factors may include those that indicate poor health or undue stress, such as body temperature, heart rate and body condition score (PetitClerc & Solberg, 1987; Solberg, 1987a). Further testing may be carried out as indicated by the initial selection criteria. Sample numbers should be as high as possible; however, the use of parametric or other statistical methods (bootstrap or robust) where appropriate, in combination with low data skewness, enables the use of fewer than the recommended 120 values to generate reference intervals (Linnet, 1987; Poulsen et al., 1997;

Jennen-Steinmetz & Wellek, 2005). Outlier values should be detected and removed using standard outlier tests (Tukey's or Dixon-Reed's), and partitioning of reference intervals into subclasses should be considered if clinically useful or physiologically indicated (Solberg, 1987b; Lahti, 2004).

#### **2.4.4 Immunosuppression**

A number of inherited primary immunodeficiency syndromes have been described in humans and, although quite rare, can predispose an individual to repeated microbial infections (Rosen et al., 1995; Roitt, 2006). Innate immune mechanism deficiencies include phagocytic cell and complement system defects. Deficiencies in adaptive immune mechanisms, affecting T- and B-lymphocytes alone or in combination, can cause cellular and humoral immunodeficiencies (Chandra, 1983; Rosen et al., 1995; Roitt, 2006). A variety of factors can non-specifically suppress the immune system, causing a secondary, or acquired, immunodeficiency. These factors include metabolic diseases (e.g. malnutrition, diabetes mellitus, renal failure), certain microbial infections, neoplasia, drugs, radiation exposure and acquired lymphoproliferative disorders (Chandra, 1983; Sellon, 1999; Roitt, 2006).

Immunosuppression manifests clinically as increased susceptibility to, and recurrence of, bacterial infections (complement, phagocytic cell, B-lymphocyte and stem cell deficiencies) and fungal and viral infections (T-lymphocyte and stem cell deficiencies) (Roitt, 2006). A relatively high incidence of neoplasia, and of autoantibody production with or without clinically apparent autoimmune disease, occurs in association with immune deficiencies (Day et al., 1980; Haraguchi et al., 1997; Roitt, 2006). Humoral responses to vaccinations and cellular responses to intradermal antigen tests may be impaired (Roitt, 2006). Clinical pathological indicators of immune deficiency include altered peripheral circulating cell counts and morphology, altered immune organ cellularity and morphology, hypoglobulinaemias, and impaired *in vitro* monoclonal antibody staining, haemagglutination, enzymatic and functional cellular tests (Sher et al., 1992; Haraguchi et al., 1997; Roitt, 2006).

Viral infections are commonly immunosuppressive, the prototypical agent being human immunodeficiency virus, an exogenous retrovirus (Roitt, 2006). This virus has a direct cytotoxic effect on lymphoid cells and causes dramatic and often complete destruction of the T-helper cell population as the infection progresses (Rosenberg & Fauci, 1990). Affected individuals are highly susceptible to secondary infections by opportunistic pathogens that would be nonpathogenic in an immunocompetent individual (Roitt, 2006).

Exogenous infectious viruses belonging to the *Retroviridae* family cause neurological and immunological diseases, neoplasia and immunodeficiency syndromes in a wide variety of species other than humans, including non-human primates, rodents, felids and birds (Herniou et al., 1998; Kennedy-Stoskopf, 1999; Bielitzki, 1999; Denicourt et al., 2003). Profound immunodeficiencies in animals infected with pathogenic retroviruses manifest as increased morbidity and mortality attributable to opportunistic viral and bacterial infections and, in some cases, neoplasia (Anderson et al., 1971; Haraguchi et al., 1997). Affected animals often demonstrate markedly reduced lymphocyte and/or neutrophil numbers and activity, complement system suppression, high levels of circulating immune complexes, imbalanced cytokine production, and reduced levels of circulating interferon- $\gamma$  and retrovirus-specific immunoglobulins (Kobilinsky et al., 1979; Day et al., 1980; Olsen et al., 1981; Liu et al., 1984; Mertens et al., 1990; Sher et al., 1992; Roitt, 2006). Pyrexia, lymphadenopathy and weight loss with terminal wasting are common clinical signs associated with progressive pathogenic retrovirus infections (Anderson et al., 1971; Olsen et al., 1981; Roitt, 2006).

Endogenous retroviruses have been identified within the genomes of a wide range of poikilothermic vertebrates including amphibians, but to date have not been confirmed to cause disease in amphibians (Herniou et al., 1998; Kambol et al., 2003). Retroviral nucleic acid has been amplified from various amphibians including: two urodele species, the palmate newt (*Triturus helveticus*) and tiger salamander (*Ambystoma tigrinum*); one caecilian species, the rhinatremid caecilian (*Epicrionops marmoratus*); and eight anuran species, the Amazonian poison dart frog (*Dendrobates ventrimaculatus*), rocket frog (*Colostethus talamancae*), edible frog (*Rana esculenta*), leopard frog (*R. pipiens*), Iberian frog (*R. iberica*), European common frog (*R. temporaria*), African clawed toad (*Xenopus laevis*) and painted frog (*Discoglossus galganoi*) (Tristem, 1996; Herniou et al., 1998). The vast majority of endogenous retroviruses characterised in lower vertebrates cluster with the murine leukaemia virus-related genus (Herniou et al., 1998). Members of this genus have also been isolated from humans, mammals, birds and reptiles (Zeigel & Clark, 1969; Payne, 1992; Wilkinson et al., 1994; Chen et al., 1987), indicating an extremely complex lineage.

Importation of wild-caught Chinese pond frogs (*Rana nigromaculata* and *R. plancyi plancyi*) into a research laboratory for hybridisation with resident Japanese, Korean and Taiwanese pond frogs (*R. nigromaculata*, *R. plancyi fukienensis* and *R. brevipoda*) resulted in the frequent occurrence of pancreatic carcinomas in subsequent generation hybrids. C-type retrovirus particles were identified ultrastructurally in the extracellular spaces and budding from cytoplasmic membranes of the carcinoma cells (Masahito et al., 1995). However, a direct causal



association between the virus and carcinoma occurrence was not demonstrated and the finding may have been incidental.

## **2.5 Wildlife disease surveillance in Australia**

Community wildlife care groups exist in many countries for wildlife rescue and rehabilitation, and groups are active in every state within Australia. In conjunction with other groups including conservation charities and environmental consultancies, they are a valuable source of wildlife information and play an important but under-utilised role, both directly and indirectly, in wildlife disease surveillance. While wildlife rehabilitators can amass diverse and important passive surveillance data, its use is greatly limited by its inaccessibility, the lack of uniform data presentation and the inherent bias in the population sample (Harden et al. 2006).

### **2.5.1 *The Australian Wildlife Health Network***

The growing importance of wildlife disease as a threat to biodiversity, human health, agriculture, aquaculture and trade has been demonstrated by recent disease outbreaks, mass mortalities and emergence of new diseases in humans and wildlife (Daszak et al., 2000, 2001). Following nationwide studies in Australia during 1999 and 2000, the establishment of a national network to coordinate wildlife disease surveillance, diagnosis, preparedness and response was considered vital. The Australian Wildlife Health Network (AWHN) was set up, a system funded and supported by the Australian Wildlife Exotic Disease Preparedness Program and hosted by the Zoological Parks Board of New South Wales (NSW) and NSW Agriculture. The aim of the network is to promote and facilitate collaborative efforts in the investigation and management of wildlife disease in support of human and animal health, biodiversity and trade. It is supported by a national coordinator, website and list server, and maintains a national database of wildlife health surveillance and diagnostic information, along with a registry of wildlife expertise. State and territory wildlife coordinators are appointed by their jurisdictional Chief Veterinary Officer and collate information to support Australia's National Animal Health Information System. The network also aims to develop wildlife management protocols, coordinate information in emergencies, advance training and education, and prioritise and promote surveillance and research activities (Australian Government Department of Agriculture, Fisheries & Forestry, 2004).

### **2.5.2 *Community surveillance for wildlife diseases***

One of the greatest challenges in wildlife disease investigations is accessing information

collated by community groups and integrating it into a national database such as that maintained by the AWHN. These groups have had limited success to date in developing suitable techniques to collect health data relevant for the surveillance of emerging and endemic wildlife diseases, and in transmitting this data in a practical and cost-effective way to databases maintained by trained professionals in the field (Harden et al., 2006).

## **2.6 Experimental subjects used in the research**

Free-ranging clinically healthy adult common green (*Litoria caerulea*) and white-lipped (*L. infrafrenata*) tree frogs were used for the experimental research reported in this thesis. Individuals were captured from widespread residential and semi-rural coastal and adjacent locations within a 3000 km<sup>2</sup> area between Cairns and Townsville in far northern Queensland, Australia. The two species were selected based on their large mature body size, relative ease of capture, endemicity, wide distribution, and stable conservation status (Least Concern; IUCN, 2001).

*Litoria caerulea* is widely distributed throughout northern and eastern coastal and interior areas of Australia, across five states and territories (Cogger, 2000). It is also found in Indonesia and Papua New Guinea. This species has broad habitat tolerance with frequent close human association. In Australia it is found predominantly in dry forest, woodland, grassland and disturbed urban environments, and rarely in wet forest or near streams and swamps (Hero et al., 2004). Mean mature adult body (snout-urostyle) length is 100 mm (Cogger, 2000). Breeding occurs in still water, including temporarily flooded ditches and ponds (Hero et al., 2004).

*Litoria infrafrenata* has a more limited Australian distribution in coastal and adjacent areas of northeastern Queensland, north of Townsville and extending around Cape York Peninsula into the Gulf of Carpentaria (Cogger, 2000). It is also found in Indonesia, Papua New Guinea, the Solomon Islands and East Timor (Iskandar et al., 2004). Habitat preference varies widely within more humid microclimates, including tropical rainforest, wet sclerophyll forest and cultivated areas; it also persists in heavily modified urban environments (Iskandar et al., 2004). Mean mature adult body (snout-urostyle) length is 110 mm (Cogger, 2000). Breeding occurs in forest pools, deep and slow streams, and ditches and pools in disturbed and urban areas (Iskandar et al., 2004).

## **2.7 Aims of the thesis**

- i) To review the literature current to August 2006 relevant to my research objectives.
- ii) To survey for and describe emerging and endemic amphibians diseases in Queensland with a focus on the port city of Cairns.
- iii) To analyse community surveillance data for amphibian diseases in the Cairns region.
- iv) To investigate the aetiology and epidemiology of the irreversible emaciation syndrome in white-lipped tree frogs.
- v) To validate haematologic and biochemical diagnostic tests in tree frogs.
- vi) To develop blood reference intervals for two species of tree frogs.
- vii) To develop and validate tests for immune function in tree frogs.
- viii) To investigate systemic adaptive immunity in tree frogs with chytridiomycosis using the validated tests.
- ix) To contribute to knowledge about the pathogenesis of chytridiomycosis.
- x) To develop strategies for captive management of chytridiomycosis.
- xi) To develop treatment protocols to cure terminally ill frogs with chytridiomycosis.
- xii) To make recommendations for future amphibian disease research directions.

## CHAPTER 3

### Surveillance for new and endemic amphibian diseases in Queensland

#### 3.1 Introduction

My research was funded primarily by the Australian Government Department of the Environment, Water, Heritage and the Arts (DEWHA). I aimed to further the knowledge base regarding amphibian diseases and the risks they pose to amphibian populations globally by investigating new and emerging amphibian diseases in Queensland, with a particular focus in the region of the port city of Cairns. Identification of new wildlife diseases in the Cairns region was considered of particular importance since entry of emerging diseases into countries often occurs through ports, and northern Queensland is considered a high national biosecurity risk for disease and invasive species incursion and emergence (Northern Australia Quarantine Service, 2004; Murray et al., 2012).

The growing importance of wildlife disease as a threat to biodiversity, human health, agriculture, aquaculture and trade has been demonstrated by recent disease outbreaks, mass mortalities and emergence of new diseases. The Australian Wildlife Health Network (AWHN) aims to facilitate collaborative efforts in the investigation and management of wildlife disease in support of human and animal health, biodiversity and trade. Community wildlife care groups exist in many countries throughout the world for wildlife rescue and rehabilitation. A number of these groups are active in every state within Australia. They play an important but under-utilised role, both directly and indirectly, in wildlife disease surveillance. One of the greatest challenges is accessing wildlife disease information collected by community groups and integrating it into a national database such as that maintained by the AWHN.

Evaluating disease surveillance techniques and integration of community surveillance into the AWHN benefits a number of government organisations, community groups, amphibian ecologists, scientists and veterinarians. The outcomes from this study will ultimately enhance both the capacity of community groups to deal with amphibian diseases, and the ability of the AWHN to monitor and diagnose important and emerging diseases affecting these species.

Chapter 3 consists of: 1) a summary of the results from the disease surveillance component of my research from the published final report to the funding body; 2) a published peer-reviewed paper detailing the use of community surveillance data to investigate amphibian diseases; and

3) a published peer-reviewed paper detailing the occurrence of cloacal prolapse in wild hylids.

### **3.2 Surveillance for amphibian diseases in Queensland with detailed pathological investigations**

Over 160 specimens were received through my passive amphibian disease surveillance system at JCU. Processing and diagnostic analysis of endangered species, live specimens with accurate historical information, and untreated individuals were prioritised. For each case, thorough physical examination, necropsy and sample collection protocols were established and implemented. A large range of diagnostic samples was collected and analysed using haematology, serum biochemistry, and parasite identification. Formalin-fixed tissues were processed from each case and histological analysis carried out where possible. Similarly, a range of tissue samples from each specimen was frozen for further diagnostic testing if required. Case details and diagnostic findings in frog specimens received during this project are summarised in Appendix 1. Significant disease and/or lesions were found in a number of cases and further diagnostic investigations were carried out. Details about the funding body report and two selected novel case reports of interest are presented below.

#### **3.2.1 Report to DEWHA**

The published final report to the funding body DEWHA was my original writing with minor editorial contributions from Rick Speare, Lee Berger, Lee Skerratt and Diana Mendez. I performed the majority of the surveillance work and specimen collection, and all of the clinical examinations, physical data collection, post mortem examinations and diagnostic sample collection. Harry Hines assisted with collection of specimens. Rebecca Webb processed the tissue samples I collected for histological examination. Stephen Garland analysed the skin swabs I collected for *B. dendrobatidis* using real-time PCR. Rick Speare, Lee Berger and Diana Mendez assisted with histopathological analysis. Ron Slocombe performed histopathological analysis and provided written pathology reports and images for cases 054 and 085. Liliana Tatarczuch performed transmission electron microscopy on tissues and provided images for these two cases, and Ron Slocombe assisted with interpretation of the electron microscopy images. Microsporidial PCR analysis was performed by Louis Weiss (case 054), poxviral PCR analysis was performed by Jianning Wang (case 085), and papillomavirus PCR was performed by Annika Antonnson (case 085). Louis Weiss and Ann Cali assisted with microsporidial identification.

Young, S., Speare, R., Berger, L., Skerratt, L. F. & Mendez, D. (2010). Emerging Amphibian Diseases and Disease Surveillance in Queensland – Stage 2 (February 2007 – April 2010). Final report for the project Emerging Amphibian Diseases and Disease Surveillance in Queensland – Stage 2 (February 2007 – April 2010) to the Australian Government Department of the Environment, Water, Heritage and the Arts. Available at: <http://www.environment.gov.au/biodiversity/invasive/publications/pubs/60776-emerging-amphibian-diseases.pdf>

### **3.2.2 Case report: papovavirus-associated dermal squamous papilloma**

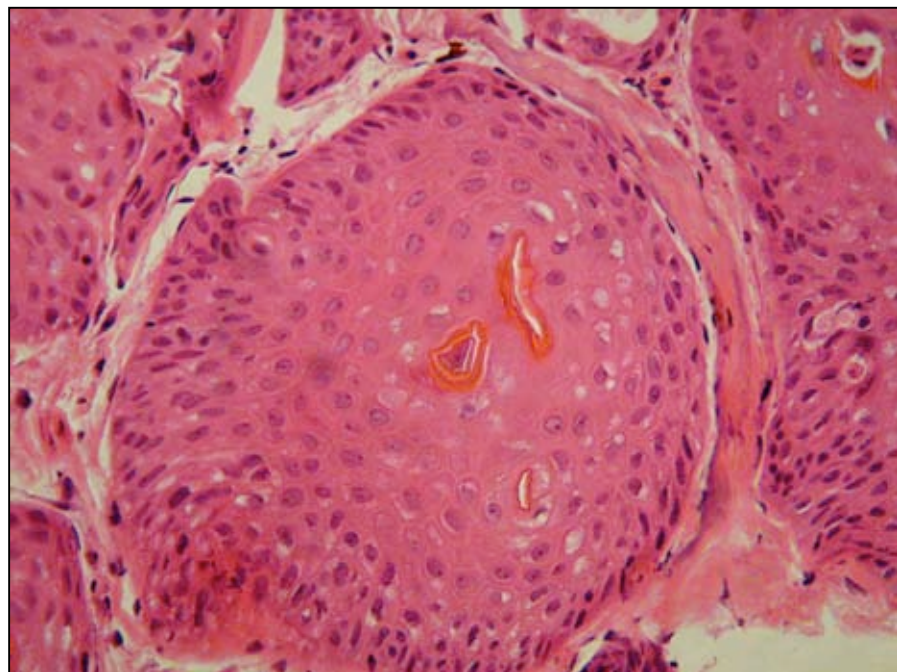
A large dermal squamous papilloma was diagnosed histologically following excisional biopsy of a cutaneous mass in a white-lipped tree frog that was otherwise clinically normal (*Litoria infrafrenata*), case 085 (Figures 3.1 and 3.2). Tissue was submitted for transmission electron microscopy and PCR analysis for further aetiological classification. Electron microscopy identified abundant large intracellular virus particles consistent with a member of the Papovaviridae family (Figure 3.3).

Poxvirus PCR testing was initiated prior to receiving the electron microscopy results. Exhaustive exclusionary PCR testing using avian poxvirus primers on preserved and fresh tissues failed to isolate viral nucleic acid. No poxviruses have been found in amphibians to date (Johnson & Wellehan, 2005).

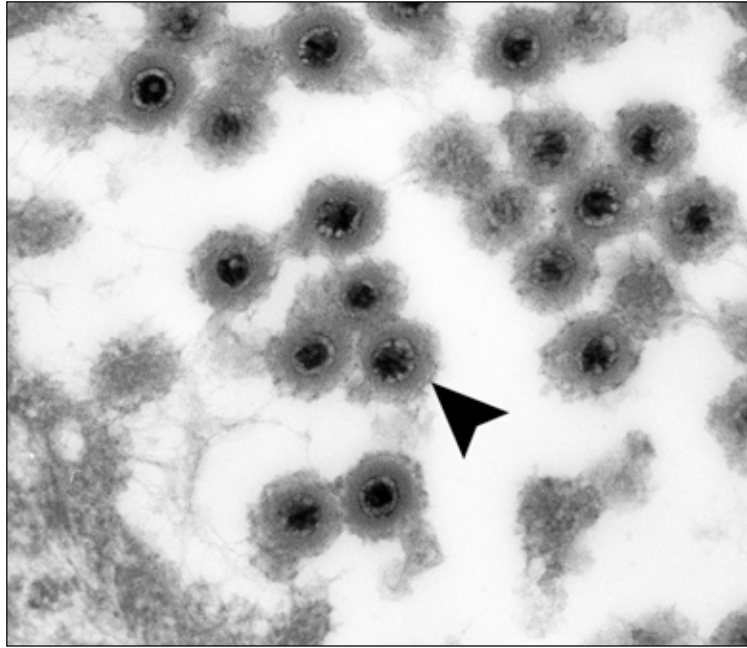
Rolling circular amplification PCR testing using human skin papillomavirus primers isolated a viral nucleic acid fragment from fresh tissue, but subsequent cloning and amplification of the fragment was unsuccessful. Papillomaviruses are commonly isolated from the healthy skin of humans, along with many other vertebrate species including mammals, birds, reptiles and amphibians, and are assumed to be commensals (Antonsson & Hansson, 2002; Antonsson & McMillan, 2006). The non-human papillomaviruses are sufficiently genetically related to their human counterparts to be identified by a human skin papillomavirus primer set (Forslund et al., 1999). Infection with oncogenic papillomaviruses causes epithelial proliferations in skin and mucosa (Bernard, 1994; Antonsson & McMillan, 2006). Epidermal papillomas have been relatively commonly reported in urodeles (Asashima et al., 1982, 1987; Pfeiffer et al., 1989; Trauth et al., 2002), and herpes-like virus bodies have been identified in papilloma cells from the Japanese newt (*Cynops pyrrhogaster*) (Asashima et al., 1982). There are no reports describing a viral aetiology for anuran papillomas to date.



**Figure 3.1** A large dermal squamous papilloma on the left hindleg of a white-lipped tree frog (*Litoria infrafrenata*, case 085). Scale = mm. Photo by Sam Young.



**Figure 3.2** Histological section of the dermal squamous papilloma from case 085. Stained with haematoxylin and eosin, 400x magnification. Photo by Ron Slocombe.



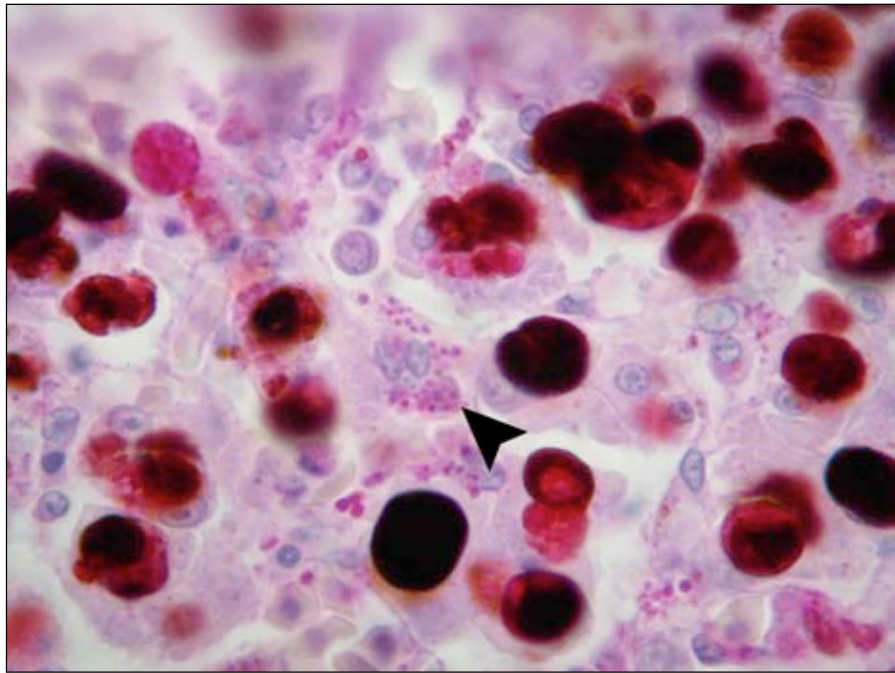
**Figure 3.3** Transmission electron micrograph of the dermal papilloma from case 085, showing multiple intracellular virus particles (arrow). 144,000x magnification. Photo by Liliana Tatarczuch.



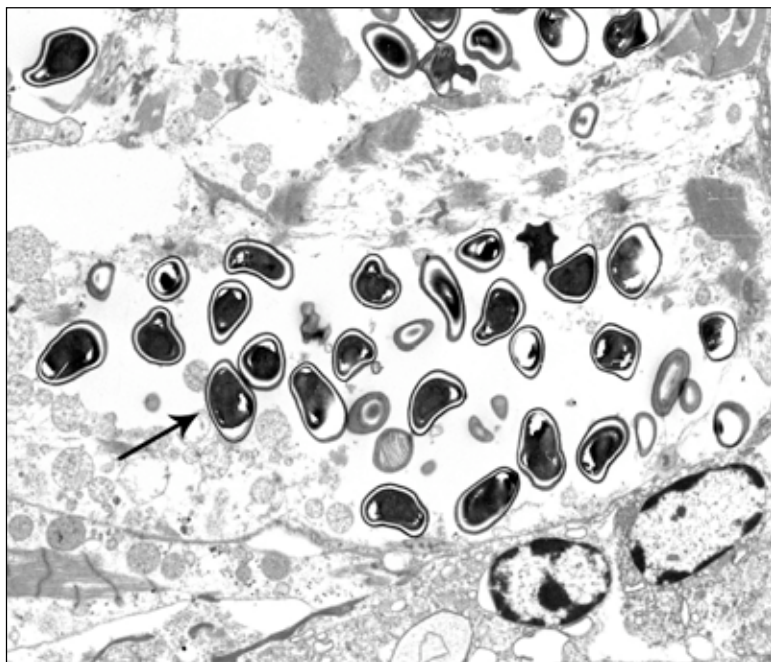
### 3.2.3 Case report: systemic microsporidiosis

Systemic infection with a protozoal-like organism was diagnosed histologically in a common green tree frog (*L. caerulea*), case 054 (Figure 3.4). The frog was found dead and submitted as a whole carcass preserved in neutral-buffered formalin. Heart, lung, liver, spleen, kidney, gastrointestinal tract, pancreas, skin, gonad, muscle and bladder were examined with light microscopy. Heart and liver were then submitted for transmission electron microscopy and PCR analysis for further aetiological classification. Electron microscopy identified a microsporidian as the causative agent (Figure 3.5), and expert assistance enabled further classification of the organism as one of two species from the genus *Anncaliia* (*A. algerae* or *A. vesicularum*; formerly genus *Brachiola*), most likely *A. algerae*. Nucleic acid extraction and amplification from preserved tissue samples was attempted but was unsuccessful.

*Anncaliia algerae* is a mosquito pathogen with a broad host range that has previously been associated with fatal myositis in humans (Coyle et al., 2004). With the exception of taxonomy, little work has been done on the biology or pathogenicity of Microsporidia in amphibians. *Pleistophora myotrophica* has been reported to cause high mortality rates in captive *Bufo bufo* following ingestion of infected prey and subsequent pathogen invasion of the musculoskeletal system via the blood stream (Canning et al., 1964). Ulcerative dermatitis has been reported in a single captive tree frog (*Phyllomedusa bicolor*), and microsporidian spores along with intracellular protozoal organisms were identified from stained impression smears of lesions with light microscopy (Graczyk et al., 1996). The organisms were not further classified, and the lesions resolved with a mixed antimicrobial treatment regime. This is the first known report of *Anncaliia* species infection in amphibians and only the third published report of a pathogenic microsporidian infection in this taxonomic host group.



**Figure 3.4** Histological section of liver from a common green tree frog (*Litoria caerulea*, case 054), showing multiple intracellular protozoal-like organisms (arrow). Stained with Periodic Acid Schiff, 1000x magnification. Photo by Ron Slocombe.



**Figure 3.5** Transmission electron micrograph of cardiac muscle from case 054, showing multiple intracellular microsporidial organisms (arrow). 7,360x magnification. Photo by Liliana Tatarczuch.

#### **3.2.4 *ARWH clinical pathology reporting parameters***

A detailed set of amphibian clinical pathology reporting parameters was developed during this study, representing part of the James Cook University (JCU) node in the Australian Registry of Wildlife Health (ARWH) (Table 3.1). I developed the original set of clinical pathology reporting parameters as part of a much larger joint project. This information was integrated into the database maintained by the Registry and developed further as an amphibian disease-reporting template. The project also involved setting up the Diagnostic Imaging Network System, a national collaborative diagnostic pathology network involving JCU, the Commonwealth Scientific and Industrial Research Organisation (CSIRO) and ARWH. Compilation and dissemination of this information was a collaborative effort between myself, Rick Speare, Lee Berger, Lee Skerratt, Diana Mendez, Karrie Rose, Alex Hyatt and many other colleagues affiliated with industry stakeholders.

Clinical Pathology Parameters	Reporting Categories
Morphometrics and clinical examination	body weight, snout-urostyle length, body condition, behaviour, posture, heart rate, respiratory rate, body temperature, mucous membrane colour, blood oxygen saturation, physical abnormalities
Haematology	blood collection method, volume collected, packed cell volume, buffy coat, total protein (refractometry), haemoglobin, serum/plasma colour, haemoparasites, red blood cell count, platelet count, total white blood cell count, differential white blood cell count
Serum/plasma biochemistry	aspartate aminotransferase, bile acids, creatinine kinase, uric acid, glucose, calcium, phosphorus, total protein, albumin, globulins, potassium, sodium, haemolysis, lipaemia, icteric index, comments
Serum protein electrophoresis	total protein, albumin, total globulins, globulin fractions
Urinalysis	urine specific gravity, dipstick (glucose, bilirubin, ketones, blood, pH, protein, urobilinogen), cytology, other, comments
Faecal analysis	form/colour, direct saline preparation, floatation, cytology (diff quik, gram, other), comments
General cytology	sample type, method, results, comments
Culture and sensitivity	sample type, method, results, comments
Chytrid qPCR	sample type, qPCR JCU ID, number wells positive, sample concentration
Other PCR assays	sample type, method, results, comments
Electron microscopy	sample type, method, results, comments
Necropsy	gross findings
Histology	findings by tissue type

**Table 3.1** Summary of the range of clinical pathology and anatomical reporting parameters for amphibian disease investigations developed during this study.

### **3.3 Using community surveillance data to investigate amphibian diseases**

This published peer-reviewed paper represents my original data organisation, classification, analysis and writing. Rick Speare, Lee Skerratt and Lee Berger assisted with data interpretation. Diana Mendez performed the histological analysis. Mike Steele performed the original statistical analysis and I performed additional revised analyses with advice on methodology from Lee Skerratt. Lee Berger, Lee Skerratt, Diana Mendez and Rick Speare provided substantial editorial input, Diana Mendez wrote the histology results section, and Mike Steele wrote the majority of the statistical methods section.

Young, S., Skerratt, L. F., Mendez, D., Speare, R., Berger, L. & Steele, M. (2012a). Using community surveillance data to differentiate between emerging and endemic amphibian diseases. *Diseases of Aquatic Organisms* 98: 1-10.

Since the journal uses American English, spelling follows this convention.

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Young, S., Skerratt, L. F., Mendez, D., Speare, R., Berger, L.  
& Steele, M. (2012a). Using community surveillance data to  
differentiate between emerging and endemic amphibian  
diseases. *Diseases of Aquatic Organisms* 98: 1-10.





















### **3.4 Occurrence of cloacal prolapse in wild hylid frogs**

This published peer-reviewed paper represents original surveillance data collection, analysis and writing by Andrea Phillott. My role in the paper included provision of veterinary technical expertise for results interpretation and substantial written contributions to the introduction and discussion sections.

Phillot, A. D. & Young, S. (2009). Occurrence of cloacal prolapse in wild hylids in the Wet Tropics, Australia. *Diseases of Aquatic Organisms* 86: 77-80.

Since the journal uses American English, spelling follows this convention.



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Phillot, A. D. & Young, S. (2009). Occurrence of cloacal prolapse in wild hylids in the Wet Tropics, Australia. *Diseases of Aquatic Organisms* 86: 77-80.







### **3.5 Progression of the surveillance research**

Results from my amphibian disease surveillance network, in conjunction with limited specimen availability for further investigation of the specific disease syndromes described in this chapter, led to a natural shift in the focus of my research. Efforts evolved away from passive disease surveillance towards the development of diagnostic tools to improve amphibian disease investigations in general. This then progressed further to specific research into amphibian immunity and chytridiomycosis, one of the most common diseases diagnosed in the frogs submitted to this surveillance network.

## CHAPTER 4

### Blood reference intervals for Australian tree frogs

#### 4.1 Introduction

Emerging infectious diseases are a significant issue for amphibian conservation and early detection via accurate disease diagnosis and active health surveillance of populations is vital. There are currently few, if any, methods validated for monitoring the health of amphibians. Blood parameters can provide valuable health information in a range of vertebrate taxa. Few studies exist that define amphibian haematologic and biochemical reference intervals.

This chapter consists of one peer-reviewed paper accepted for publication that establishes haematologic and plasma biochemistry reference intervals for two frog species (*L. caerulea* and *L. infrafrenata*), describes their leucocyte morphology, and analyses the effects of season, year and parasite status on blood values. Wide inter-species and seasonal variations were found, highlighting the need to establish species- and season-specific reference intervals in amphibians. This is the first known report establishing comprehensive haematologic and biochemical reference values for: (i) clinically normal free-ranging anurans, (ii) Australian frog species; and (iii) different seasons in anurans, and is the first study of this magnitude in amphibians. Results demonstrate that haematology and plasma biochemistry are useful for assessing the health status of wild amphibians, and may assist in the future detection of emerging diseases.

#### 4.2 Blood reference intervals for health monitoring of wild Australian tree frogs

This peer-reviewed paper represents my original research and writing and has been accepted for publication in the journal *Veterinary Clinical Pathology*. I received study design advice from Rick Speare, Lee Berger and Lee Skerratt, technical methodology advice and cell identification assistance from Jeff Warner, and editorial contributions from Jeff Warner, Lee Berger and Lee Skerratt. Reinhold Muller performed all of the statistical analyses. Helen Martin and Deb Buckett at Gribbles Veterinary Pathology Laboratory performed the serum protein electrophoresis. Jamie Voyles, Rob Puschendorf and Paul Whitehorn helped with field collection of frogs.

Standard abbreviations used by the journal *Veterinary Clinical Pathology* are defined below.

ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANOVA	analysis of variance
AST	asparate aminotransferase
ATP	adenosine triphosphate
CBC	complete blood count
CK	creatine kinase
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
GGT	gamma-glutamyl tranferase
HCT	haematocrit
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
MCV	mean corpuscular volume
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
PCR	polymerase chain reaction
PCV	packed cell volume
RBC	red blood cell
RNA	ribonucleic acid
SD	standard deviation
SEM	standard error of the mean
WBC	white blood cell
H&E	haematoxylin and eosin

Young, S., Warner, J., Speare, R., Berger, L., Skerratt, L. F. & Muller, R. Hematologic and plasma biochemical reference intervals for health monitoring of wild Australian tree frogs. *Veterinary Clinical Pathology* (In press).

The following paper is an exact, word-for-word copy of the manuscript accepted for publication. It is scheduled for print in the December 2012 volume of *Veterinary Clinical Pathology*. Table and figure numbering has been reformatted for this thesis and section numbering has been added for ease of reference. Since the journal uses American English, spelling follows this convention.



ORIGINAL RESEARCH

**Hematologic and plasma biochemical reference intervals for health monitoring of wild Australian tree frogs**

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**Key Words**

Biochemistry, health monitoring, hematology, *Litoria caerulea*, *Litoria infrafrenata*, reference values

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**Running short title:** Blood values for Australian tree frogs

**Running authors:** Young et al

#### 4.2.1 Abstract

**Background:** Few hematologic and biochemical reference intervals for wild amphibians have been established. Reference values would aid in early detection of emerging infectious diseases, which are a significant problem for amphibian conservation efforts.

**Objective:** We aimed to establish reference intervals for a wide range of hematologic and plasma biochemistry variables for 2 species of Australian tree frogs, describe morphologic features of leukocytes, and analyze the effects of season, year, and parasite status on blood values.

**Methods:** Blood samples were collected from reference populations of wild adult Australian tree frogs, *Litoria caerulea* and *L. infrafrenata*, for analysis of hematologic (manual) and plasma biochemical (automated) variables and proteins by automated methods, refractometry, and electrophoresis.

**Results:** Inter- and intra-species differences were found in *L. caerulea* ( $n = 80$ ) and *L. infrafrenata* ( $n = 66$ ) frogs for hematologic and biochemical variables. Intra-species differences were largely associated with seasonal variations. In the dry season, both species had higher WBC counts, with higher lymphocyte counts in *L. caerulea* and higher neutrophil counts in *L. infrafrenata*, and uric acid concentrations. In the wet season, both species had higher glucose and potassium concentrations, *L. caerulea* frogs had higher neutrophil counts, and *L. infrafrenata* frogs had higher total protein, phosphorus and sodium concentrations, AST activity, PCV, hemoglobin concentration, and RBC, thrombocyte, and basophil counts. Hemogregarines were identified in 19% of blood samples from *L. infrafrenata* frogs; multiple hematologic and biochemical variables were altered in infected frogs.

**Conclusions:** Wide inter-species and seasonal variations highlight the need to establish species- and season-specific reference intervals in amphibians. Hematologic and plasma biochemical reference values should be useful in assessing the health status and in detecting emerging diseases in wild amphibians.

#### 4.2.2 Introduction

Emerging infectious diseases are a significant issue for amphibian conservation; early detection based on accurate diagnosis and active health surveillance of populations is vital. Currently, few methods have been validated for monitoring the health of amphibians. Evaluation of hematologic and biochemical variables can provide valuable diagnostic information and aid in understanding pathogenesis of disease, evaluating nutritional status, detecting exposure to toxins and pollutants, and monitoring the health of vertebrate populations.<sup>1</sup> Little information is available on hematologic and biochemical reference values in clinically healthy frogs, and information is lacking for free-ranging amphibians, Australian frogs, and seasonal variations within species.

Three comprehensive reports on morphologic, cytochemical, and ultrastructural features of amphibian blood cells are limited to anurans of commercial importance, primarily *Bufo*, *Rana*, and *Xenopus* species,<sup>2,3,4</sup> but provide a solid foundation for identification of amphibian blood cells and for understanding hematopoiesis. The few reports on amphibian hematologic and biochemical reference values are dated, difficult to obtain, or of limited clinical value owing to small sample sizes.<sup>5,6</sup> A recent report of normal values for the American bullfrog, *Rana catesbeiana*, was limited to 14 anesthetized adult laboratory frogs with 7 individuals of each sex.<sup>5</sup>

Establishing reference intervals in reptiles and amphibians is complicated by difficulties associated with specimen collection and variations in analytes related to age, sex, nutritional status, health status, temperature, season, handling, and analytical methods.<sup>6-11</sup> Several biochemical analytes, including plasma protein, sodium, calcium, glucose, and uric acid concentrations, have been reported to vary widely depending on intrinsic and extrinsic factors.<sup>5,6,12-17</sup> Plasma potassium concentration was relatively stable throughout the year in one anuran species.<sup>8</sup>

There have been fewer studies on factors affecting specific amphibian hematologic values.<sup>5,6,10,11</sup> Environmental temperature can affect PCV, hemoglobin (Hgb) concentration, and RBC count in reptiles and amphibians.<sup>7,16,18</sup> Considerable expertise is needed for accurate WBC differential counts and their interpretation in amphibians, as granulocytes have been poorly described and considerable inter-species variations in staining and morphologic characteristics occur.<sup>2,4,6,19</sup> Although amphibian granulocytes are thought to function similarly to mammalian counterparts, this has not been proven.<sup>6</sup> Amphibian and reptilian neutrophil and lymphocyte counts may be affected by various factors, including sex, age, season, stress, pollutants,

hematozoa, and viral infections.<sup>1,10,12,16,20</sup> Although the typical mammalian stress leukogram has been reported in one anuran species,<sup>21</sup> the stress response of lymphocytes in reptiles and amphibians is unclear. Amphibian eosinophils are thought to respond to stimuli such as parasites and pollutants.<sup>6,10,22</sup> Basophil numbers vary widely between amphibian species, and degranulation is relatively common in some species;<sup>3,6,19,23</sup> they may play a significant role in immunosurveillance and host response to helminth infections and allergens, as in other vertebrates.<sup>3,6,19</sup> Common intraerythrocytic parasites found in amphibians include hemogregarines and *Aegyptianella* rickettsial species.<sup>6,24-26</sup>

The objectives of this study were to 1) establish reference intervals for a wide range of hematologic and plasma biochemical variables for 2 species of Australian tree frogs, describe light microscopic morphologic features of leukocytes, and identify differences between species, and 2) identify the effect of season (wet versus dry), year (2007 versus 2008), and endemic parasite status on blood values within each species. We hypothesized that both extrinsic and intrinsic factors would affect measured analytes.

### 4.2.3 *Materials and methods*

#### *Study period and experimental animals*

All procedures involving animals were approved by the James Cook University Animal Ethics Committee (approval number A1085) and Queensland Parks and Wildlife Service (Scientific Purposes Permit number WISP03866106). Free-ranging clinically healthy adult\* common green (*Litoria caerulea*) and white-lipped (*L infrafrenata*) tree frogs were captured from widespread residential and semi-rural areas in and around Cairns and Townsville in far northern Queensland, Australia. The 2 species were selected based on their large mature body size, relative ease of capture, endemism, wide distribution, and stable conservation status (Least Concern).<sup>27</sup> Blood was collected during 2 consecutive wet (November to April) and dry (May to October) seasons in 2007 and 2008; individual frogs were sampled only once during the study period. Handlers wore a new powder-free nitrile medical examination glove (Supergloves Australia Pty Ltd, Gold Coast, Queensland, Australia) for each frog procedure. Each frog was manually placed into an individual plastic holding container, 70 × 95 × 150 mm<sup>3</sup>, for transport. Frogs were housed in individual plastic containers, 230 × 230 × 350 mm<sup>3</sup>, in quarantine facilities at James Cook University, Cairns, Australia that were controlled for temperature (20–22°C) and light (12 hours of light/12 hours of darkness) for a maximum of 48 hours before release back into the wild. Aged tap water was changed daily and frogs were fed large domestic crickets (*Acheta domestica*, Pisces Enterprises Inc., Kenmore, Queensland, Australia) dusted with superfine calcium carbonate (Cattlekare, Dandenong, Victoria, Australia) and multivitamin powder (Reptivite, Zoo Med Laboratories Inc., San Luis Obispo, CA, USA) ad libitum each day.

#### *Specimen collection and processing*

Blood in a volume of 250–500 µL, comprising < 1 % of a frog's body weight, was collected from dorsally recumbent anesthetized frogs by cardiocentesis with a 1-mL syringe and 25-gauge needle (Terumo Corporation, Binan, Laguna, Philippines) following shallow immersion in 0.20 % (*L infrafrenata*) or 0.25 % (*L caerulea*) ethyl 3-aminobenzoate methanesulfonic acid solution (tricaine methanesulfonate, Sigma-Aldrich Inc., St Louis, MO, USA) buffered with 10 mmol/L sodium bicarbonate solution (8.4 %, Pro Care Animal Health, Dandenong, Victoria, Australia). Fresh blood smears were made immediately after collection and were air-dried and immediately fixed in 100% methanol. An aliquot of blood was placed in a 200-µL pediatric lithium heparin tube (Microtainer, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). When possible,

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\* Frogs were classified as adult if they had markings and other physical characteristics consistent with maturity, and a snout-urostyle length > 60 mm.

depending on the volume of blood collected, 150-200  $\mu\text{L}$  were placed in a plain 1.0-mL microcentrifuge tube (Eppendorf AG, Hamburg, Germany), and immediately centrifuged at 5590g for 10 minutes; serum was removed and refrigerated at 4°C before analysis within 24-48 hours.

### ***Clinical examination and parasitology***

Body weight, snout-urostyle length, body condition score (1 = poor, 2 = fair, 3 = good, 4 = very good, 5 = obese), body temperature<sup>†</sup>, manual heart rate, and pulse oximetry (heart rate and oxygen saturation) measurements were recorded for each frog. All individuals were examined by a veterinarian (SY); abnormalities were recorded and each frog received a physical examination score: 0 = no abnormalities detected, 1 = insignificant healed injury, 2 = mild superficial localized lesion, 3 = moderate/severe superficial localized lesion, 4 = multiple superficial abnormalities, and 5 = generalized/systemic abnormality.

A swab sample was collected from the ventral skin surface of each anesthetized frog for determination of *Batrachochytrium dendrobatidis* zoospore equivalents by real-time PCR analysis (School of Public Health, Tropical Medicine and Rehabilitation Sciences, James Cook University).<sup>28</sup> When possible, a fecal sample was collected and a standard fecal floatation performed.<sup>29</sup> Gastrointestinal and pulmonary parasite ova and larvae were classified by morphologic light microscopic characteristics.<sup>29</sup> Fecal parasite burdens were scored at 50 $\times$  magnification as: 0 = no parasitic ova/larvae present, 1 = < 10 per slide, 2 = up to 1/field, and 3 = > 1/field. Hemoparasites were evaluated as part of the hematologic analysis.

### ***Hematologic analysis***

Total RBC, WBC, and thrombocyte counts were performed manually by a veterinary clinical pathologist (SY) at 400 $\times$  magnification using a modified Neubauer hemocytometer (Brand, Wertheim, Germany) with Natt-Herrick's solution as the diluent as previously described.<sup>6,30</sup> For differential leukocyte counts, WBCs were counted at 1000 $\times$  magnification on Wright's-stained blood smears (Clinipure Wright's Stain and Wright's Buffer Concentrate, HD Scientific Supplies Pty Ltd, Wetherill Park, New South Wales, Australia). Polychromatophilic RBCs, basophilic erythroblasts, mitotic RBCs, anucleate RBCs, progranulocytes, and promononuclear cells were counted and recorded per 100 mature WBC counted. Intracytoplasmic RBC parasites and inclusions were counted and recorded as % of total RBC count. On each blood smear, dimensions of RBCs, WBCs, and thrombocytes were measured using a calibrated micrometer. Well-mixed whole non-anticoagulated blood (5  $\mu\text{L}$ ) was drawn into a pediatric microhematocrit

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<sup>†</sup> Surface body temperature was measured over the dorsum using a hand-held infrared temperature sensor.

tube (Becton, Dickinson and Company), scanned at 40× magnification for microfilarial larvae, and centrifuged at 112g for 2 minutes) for determination of PCV and buffy coat measurements.

Hemoglobin concentration was measured manually using the cyanomethemoglobin method modified for species with nucleated RBC.<sup>32,33</sup> Absorbance was measured at 540 nm with a microplate absorbance spectrophotometer (Bio-Rad Laboratories Pty Ltd, Hercules, CA, USA). Assayed whole blood Meter Trax Control solutions for Hgb testing (Bio-Rad Laboratories Pty Ltd, Regent's Park, NSW, Australia) were used to establish a standard curve, which was used to determine Hgb concentrations of blood collected from frogs. MCV, MCH, and MCHC were calculated from Hgb concentration, PCV, and RBC counts using standard formulae.<sup>1</sup>

### ***Plasma biochemical analysis***

Plasma biochemical analytes were measured in 100 µL of heparinized whole blood using the automated bench-top VetScan VS2 Chemistry Analyzer and VetScan Avian/Reptilian Profile Plus rotor (Abaxis Inc., Union City, CA, USA). Measurements included activities of aspartate aminotransferase (AST) and creatine kinase (CK) and concentrations of uric acid, glucose, calcium, phosphorus, potassium, sodium, total protein (TP), albumin, globulin, and quantitative Hgb as an index of plasma hemolysis. VetScan TP and albumin concentrations are measured using the biuret and bromocresol green dye-binding methods, respectively. TP in serum from the centrifuged microhematocrit tube was also measured manually using a commercially available temperature-compensated hand-held refractometer (VetQuip, Castle Hill, New South Wales, Australia).

### ***Serum proteins***

Available serum samples were submitted to a commercial reference laboratory (Gribbles Veterinary Pathology, Clayton, Victoria, Australia) for measurement of TP concentration by the biuret method and albumin and globulin by agarose gel electrophoresis. These values were compared with values obtained for heparinized whole blood from the VetScan chemistry analyzer (TP and albumin concentrations with calculation of globulin concentration) and for serum values obtained by refractometry (TP concentration) to assess method concordance.

### ***Statistical analysis***

Up to 98 variables were measured for each frog, including spatial, temporal, physical, and laboratory values. Reference limits calculated as the 2.5th and 97.5th percentiles, together with their 95% confidence intervals (CI), of hematologic and plasma biochemical variables were established from the distribution of reference values using a nonparametric bootstrap approach (with 10,000 replicates) in accordance with current international guidelines using Stata v.

11/2011 (StataCorp LP, College Station, TX, USA).<sup>33,34</sup> As a strictly nonparametric approach was used to generate reference limits, data transformations or exclusions of extremes were not applied, or considered necessary.

Thirty-eight variables were selected and analyzed for inter- and intra-species differences with respect to season (wet/dry) and year (2007/2008): 2 physical (body condition and fecal parasite burden scores), 23 hematologic, 10 biochemical, and 3 protein variables determined by biuret and electrophoretic methods. As distributions of most variables proved to be skewed when examined with Kolmogorov-Smirnov tests and simple median/mean comparisons, median and inter-quartile ranges were reported for descriptive purposes and unpaired nonparametric Mann-Whitney tests were used for inter- and intra-species statistical comparisons. The physical variables proved to be normally distributed and consequently were reported as mean +/- SD. Three physical variables (body weight, body condition, and fecal parasite burden scores), 21 hematologic variables (including % polychromatophilic RBCs, basophilic erythroblasts, and mitotic RBCs), and 2 biochemical variables (TP and hemolysis index) were analyzed using unpaired nonparametric Mann-Whitney tests to determine intra-species effects of gastrointestinal and pulmonary parasites (both species) and intraerythrocytic hemogregarines (*L. infrafrenata*) on blood values.

Concordance of analytic methods for TP, albumin, and globulin was assessed by scatterplots, including lines of identity, and analysis of variance-based intra-class correlation coefficients.<sup>35</sup> The software package PASW Statistics v. 18/2009 (SPSS Inc., Chicago, IL, USA) was used for concordance analyses. Statistical significance was set at  $P \leq .050$  for all analyses.



#### 4.2.4 Results

##### *Clinical examination and parasitology*

The number of frogs sampled was 161. Physical data values (mean +/- SD) for *L caerulea* ( $n = 80$  except for fecal parasite burden score) and *L infrafrenata* ( $n = 81$  except for fecal parasite burden score), respectively, were: body weight, 51.5 +/- 17.0 g and 54.2 +/- 19.5 g; snout-urostyle length, 81.6 +/- 8.6 mm and 91.5 +/- 9.3 mm; body condition score, 3 +/- 0.4 and 2.8 +/- 0.5; physical examination score, 0.5 +/- 0.8 and 0.6 +/- 0.9; and fecal parasite burden score, 0.8 +/- 0.9 ( $n = 71$ ) and 1.1 +/- 0.9 ( $n = 65$ ). For all 161 frogs, body temperature at the time of sample collection ranged from 20.4-23.8°C, and all frogs were negative by PCR for *B. dendrobatidis* infection.

Hemogregarines and microfilaria were not detected in *L caerulea*. Intracytoplasmic RBC hemogregarine gametocytes were identified in 19% (15/81) of samples from *L infrafrenata*, with organisms found in 0.1-10% of total RBC (Figure 4.1). In a single sample from *L infrafrenata*, 2 extra-erythrocytic microfilaria were detected; this sample also contained hemogregarine organisms. Although hemogregarine-infected *L infrafrenata* were clinically healthy, differences in 8 of 26 variables differed between infected and uninfected frogs, and infected frogs were excluded retrospectively from further analyses.

There were no differences in median body condition score ( $P = .052$ ) or fecal parasite burden score ( $P = .330$ ) between the 2 species or within each species between season (wet versus dry) or year (2007 versus 2008) ( $P > .050$  in all cases). For both species, there were no differences in body condition score or any of the hematologic variables between frogs positive for gastrointestinal and pulmonary parasites (41/71 *L caerulea*, 38/53 *L infrafrenata*), most commonly *Rhabdias* spp., and those negative for these parasites (30/71 *L caerulea*, 15/53 *L infrafrenata*) ( $P > .010$  in all cases). Clinical findings included healed injuries without physical impairment in 6/80 *L caerulea* and 6/66 *L infrafrenata*, and mild superficial localized skin abrasions in 15/80 *L caerulea* and 9/66 *L infrafrenata*. None of the frogs had a physical examination score  $> 2$ .

##### *Hematology*

Hematologic reference intervals for *L caerulea* ( $n = 80$ ) and *L infrafrenata* ( $n = 66$ ) frogs were established (Table 4.1) with inter-species differences found for 15 of 23 variables. Serum and plasma were clear and straw-colored from *L caerulea* frogs and clear and bright blue from *L infrafrenata* frogs.

Lymphocytes were the most abundant WBC present in both frog species, followed by neutrophils, monocytes, eosinophils, and basophils. Lymphocytes were morphologically similar to those of other vertebrate taxa. Two distinct neutrophil phenotypes were identified in both species: large round cells with no or indistinct cytoplasmic staining, consistent with the mammalian counterpart and classified as large neutrophils (Figure 4.2) and smaller round cells with distinctly eosinophilic cytoplasmic staining with or without granular detail and classified as small neutrophils (Figure 4.3). Small neutrophils predominated in *L caerulea* and were present in 100% (80/80) of frogs; large neutrophils predominated in *L infrafrenata* and were present in 71% (54/66) of frogs. Both phenotypes were present in 2.5% (2/80) of *L caerulea* and 27% (18/66) of *L infrafrenata* frogs. Prominent Döhle body-like intracytoplasmic inclusions were present in all *L caerulea* frogs (80) in 97.9 +/- 5.1% (mean +/- SD) of total neutrophils and in all *L infrafrenata* frogs (66) in 86.0 +/- 15.9% of total neutrophils (Figure 4.2). There were also two distinct monocyte phenotypes identified in both species: large round cells with abundant pale blue/grey cytoplasm, consistent with the mammalian counterpart and classified as large monocytes (Figure 4.4) and smaller round cells with less abundant more densely basophilic cytoplasm and classified as small monocytes<sup>‡</sup> (Figure 4.5). Small monocytes predominated and were present in 100% (80/80) *L caerulea* and 88% (58/66) *L infrafrenata* frogs. Both phenotypes were present in 10% (8/80) of *L caerulea* and 17% (11/66) of *L infrafrenata* frogs. Cytoplasmic vacuolation was noted occasionally in neutrophils and less frequently in monocytes.

Eosinophils were distinctive large round cells with deeply eosinophilic, usually refractile, round cytoplasmic granules and were present in 78% (62/80) *L caerulea* and 38% (25/66) *L infrafrenata* frogs. In *L caerulea*, granules were abundant and regular in size and stain uptake (Figure 4.6); in *L infrafrenata*, granules varied in both size and stain uptake (Figure 4.7). Degranulation of eosinophils occurred in 5% (3/62) *L caerulea* and 16% (4/25) *L infrafrenata* frogs in up to 50 and 100% of cells, respectively. Basophils were distinctive, irregular ovoid cells with large densely staining purple-black round cytoplasmic granules, typically obscuring all nuclear and cytoplasmic detail, and were present in 5% (4/80) *L caerulea* and 30% (20/66) *L infrafrenata* frogs (Figures 4.2 and 4.8). Degranulation of basophils occurred in 75% (3/4) *L caerulea* and 25% (5/20) *L infrafrenata* frogs in up to 100 and 40% of cells, respectively. A subset of basophils with poor granular stain uptake, classified as poorly differentiated basophils, was identified in 45% (9/20) of *L infrafrenata* frogs in up to 100% of their basophils. Condensed karyorrhectic granulocytes were unable to be further classified in samples from 40%

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<sup>‡</sup> Small monocytes were differentiated from lymphocytes based on cytoplasm colour, nucleus shape and cytoplasm-nucleus ratio.

(32/80, 0.7 +/- 1.0% [mean +/- SD] total WBC) *L caerulea* and 26% (17/66, 0.8 +/- 1.6% total WBC) *L infrafrenata* frogs.

Based on median sizes of blood cells from each species (Table 4.2), mature RBC were the largest cells for both species, followed by eosinophils, large monocytes, large neutrophils, small neutrophils, basophils, small monocytes, and lymphocytes for *L caerulea* and large neutrophils, eosinophils, large monocytes, small neutrophils, small monocytes, basophils, and lymphocytes for *L infrafrenata*. As cytoplasmic membranes of thrombocytes were rarely clearly defined, only nuclear dimensions were recorded for the majority of samples; whole thrombocytes were distinctive in samples from 2 *L caerulea* frogs and measured  $4.0 \times 13.0$  and  $6.0 \times 12.0$   $\mu\text{m}$ . Significant differences in sizes of mature RBCs, thrombocyte nuclei, lymphocytes, small monocytes, large monocytes, and eosinophils were found between the 2 species ( $P \leq .050$  in all cases). Polychromatophilic RBCs were present in 100% (80/80, up to 25% of total RBC) *L caerulea* and 96% (63/66, up to 15% of total RBC) *L infrafrenata* frogs (Figure 4.9). Basophilic erythroblasts were identified in 59% (47/80, up to 19 cells per 100 WBCs) *L caerulea* and 62% (41/66, up to 18 cells per 100 WBCs) *L infrafrenata* frogs (Figure 4.9). Mitotic and anucleate RBC, respectively, were identified in low numbers in samples from 9% *L caerulea* and 17% *L infrafrenata* frogs and from 9% *L caerulea* and 9% *L infrafrenata* frogs.

For *L caerulea* frogs, differences were found between samples collected in the wet and dry seasons for 4 of 20 hematologic variables (Table 4.3). MCH was significantly greater in *L caerulea* samples from 2007 ( $n = 39$ ) with median (IQR) MCH of 138 pg (114 – 156) compared with 2008 ( $n = 41$ ) with median (IQR) MCH of 121 pg (109 – 138) ( $P = .038$ ). There were no differences between 2007 and 2008 for samples from *L caerulea* for any of the other hematologic variables ( $P > .050$  in all cases). For *L infrafrenata* frogs, seasonal differences were found for 10 of 20 hematologic variables (Table 4.3). There were no differences between 2007 ( $n = 26$ ) and 2008 ( $n = 40$ ) for samples from *L infrafrenata* for any of the hematologic variables ( $P > .050$  in all cases).

### ***Plasma biochemical analytes***

Reference intervals for plasma biochemical analytes were established for *L caerulea* ( $n = 80$ ) and *L infrafrenata* ( $n = 66$ ) frogs (Table 4.4) with inter-species differences found for 7 of 10 analytes. For *L caerulea* frogs, seasonal differences were found for 3 of 10 analytes (Table 4.5). Potassium concentration and CK activity were significantly higher in samples from 2008 compared with 2007: for potassium, median concentration (IQR) was 6.7 mmol/L (5.1 – 8.2) in 2008 ( $n = 41$ ) and 5.6 mmol/L (4.5 – 6.5) in 2007 ( $n = 39$ ) ( $P = .018$ ); for CK, median activity (IQR) was 557 U/L (410 – 720) in 2008 ( $n = 39$ ) and 428 U/L (236 – 670) in 2007 ( $n = 39$ ) ( $P =$

.034). There were no differences between 2007 and 2008 in *L caerulea* for any other analytes ( $P > .050$  in all cases). For *L infrafrenata* frogs, seasonal differences were found for 8 of 10 analytes (Table 4.5). Calcium:phosphorus ratio was significantly higher in samples from 2008 ( $n = 40$ ) with a median ratio (IQR) of 2.00 (1.71 – 2.37) compared with 2007 ( $n = 26$ ) with a median ratio (IQR) of 1.80 (1.46 – 1.99) ( $P = 0.017$ ). There were no differences between 2007 and 2008 for any other analytes ( $P > .050$  in all cases).

### ***Serum proteins***

Reference intervals for serum TP measured by the reference laboratory and by protein electrophoresis were established for the 2 frog species (Table 4.6). Median TP and albumin concentrations were significantly higher in *L caerulea* ( $n = 27$ ) compared with *L infrafrenata* ( $n = 13$ ). TP, albumin, and globulin concentrations determined by a combination of the biuret method and electrophoresis, representing the reference (gold standard) methods, were compared with concentrations obtained using the VetScan and refractometry to assess agreement. All 51 serum samples that had been collected, including those from hemogregarine-infected *L infrafrenata* excluded from the reference population ( $n = 11$ ), were included in the analytic method concordance analysis. For TP, relatively good correlation was found between protein electrophoresis and the VetScan (intra-class correlation coefficient 0.93,  $P < .001$ ,  $R^2$  -0.78) and refractometer (intra-class correlation coefficient 0.86,  $P < .001$ ,  $R^2$  -0.77) methods. Conversely, protein electrophoresis and VetScan methods for concentrations of both albumin (intra-class correlation coefficient 0.76,  $P < .001$ ,  $R^2$  -0.70) and globulin (intra-class correlation coefficient 0.25,  $P < .001$ ,  $R^2$  -0.44) were only weakly correlated.

### ***Effect of Hemogregarine infection in Litoria infrafrenata***

Hemogregarine infection occurred more commonly in *L infrafrenata* ( $n = 81$ ) in the wet than in the dry season ( $P = .050$ ). Hemogregarine-infected (15/81) and uninfected (66/81) *L infrafrenata* frogs had differences in 8 of 26 variables: median thrombocyte, total WBC and absolute lymphocyte counts and percent polychromatophilic cells were significantly higher, whereas median body weight, TP concentration (refractometry), relative neutrophil count, and hemolysis index were significantly lower in infected individuals ( $P \leq .050$  in all cases) (Table 4.7).

**Table 4.1.** Hematologic reference intervals for common green (*Litoria caerulea*) and white-lipped (*L. infrafrenata*) tree frogs.

Species	<i>Litoria caerulea</i> (n = 80)						<i>Litoria infrafrenata</i> (n = 66)						P - value*
	Median	IQR	2.5th Quantile	95% CI	97.5th Quantile	95% CI	Median	IQR	2.5th Quantile	95% CI	97.5th Quantile	95% CI	
PCV (%)	38.0	34.0-40.8	23.0	14.0-32.1	48.0	46.7-49.3	30.0	26.0-34.0	19.4	16.8-21.9	48.6	41.5-55.7	<.001
Buffy Coat (%)	2.0	2.0-2.0	1.0	ND	3.0	ND	2.0	1.0-2.0	1.0	ND	4.0	ND	.831
Hemoglobin (g/dL)	9.3	8.0-10.6	4.1	2.7-5.6	12.6	11.5-13.6	7.0	6.1-8.2	3.3	2.4-4.2	11.7	10.2-13.2	<.001
RBC ( $\times 10^9/L$ )	735	623-818	420	300-540	1018	849-1187	720	628-820	401	321-480	1123	1048-1198	.959
MCV (fL)	507	461-602	349	328-370	737	595-878	418	374-486	284	268-301	625	537-714	<.001
MCH (pg)	126	111-148	75	58-93	198	155-241	99	84-115	51	42-61	155	119-191	<.001
MCHC (g/L)	255	236-268	139	113-165	323	269-378	236	210-250	153	133-172	315	291-339	<.001
Thrombocyte ( $\times 10^9/L$ )	27.3	23.2-33.5	13.3	9.6-17.0	49.1	44.0-54.1	31.9	25.8-38.8	20.0	17.3-22.8	62.5	57.2-67.8	.004
WBC ( $\times 10^9/L$ )	15.9	12.4-22.1	6.7	4.9-8.5	34.9	29.8-40.0	21.0	14.2-29.1	6.5	2.8-10.2	47.9	40.6-55.1	.011
Neutrophil (%)	21.5	14.0-27.0	7.0	2.9-11.2	42.0	36.7-47.2	20.0	15.0-32.0	5.0	1.6-8.4	50.3	45.5-55.1	.406
Neutrophil ( $\times 10^9/L$ )	3.3	2.2-4.8	0.93	0.58-1.27	7.7	6.6-8.7	4.2	2.4-7.4	0.63	0.13-1.12	17.1	14.0-20.3	.024
Lymphocyte (%)	67.5	61.0-76.0	40.2	28.0-52.4	88.0	85.4-90.5	70.0	57.0-78.3	33.4	25.4-41.4	85.0	84.3-85.7	.926
Lymphocyte ( $\times 10^9/L$ )	10.7	7.8-15.2	3.9	3.0-4.8	27.1	23.6-30.5	12.2	8.7-19.4	3.2	1.8-4.7	34.7	25.5-43.8	.065
Monocyte (%)	7.0	5.0-10.0	2.0	0.92-3.13	18.0	10.8-25.1	6.0	4.0-8.0	1.0	0.58-1.42	21.3	16.9-25.7	.021
Monocyte ( $\times 10^9/L$ )	1.3	0.8-1.8	0.31	0.24-0.37	4.7	3.5-5.9	1.1	0.6-2.0	0.1	0.05-0.20	6.9	4.9-8.8	.681

Eosinophil (%)	2.0	1.0-5.0	0.0	ND	11.0	ND	0.0	0.0-1.3	0.0	ND	10.6	ND	<.001
Eosinophil ( $\times 10^9/L$ )	0.4	0.1-0.7	0.0	ND	3.1	ND	0.0	0.0-0.3	0.0	ND	2.0	ND	<.001
Basophil (%)	0.0	0.0-0.0	0.0	ND	7.0	ND	0.0	0.0-1.0	0.0	ND	31.0	ND	<.001
Basophil ( $\times 10^9/L$ )	0.0	0.0-0.0	0.0	ND	1.1	ND	0.0	0.0-0.1	0.0	ND	4.0	ND	<.001
Polychromatophilic Cells (%)	4.0	3.0-7.0	1.0	ND	15.0	ND	1.0	0.4-3.3	0.0	ND	15.0	ND	<.001
Basophilic Erythroblasts†	1.0	0.0-2.0	0.0	ND	7.0	ND	1.0	0.0-3.0	0.0	ND	17.3	ND	.553
Progranulocytes†	0.0	0.0-0.0	0.0	ND	1.0	ND	0.0	0.0-0.0	0.0	ND	1.0	ND	.250
Promononuclear WBC†	1.0	0.0-1.0	0.0	ND	4.0	ND	0.0	0.0-1.0	0.0	ND	3.3	ND	.003

Data are bootstrapped 2.5th and 97.5th quantiles, 95% confidence intervals (CI), medians, and inter-quartile ranges (IQR).

\**P*-values refer to nonparametric Mann-Whitney tests between species.

†Cells/100 WBCs.

ND indicates not determined.

**Table 4.2.** Reference intervals for dimensions of blood cells from common green (*Litoria caerulea*) and white-lipped (*L. infrafrenata*) tree frogs.

Species	<i>Litoria caerulea</i>							<i>Litoria infrafrenata</i>						
	Median	IQR	2.5th Quantile	95% CI	97.5th Quantile	95% CI	<i>n</i>	Median	IQR	2.5th Quantile	95% CI	97.5th Quantile	95% CI	<i>n</i>
RBC length	19.0	18.0-20.0	17.0	16.1-17.9	22.0	21.8-22.2	80	18.0	18.0-19.0	16.0	15.2-16.8	20.0	19.2-20.8	66
RBC width	12.0	12.0-13.0	11.0	11.0-11.0	14.0	13.0-15.0	80	11.0	11.0-11.3	9.7	8.9-10.4	12.3	11.6-13.1	66
Thrombocyte length*	9.0	8.0-10.0	6.0	5.5-6.5	11.0	11.0-11.0	80	10.0	9.0-11.0	7.0	6.4-7.6	12.3	11.6-13.1	66
Thrombocyte width*	5.0	4.0-6.0	3.0	2.1-4.0	7.0	6.0-8.0	80	5.0	5.0-6.0	4.0	3.9-4.1	7.0	6.2-7.8	66
Neutrophil (large) length	13.0	12.0-ND	ND	ND	ND	ND	2	17.0	15.8-18.0	14.0	13.8-14.2	19.0	18.7-19.3	54
Neutrophil (large) width	12.5	12.0-ND	ND	ND	ND	ND	2	16.0	15.0-17.0	13.0	12.7-13.3	18.0	17.8-18.2	54
Neutrophil (small) length	12.0	11.0-13.0	9.0	7.7-10.3	14.0	13.2-14.8	80	12.0	11.0-13.0	9.0	8.6-9.4	13.0	13.0-13.0	30
Neutrophil (small) width	11.0	10.3-12.0	8.0	6.7-9.3	13.0	12.2-13.8	80	11.0	10.0-12.0	8.0	7.8-8.2	13.0	12.1-13.9	30
Lymphocyte length	8.0	7.0-8.0	6.0	5.4-6.6	9.0	9.0-9.0	80	9.0	8.0-9.0	7.0	6.9-7.1	10.0	10.0-10.0	66
Lymphocyte width	8.0	7.0-8.0	6.0	5.9-6.1	9.0	8.9-9.1	80	8.0	7.0-9.0	7.0	7.0-7.0	10.0	9.2-10.8	66
Monocyte (large) length	13.0	13.0-14.0	ND	ND	ND	ND	8	15.0	14.0-16.0	ND	ND	ND	ND	19
Monocyte (large) width	12.5	12.0-13.0	ND	ND	ND	ND	8	14.0	13.0-16.0	ND	ND	ND	ND	19
Monocyte (small) length	10.0	10.0-11.0	9.0	8.2-9.8	12.0	11.9-12.1	80	11.0	10.0-12.0	10.0	10.0-10.0	13.5	12.7-14.3	58
Monocyte (small) width	10.0	9.0-10.0	8.0	7.1-8.9	12.0	11.6-12.4	80	11.0	10.0-11.0	9.0	8.9-9.1	13.0	12.5-13.5	58
Eosinophil length	13.5	12.0-15.0	9.6	8.5-10.7	17.0	16.6-17.4	62	16.0	15.0-17.0	ND	ND	ND	ND	24

Eosinophil width	13.0	12.0-14.0	9.6	8.7-10.4	16.0	15.7-16.3	62	15.0	14.0-16.0	ND	ND	ND	ND	24
Basophil length	11.5	9.3-13.0	ND	ND	ND	ND	4	12.0	11.0-13.0	ND	ND	ND	ND	20
Basophil width	10.0	8.3-11.0	ND	ND	ND	ND	4	10.0	9.0-11.0	ND	ND	ND	ND	20

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Data are bootstrapped 2.5th and 97.5th quantiles, 95% confidence intervals (CI), medians, and inter-quartile ranges (IQR).

\*Nuclear dimensions.

ND indicates not determined.



**Table 4.3.** Hematologic variables for common green (*Litoria caerulea*) and white-lipped (*L. infrafrenata*) tree frogs during the wet and dry seasons.

Species	<i>Litoria caerulea</i>					<i>Litoria infrafrenata</i>				
Season	Wet (n = 39)		Dry (n = 41)			Wet (n = 27)		Dry (n = 39)		
Hematologic Variable	Median	IQR	Median	IQR	P-value*	Median	IQR	Median	IQR	P-value*
PCV (%)	38.0	34.0-42.0	37.0	33.5-40.0	.508	34.0	30.0-37.0	28.0	24.0-32.0	<.001
Hemoglobin (g/dL)	9.7	8.5-10.9	8.6	7.6-10.2	.052	8.2	7.0-9.1	6.5	5.5-7.2	<.001
RBC ( $\times 10^9/L$ )	750	590-830	720	635-810	.675	800	720-860	650	590-770	.001
MCV (fL)	508	463-609	494	459-595	.654	420	383-507	417	359-480	.629
MCH (pg)	134	112-153	122	110-145	.142	100	84-119	97	84-114	.379
MCHC (g/L)	255	246-268	252	213-267	.202	241	210-268	235	210-243	.473
Thrombocyte ( $\times 10^9/L$ )	28.2	23.8-33.1	25.8	22.2-33.9	.464	36.8	29.1-46.4	28.2	23.5-33.7	.002
WBC ( $\times 10^9/L$ )	13.8	11.8-20.2	17.9	13.8-26.5	.022	15.3	11.5-23.9	23.1	15.1-34.0	.016
Neutrophil (%)	25.0	17.0-33.0	17.0	12.5-24.0	<.001	16.0	11.0-24.0	25.0	18.0-34.0	.002
Neutrophil ( $\times 10^9/L$ )	3.5	2.2-5.6	3.2	2.1-4.3	.163	2.7	1.7-4.0	5.8	3.5-8.4	<.001
Lymphocyte (%)	65.0	57.0-70.0	70.0	65.0-79.5	.004	73.0	50.0-80.0	66.0	58.0-75.0	.232
Lymphocyte ( $\times 10^9/L$ )	8.4	7.0-13.9	12.5	9.3-20.8	.002	10.7	8.0-17.1	14.3	10.4-20.2	.041
Monocyte (%)	7.0	5.0-12.0	7.0	5.0-10.0	.368	5.0	3.0-8.0	6.0	4.0-8.0	.633

Monocyte ( $\times 10^9/L$ )	1.3	0.6-1.8	1.2	0.9-1.8	.654	1.1	0.3-1.5	1.2	0.7-2.2	.119
Eosinophil (%)	2.0	1.0-3.0	3.0	0.5-5.0	.170	0.0	0.0-3.0	0.0	0.0-1.0	.377
Eosinophil ( $\times 10^9/L$ )	0.3	0.1-0.5	0.5	0.1-1.1	.099	0.0	0.0-0.7	0.0	0.0-0.2	.585
Basophil (%)	0.0	0.0-0.0	0.0	0.0-0.0	.302	1.0	0.0-6.0	0.0	0.0-0.0	.001
Basophil ( $\times 10^9/L$ )	0.0	0.0-0.0	0.0	0.0-0.0	.302	0.1	0.0-1.42	0.0	0.0-0.0	.001
Polychromatophilic Cells (%)	4.0	3.0-5.0	5.0	3.0-8.0	.361	1.0	0.8-4.0	1.0	0.4-3.0	.129
Basophilic Erythroblasts†	1.0	0.0-2.0	1.0	0.0-2.5	.139	1.0	0.0-2.0	1.0	0.0-3.0	.808

Data are medians and inter-quartile ranges (IQR).

\**P*-values refer to nonparametric Mann-Whitney tests between seasons within each species.

†Cells/100 WBCs.

**Table 4.4.** Plasma biochemical reference intervals for common green (*Litoria caerulea*) and white-lipped (*L infrafrenata*) tree frogs.

Species	<i>Litoria caerulea</i> (n = 80)						<i>Litoria infrafrenata</i> (n = 66)						<i>P</i> -value*
	Median	IQR	2.5th Quantile	95% CI	97.5th Quantile	95% CI	Median	IQR	2.5th Quantile	95% CI	97.5th Quantile	95% CI	
Total Protein (g/L)	62.0	55.0-68.0	39.0	34.2-43.9	85.9	74.8-96.7	35.0	30.0-41.0	18.0	12.5-23.5	56.3	51.4-61.3	<.001
AST (U/L)	91	66-122	30	13-47	362	245-478	67	41-119	26	23-29	370	141-599	.009
CK (U/L)	470†	347-705	75	0-164	2555	1564-3546	399	233-722	73	0-162	3420	1000-5840	.214
Uric Acid (μmol/L)	25	13-44	4	2-6	86	76-96	12§	7-15	0	0-2	27	14-40	<.001
Glucose (mmol/L)	3.6	3.1-4.3	1.9	1.6-2.2	6.0	5.2-6.8	3.3	2.5-4.5	2.0	1.8-2.1	6.8	5.2-8.3	.309
Calcium (mmol/L)	2.94	2.66-3.27	2.00	1.70-2.40	4.40	4.00-4.80	2.45	2.16-2.82	1.80	1.70-1.80	4.70	3.90-5.50	<.001
Phosphorus (mmol/L)	1.33	1.06-1.61	0.72	0.65-0.79	2.64	2.11-3.16	1.30	1.04-1.59	0.60	0.40-0.80	2.70	2.40-3.00	.835
Ca:P Ratio	2.23	1.91-2.76	1.18	0.86-1.50	4.00	3.40-4.58	1.90	1.60-2.27	1.10	0.80-1.40	3.30	3.10-3.50	<.001
Potassium (mmol/L)	5.9	4.9-7.7	3.2	2.3-4.1	9.5	9.0-10.0	3.7	3.2-4.7	1.9	1.4-2.3	3.1	5.8-10.5	<.001
Sodium (mmol/L)	110	107-114	101	99-103	123	120-126	106	104-108	99	98-100	114	108-120	<.001

Data are bootstrapped 2.5th and 97.5th quantiles, 95% confidence intervals (CI), medians, and inter-quartile ranges (IQR).

\**P*-values refer to nonparametric Mann-Whitney tests between species.

†*n* = 78.

§*n* = 65.

**Table 4.5.** Plasma biochemical analytes in common green (*Litoria caerulea*) and white-lipped (*L. infrafrenata*) tree frogs during the wet and dry seasons.

Species	<i>Litoria caerulea</i>					<i>Litoria infrafrenata</i>				
Season	Wet ( <i>n</i> = 39)		Dry ( <i>n</i> = 41)			Wet ( <i>n</i> = 27)		Dry ( <i>n</i> = 39)		
Biochemical Analyte	Median	IQR	Median	IQR	<i>P</i> -value*	Median	IQR	Median	IQR	<i>P</i> -value*
Total Protein (g/L)	61.0	51.0-68.0	62.0	57.0-71.5	0.187	39.0	34.0-45.0	32.0	28.0-37.0	.001
AST (U/L)	90	67-136	91	64-120	0.630	100	53-160	49	37-86	.005
CK (U/L)	449 <sup>†</sup>	186-744	491	390-691	0.347	375	233-708	431	226-763	.881
Uric Acid (μmol/L)	16	11-32	34	19-56	0.004	6	5-11	15 <sup>§</sup>	12-16	<.001
Glucose (mmol/L)	3.9	3.4-4.9	3.4	2.7-3.8	0.001	4.9	3.9-5.5	2.7	2.4-3.3	<.001
Calcium (mmol/L)	2.97	2.63-3.38	2.93	2.71-3.12	0.686	2.51	2.13-2.96	2.42	2.16-2.74	.616
Phosphorus (mmol/L)	1.41	1.08-1.59	1.24	1.01-1.69	0.634	1.42	1.15-2.08	1.14	0.98-1.48	.010
Ca:P Ratio	2.20	1.99-2.64	2.31	1.89-2.99	0.651	1.80	1.48-1.92	2.03	1.74-2.45	.001
Potassium (mmol/L)	6.2	5.2-8.1	5.5	4.0-7.0	0.049	4.3	3.5-5.2	3.5	3.0-4.2	.008
Sodium (mmol/L)	110	107-113	110	107-114	0.919	108	105-109	106	103-108	.040

Data are medians and inter-quartile ranges (IQR).

\**P*-values refer to nonparametric Mann-Whitney tests between seasons within each species.

<sup>†</sup>*n* = 37; <sup>§</sup>*n* = 38.

**Table 4.6.** Reference laboratory biuret total protein and electrophoretic albumin and globulin measurements (serum) for common green (*Litoria caerulea*) and white-lipped (*L. infrafrenata*) tree frogs. Values obtained using 2 in-house analytical methods (VetScan chemistry analyser/heparinized whole blood and refractometry/serum) are shown for comparison.

Analytical Method	<i>L. caerulea</i> (n = 27)		<i>L. infrafrenata</i> (n = 13)		P-value*
	Median	IQR	Median	IQR	
Biuret/Electrophoresis					
Total Protein (g/L)	57.0	48.0-63.0	40.0	35.0-46.5	<.001
Albumin (g/L)	36.0	32.0-40.0	22.0	18.0-24.5	<.001
Globulin (g/L)	21.0	17.0-25.0	18.0	15.5-21.5	.064
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VetScan					
Total Protein (g/L)	62.0	55.0-69.0	42.0	34.0-49.5	<.001
Albumin (g/L)	27.0	25.0-31.0	12.0	9.0-18.0	NV
Globulin (g/L)	34.0	29.0-40.0	27.0	23.0-33.0	NV
<hr/>					
Refractometer					
Total Protein (g/L)	56.0	46.0-59.0	36.0	33.0-46.0	<.001

\*P-values refer to nonparametric Mann-Whitney tests between species.

NV indicates results not valid using this analytic method (see text for details).

**Table 4.7.** Selected physical, hematologic, and biochemical variables for white-lipped tree frogs (*Litoria infrafrenata*) negative or positive for intraerythrocytic hemogregarine infection.

Infection Status	Negative ( <i>n</i> = 66)		Positive ( <i>n</i> = 15)		
Parameter	Median	IQR	Median	IQR	<i>P</i> -value*
Body Weight (g)	54.1	42.4-64.0	37.1	31.2-58.8	.021
Body Condition Score (1-5)	3.0	2.0-3.0	3.0	3.0-3.0	.161
Fecal Parasite Score (0-3)	1.0 <sup>§</sup>	0.0-1.5	1.5 <sup>‡</sup>	0.0-2.0	.371
PCV (%)	30.0	26.0-34.0	27.0	24.0-30.0	.060
Hemoglobin (g/dL)	7.0	6.1-8.2	7.1	5.3-9.2	.794
RBC ( $\times 10^9/L$ )	720	628-820	710	620-800	.688
MCV (fL)	418	374-486	353	286-484	.200
MCH (pg)	99	84-115	109	65-128	.817
MCHC (g/L)	236	210-250	241	191-381	.334
Thrombocyte ( $\times 10^9/L$ )	31.9	25.8-38.8	51.5	36.6-54.6	<.001
WBC ( $\times 10^9/L$ )	21.0	14.2-29.1	28.2	24.4-38.9	.009
Neutrophil (%)	20.0	15.0-32.0	13.0	7.0-28.0	.042
Neutrophil ( $\times 10^9/L$ )	4.2	2.4-7.4	2.9	2.2-8.6	.697
Lymphocyte (%)	70.0	57.0-78.3	74.0	66.0-85.0	.057
Lymphocyte ( $\times 10^9/L$ )	12.2	8.7-19.4	20.7	18.0-25.1	.002
Monocyte (%)	6.0	4.0-8.0	6.0	4.0-8.0	.859
Monocyte ( $\times 10^9/L$ )	1.1	0.6-2.0	1.6	1.0-2.0	.109
Eosinophil (%)	0.0	0.0-1.3	0.0	0.0-2.0	.878
Eosinophil ( $\times 10^9/L$ )	0.0	0.0-0.3	0.0	0.0-0.7	.754
Basophil (%)	0.0	0.0-1.0	0.0	0.0-1.0	.659
Basophil ( $\times 10^9/L$ )	0.0	0.0-0.1	0.0	0.0-0.5	.486
Polychromatophilic Cells (%)	1.0	0.4-3.3	3.0	3.0-8.0	.001
Basophilic Erythroblasts <sup>†</sup>	1.0	0.0-3.0	1.0	0.0-2.0	.290
Mitotic RBC <sup>†</sup>	0.0	0.0-0.0	0.0	0.0-0.0	.319
Hemolysis Index (mg/dL hemoglobin)	13.0	9.0-20.0	5.0	0.0-11.0	<.001

Total Protein (g/L)	32.0	28.0-38.0	24.0	18.0-33.0	.008
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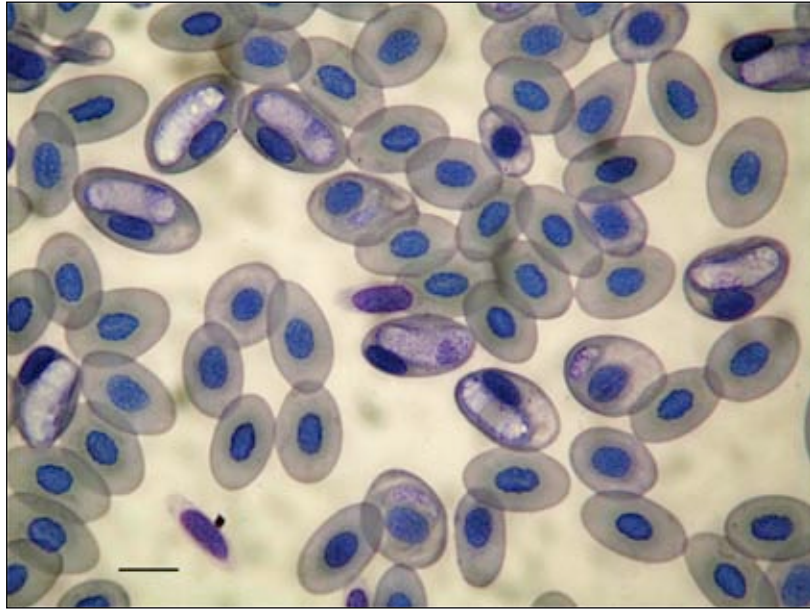
Data are medians and inter-quartile ranges (IQR)

\**P*-values refer to nonparametric Mann-Whitney tests between groups.

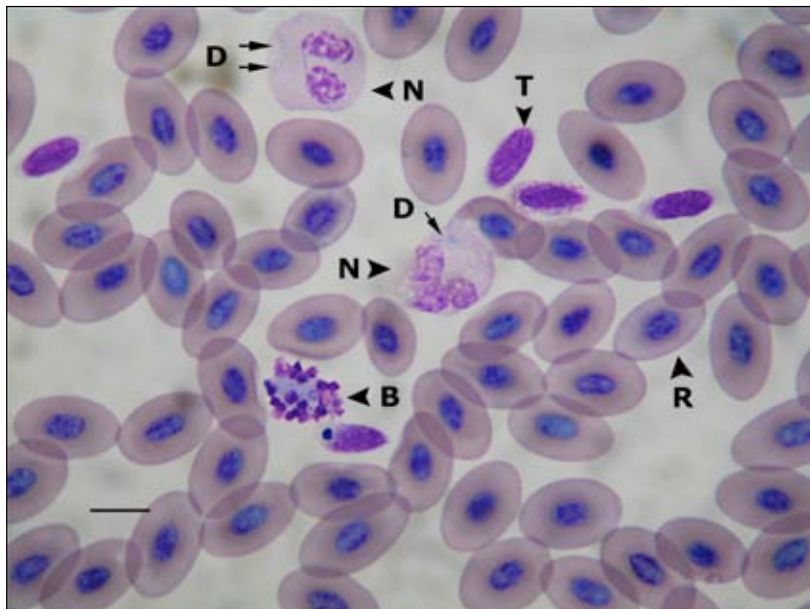
†Cells/100 WBCs.

§*n* = 53

‡*n* = 12.

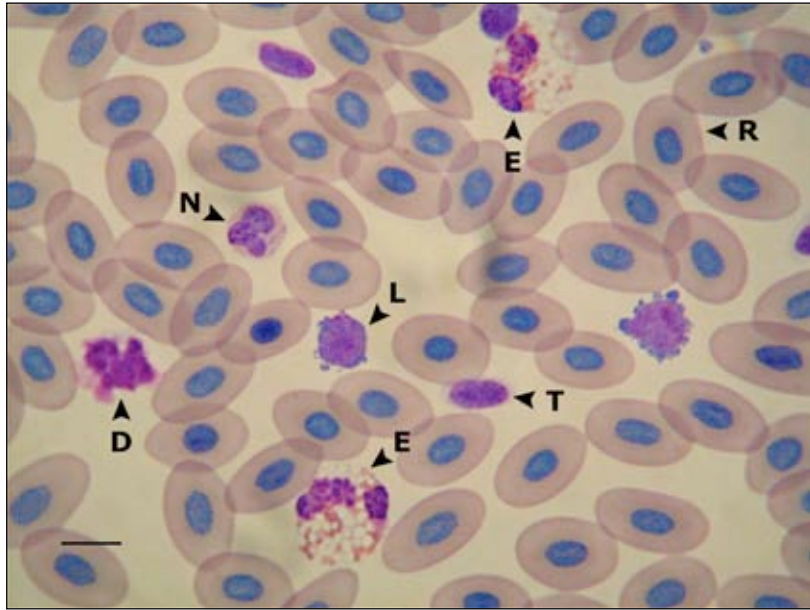


**Figure 4.1.** Wright's stained blood smear from a white-lipped tree frog (*Litoria infrafrenata*) with a heavy burden of intracytoplasmic RBC hemogregarine gametocytes and extensive polychromasia. Bar = 10  $\mu$ m.

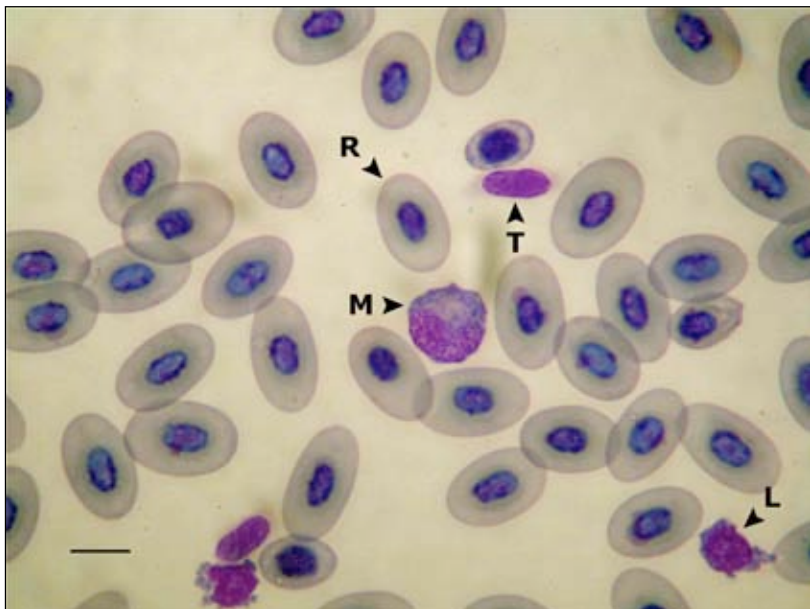


**Figure 4.2.** Wright's stained blood smear from a white-lipped tree frog (*Litoria infrafrenata*) showing two neutrophils (N) of the large phenotype; both have prominent cytoplasmic Dohle-like bodies (D) and one has multiple cytoplasmic vacuoles. The basophil (B) has variable granule stain uptake. R = mature RBC, T = thrombocyte. Bar = 10  $\mu$ m.

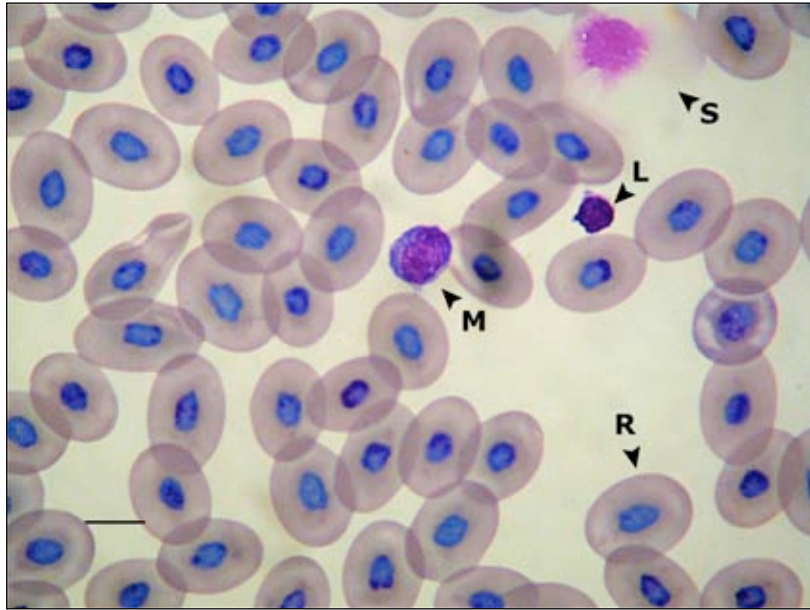




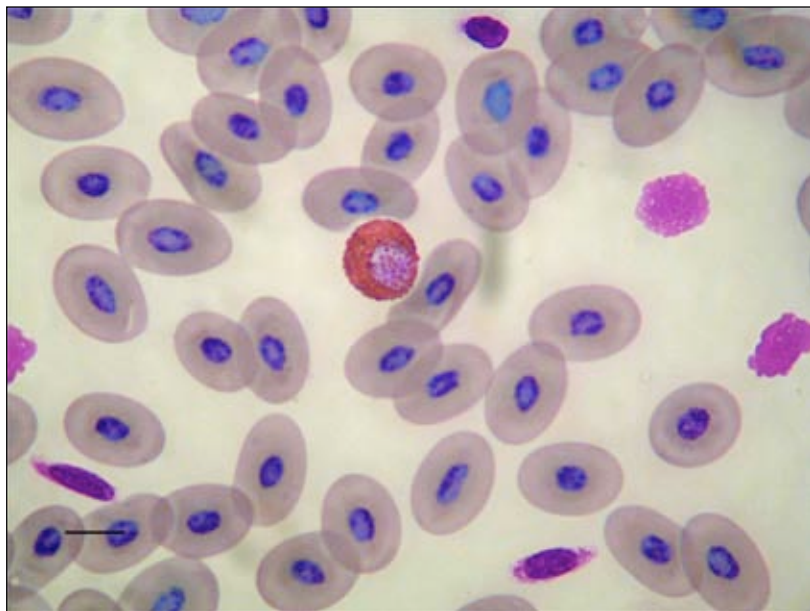
**Figure 4.3.** Wright's stained blood smear from a white-lipped tree frog (*Litoria infrafrenata*) showing a neutrophil (N) of the small phenotype. D = damaged unidentifiable cell, E = eosinophil, L = lymphocyte, R = mature RBC, T = thrombocyte. Bar = 10  $\mu\text{m}$ .



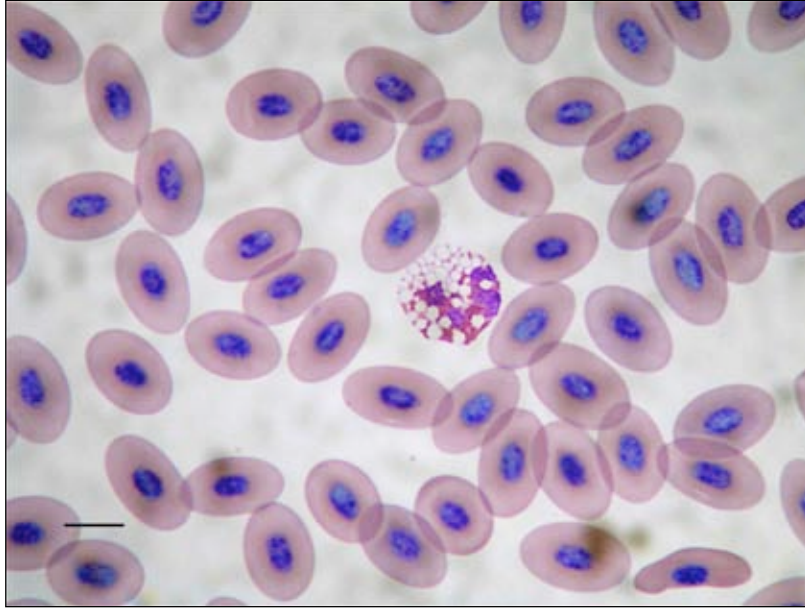
**Figure 4.4.** Wright's stained blood smear from a white-lipped tree frog (*Litoria infrafrenata*) showing a monocyte (M) of the large phenotype. L = lymphocyte, R = mature RBC, T = thrombocyte. Bar = 10  $\mu\text{m}$ .



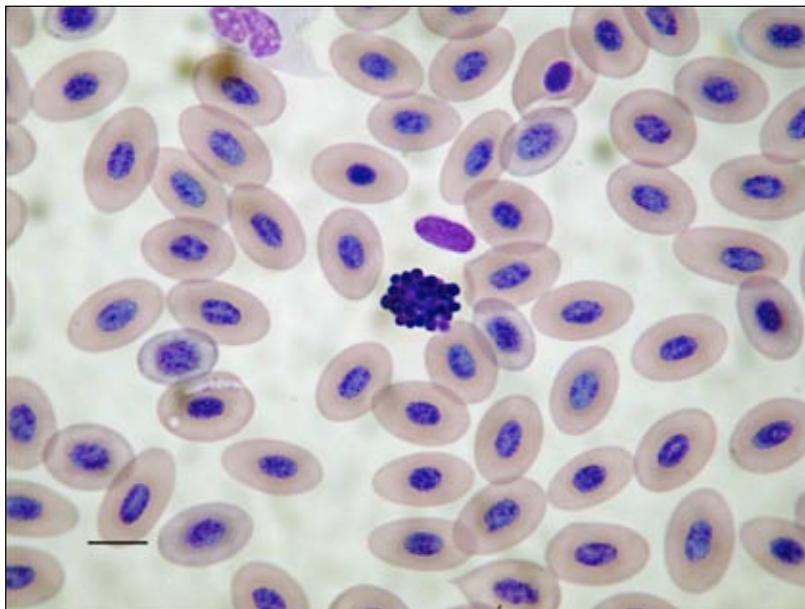
**Figure 4.5.** Wright's stained blood smear from a white-lipped tree frog (*Litoria infrafrenata*) showing a monocyte (M) of the small phenotype. L = lymphocyte, R = mature RBC, S = smudged RBC. Bar = 10  $\mu$ m.



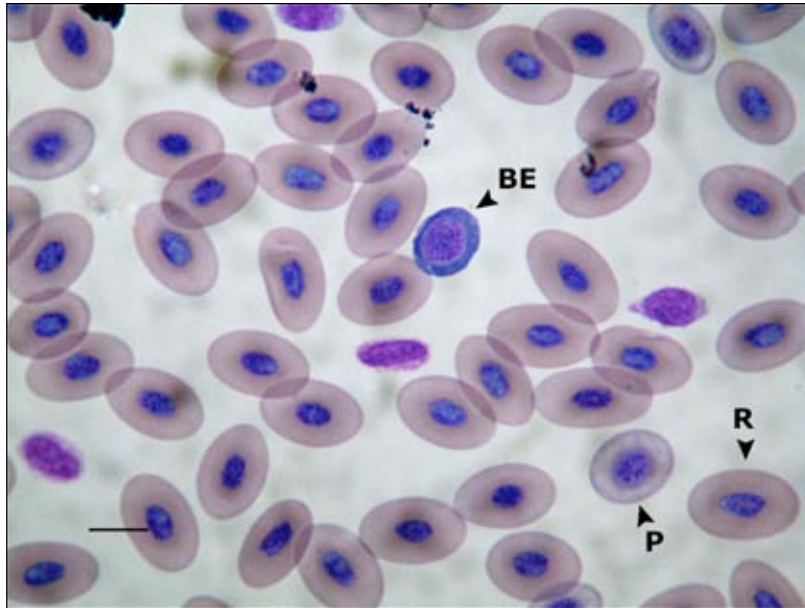
**Figure 4.6.** Wright's stained blood smear from a common green tree frog (*Litoria caerulea*) showing an eosinophil. Bar = 10  $\mu$ m.



**Figure 4.7.** Wright's stained blood smear from a white-lipped tree frog (*Litoria infrafrenata*) showing an eosinophil. Bar = 10  $\mu$ m.



**Figure 4.8.** Wright's stained blood smear from a white-lipped tree frog (*Litoria infrafrenata*) showing a basophil. Bar = 10  $\mu$ m.



**Figure 4.9.** Wright's stained blood smear from a common green tree frog (*Litoria caerulea*) showing polychromasia and a basophilic erythroblast (BE). R = mature RBC, P = polychromatophilic RBC. Bar = 10  $\mu\text{m}$ .

#### 4.2.5 Discussion

Hematologic and biochemical reference intervals for tree frogs established in this study are essential tools for use in the global effort to save amphibians. The effects of both intrinsic and extrinsic factors on analyte concentrations suggest that these reference intervals will be useful to screen for diseases and, potentially, to detect emerging diseases early. These effects support partitioning of reference intervals in tree frogs. We found significant inter- and intra-species differences for multiple hematologic and biochemical variables in *L caerulea* and *L infrafrenata* frogs, highlighting the importance of establishing both species-specific and season-specific reference intervals, even for closely related species found in similar geographical areas and environmental conditions. Compared with seasonal variations, little variation occurred in values for either species between 2007 and 2008, indicating that the health status of the populations studied may be stable on an annual basis. Higher MCH in 2007 and CK activity in 2008 in *L caerulea* frogs were likely incidental findings; higher potassium concentrations in 2008 were unexpected based on previous findings of relatively stable plasma potassium.<sup>8</sup> In *L infrafrenata* frogs, only calcium:phosphorus ratio varied between 2007 and 2008, but likely had no clinical relevance.

All frogs in our reference sample population were clinically healthy, eliminating sample bias resulting from clinical disease. Although subclinical disease could not be ruled out by physical examination, underlying diseases were not identified in any frogs with the exception of mild superficial skin abrasions and the presence of fecal parasites. Thus, these reference intervals represent true baseline values, which will be invaluable for future health monitoring and diagnostic investigations, similar to those established for other wildlife populations.<sup>1,10-12,16,36</sup> Such investigations have already contributed to significant advances in understanding the pathogenesis of chytridiomycosis, an emerging infectious disease that has caused catastrophic global declines in amphibian populations.<sup>37,38</sup>

Although some of the reference values may have been reliably interchanged between the 2 species of frogs, important variables for routinely monitoring health, such as PCV, total and differential WBC counts, concentrations of TP, uric acid, calcium, potassium, and sodium, and AST activity, were significantly different between species, and interchanging these reference values would have provided misleading diagnostic information. Leukocyte differentiation was challenging due to wide intra- and inter-species variation in morphologic and staining characteristics. Identification of different neutrophil and monocyte phenotypes within individuals of both species led to classification of these cells into subgroups. Poorly differentiated basophils and karyorrhectic granulocytes further complicated differentiation.

Although considerable expertise is needed for accurate identification of amphibian WBCs, the comprehensive descriptions provided in this study should be of great use to future studies of anuran blood cells.

Inter-species differences may have reflected adaptations associated with microhabitat preference and host-parasite co-evolution. For example, *L. infrafronata* may have more continual access to permanent water sources, reducing the need for physiologic water conservation and leading to lower PCV and TP, uric acid, and electrolyte concentrations. Differences in nutrient composition of prey items or substrate may also account for some variations. The 2 species differ in temperament, with *L. caerulea* frogs typically being calm and tolerant of handling and *L. infrafronata* frogs often exhibiting signs of stress associated with handling and confinement; this may have accounted for the increased total WBC and neutrophil counts in *L. infrafronata* frogs. Although sample collection may have been a stressful event, the large number of samples collected ensured streamlining of all processes, and anesthesia procedures were considered to be less stressful than manual restraint of conscious frogs for cardiocentesis. It is unknown why *L. caerulea* frogs had higher eosinophil counts, as eosinophils often are associated with parasitic infections<sup>6,22</sup> and there were no differences in gastrointestinal and pulmonary parasite burdens between the 2 species; also, hemoparasites were not found in *L. caerulea* frogs. The higher basophil counts found in *L. infrafronata* frogs may reflect an enhanced role of basophils in this species. Differences in eosinophil and basophil counts may have resulted from inherent differences in host tolerance to parasite infections or differential exposures to allergens or pollution.<sup>3,6,10,19</sup>

Unusually colored plasma, such as the clear blue plasma found in *L. infrafronata* frogs, has been noted rarely in other amphibians. Two Japanese giant salamanders (*Andrias japonicus*) and 1 of 4 consecutive samples from an adult female South American bullfrog (*Leptodactylus pentadactylus*) with corneal lipidosis had this same plasma appearance.<sup>6</sup> The blue coloration was a normal finding in *L. infrafronata*, but the reason for the color is unknown. Another normal finding was cytoplasmic Döhle-like bodies in neutrophils from both species; in mammals Döhle bodies are aggregations of endoplasmic reticulum usually found in animals with inflammation,<sup>1</sup> but they can also occur in low numbers in clinically healthy cats.<sup>39</sup>

In *L. caerulea* frogs during the dry season, higher concentrations of uric acid may have reflected seasonal differences in water or nutrient availability, metabolism, or immune stimulation. Higher lymphocyte counts were also found in these frogs during the dry season, but it is unknown whether the response to stress in *L. caerulea* frogs is manifest as lymphopenia and concurrent neutrophilia, similar to *R. perezii*,<sup>21</sup> or as lymphocytosis, similar to what may occur in

the Japanese newt (*Cynops pyrrhogaster*).<sup>19</sup> Seasonal increases in lymphocyte counts in *L caerulea* may have been due to the stress of reduced food or water availability linked with increased conspecific competition. Increased circulating eosinophil and lymphocyte counts in *L caerulea* during the dry season may also have been due to seasonal variations in host-parasite interactions.

In the wet season, increased neutrophil counts with lymphopenia in *L caerulea* frogs may have been associated with the stress of breeding and increased glucose concentrations may have resulted from increased intrinsic or extrinsic stressors or increased ambient temperatures.<sup>6</sup> Higher potassium concentrations may have reflected seasonal variations in nutrient availability, renal metabolism, or electrolyte homeostasis, including a requirement for sodium conservation as potassium and sodium concentrations have a direct molar relationship.<sup>8</sup> In contrast to our findings, in adult male *R. pipiens* plasma potassium concentration decreased only in the last month of spring.<sup>8</sup> However, *R. pipiens* hibernates during the winter and would be expected to have significantly different seasonal metabolic patterns compared with non-hibernating tropical frogs, such as *L caerulea*. Further study of seasonal plasma and urinary electrolyte concentrations are warranted.

Although lymphocyte counts were higher in *L infrafrrenata* frogs during the dry season, similar to what was found for *L caerulea* frogs, higher neutrophil counts were also found and may have resulted from nonspecific immune stimulation or increased environmental stressors, if this species exhibits a neutrophilic stress response. Higher calcium:phosphorus ratio was due to significantly lower phosphorus concentrations and may also have reflected other seasonal variations in metabolism or nutrient availability.

In *L infrafrrenata* frogs during the wet season, higher PCV, Hgb concentration, RBC count, and thrombocyte count may have reflected enhanced hematopoiesis associated with warmer ambient temperatures.<sup>7,16,18</sup> Higher basophil counts may have resulted from greater immune stimulation from allergens, pollution, or parasites during the wet season.<sup>3,6,10,19</sup> The rise in hemogregarine infections in *L infrafrrenata* during the wet season probably reflected increased intermediate host activity: reptilian and amphibian coccidia in the family Hemogregarinidae (Phylum Apicomplexa, Subclass Coccidiasina) have an indirect life cycle with intermediate hosts that include leeches, ticks, mosquitoes, and mites.<sup>36</sup> As with *L caerulea* frogs, higher glucose concentrations in *L infrafrrenata* frogs may have resulted from environmental stressors or increased ambient temperatures.<sup>6</sup> Higher TP, sodium, potassium, and phosphorus concentrations and AST activity may represent seasonal alterations in nutrient availability, hepatic function,

renal metabolism, and electrolyte homeostasis, including increased sodium conservation. In adult male *R. pipiens*, plasma sodium concentrations peaked during summer.<sup>8</sup>

Hemogregarine-infected *L. infrafrenata* frogs had no apparent signs of disease, but changes in some physical, hematologic, and biochemical variables suggested a subclinical impact of infection on health status. Low burdens of hemoparasites typically are considered insignificant,<sup>1,6,9</sup> but moderate to high burdens may be of clinical significance and may cause anemia, increased polychromasia, or microcytosis.<sup>6,26</sup> Increased numbers of immature RBCs may indicate erythrocytic diseases, eg, iridovirus infection, or a regenerative response.<sup>1,9,40</sup> Lymphocytosis can occur in reptiles in response to a variety of parasitic infections, including hematozoan infections.<sup>16,20</sup> Increased thrombocyte counts in infected frogs may reflect concurrent thrombopoietic and erythropoietic stimulation. Increased polychromasia without concurrent anemia or changes in RBC indices suggests an adequate regenerative response to infection. The lower hemolysis index values found in infected frogs were unlikely to be clinically relevant. Further investigation is needed for taxonomic classification, and to determine the clinical significance, of the hemogregarine infections, as well as to establish whether the parasite is endemic or emerging in Australian *L. infrafrenata* frog populations.\*

In avian species, TP concentrations obtained by refractometry can be higher than those measured by the biuret method due to the presence of nonprotein solids, such as cholesterol, urea, lipoproteins, and glucose.<sup>41</sup> In our study, the 2 methods were well correlated, even though TP concentration by refractometry was slightly lower than biuret TP concentrations for both frog species. Thus, refractometry can be recommended for rapid in-house determination of TP concentration in *L. caerulea* and *L. infrafrenata* frogs, particularly if the sample volume is small or there are cost constraints. However, the VetScan system cannot be recommended for measuring albumin, and subsequently calculating globulin, concentration in these frogs, and analysis of serum protein by electrophoresis should be used instead. The VetScan system uses the bromocresol green dye-binding method for measurement of albumin concentration, a method that is less accurate than electrophoresis in birds and turtles<sup>42-44</sup> and that is not recommended for diagnostic use in these species, particularly in diseased animals.<sup>44</sup> In both *L. caerulea* and *L. infrafrenata* frogs, albumin concentrations measured by the dye-binding method were only weakly correlated with concentrations measured by electrophoresis and were spuriously low; calculated globulin concentrations (TP-albumin) were, therefore, spuriously high. The VetScan analyzer was, however, valuable for rapid measurement of the other biochemical analytes. Advantages of the system compared with sending samples to commercial

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\* *Litoria infrafrenata* occurs in humid microclimates, closely associated with permanent water bodies, often in wet forest; this may increase the opportunity for host-vector interaction and subsequent parasite transmission.



laboratories included its compact size and portability, ease of use, rapid turn-round time for obtaining results, and relatively small sample volume requirement (100  $\mu$ L whole blood for a panel of analytes); disadvantages compared with other in-house analyzers included the inability to measure individual analytes separately where sample volumes were < 100  $\mu$ L and loss of the entire sample with rotor failure.

In conclusion, wide inter- and intra-species variations in hematologic and biochemical reference values for 2 species of Australian tree frogs were found, highlighting the importance of establishing species-specific reference values for different seasons. This is the first report to establish hematologic and biochemical reference values for clinically healthy free-ranging anurans, Australian frog species, and different seasons in anurans and is the first study of this magnitude in amphibians. Established reference intervals are applicable to the specific analytic methods used in this study and should ideally be validated before use with other tree frog populations.

### **Acknowledgments**

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***Disclosure:*** The authors have indicated that they have no affiliations or financial involvement with any organization or entity with a financial interest in, or in financial competition with, the subject matter or materials discussed in this article.

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## CHAPTER 5

### Immune function in tree frogs with chytridiomycosis

#### 5.1 Introduction

Wide variation in susceptibility to chytridiomycosis exists between species, populations and individuals. Although recent progress has been made in understanding aspects of resistance to *B. dendrobatidis*, the mechanisms of immunity appear complex and much remains unknown. Innate host defense mechanisms, such as antimicrobial skin peptides and symbiotic bacteria, may influence susceptibility to infection. Little evidence of an effective localized or systemic adaptive immune response in *B. dendrobatidis*-infected anurans has been found. Furthermore, there is a critical knowledge gap about why susceptible amphibians fail to mount an effective immune response. Negligible cellular inflammation occurs in the skin of infected frogs, suggesting sporangia may evade host immune recognition due to their intracellular location within the superficial epidermis. Recent genetic, stress hormone and *in vitro* immune function studies indirectly suggest the pathogen may actively suppress the host's immune response.

This chapter consists of one manuscript submitted for publication detailing investigations into adaptive immunity in tree frogs with chytridiomycosis. Our results provide the first direct evidence that *B. dendrobatidis* actively suppresses a systemic immune response when infecting the amphibian host. This is a major step forward in following up on previous genetic and *in vitro* studies. Host immune suppression is likely a key factor enabling chytridiomycosis to be a formidable disease with unprecedented effects on biodiversity.

#### 5.2 Suppression of immune function in tree frogs with chytridiomycosis

This manuscript represents my original research and writing and has been submitted for publication to the peer-reviewed journal *Diseases of Aquatic Organisms*. I received study design advice from Rick Speare, Lee Berger and Lee Skerratt. Paul Whitehorn assisted with field collection of frogs, along with all husbandry and some experimental procedures. Stephen Garland ran the PCR analyses, Rebecca Webb processed the tissue samples for histology and Ammar Aziz assisted with microtitre assays. Helen Martin and Deb Buckett at Gribbles Veterinary Pathology Laboratory performed the serum protein electrophoresis. I performed all of the statistical analyses with advice from Lee Skerratt. Lee Berger and Lee Skerratt gave substantial editorial input to the final written paper.

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*Batrachochytrium dendrobatidis* suppresses immune function in tree frogs. *Diseases of Aquatic Organisms* (submitted).

The following manuscript is an exact, word-for-word copy of the manuscript submitted for publication, with the exception of minor amendments recommended by thesis reviewers. Section, table and figure numbering has been reformatted for this thesis. Since the journal uses American English, spelling follows this convention.

***Batrachochytrium dendrobatidis* suppresses immune function in tree frogs**

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### 5.2.1 Abstract

The amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) (*Bd*) has caused mass mortality leading to population declines and extinctions in many frog species worldwide. We used a range of hematologic and protein electrophoresis biomarkers, along with various functional tests, to assess immune competence in common green tree frogs (*Litoria caerulea*) experimentally infected with *Bd* and in white-lipped tree frogs (*L. infrafrenata*) experimentally exposed to *Bd*. Stimulation of *Bd*-infected *L. caerulea* resulted in a minimal immune system response. Compared with uninfected frogs, *Bd* infection caused a reduction in splenic, white blood cell, acute-phase protein and immunoglobulin responses, indicating a significantly impaired ability of *Bd*-infected frogs to respond adequately to antigenic stimulation. Although *L. infrafrenata* failed to maintain *Bd* infection after exposure, sub-clinical immunologic effects occurred in recovered compared with unexposed frogs. This is the first study to show that infection with *Bd* reduces systemic adaptive immune function in frogs. This host immune suppression is likely a key factor enabling *Bd* to be a formidable disease with unprecedented effects on biodiversity.

Keywords: Adaptive immunity; Amphibian; *Batrachochytrium dendrobatidis*; Chytridiomycosis; Frog; Immune; Immunity



### 5.2.2 Introduction

The recent global spread of the emerging infectious disease chytridiomycosis has caused declines and extinctions of many amphibian species [1-3]. The causative fungal skin pathogen, *Batrachochytrium dendrobatidis* (*Bd*), has had the most devastating impact in remote and protected mountainous regions, where abundant populations crashed within months of its arrival [1,2,4]. Environmental changes such as pollution and climate change have been ruled out as contributory factors – *Bd* can clearly cause high mortality rates in healthy, immune competent populations [3]. Combined with its ability to spread rapidly through host populations and persist even at low host densities, it has had an unprecedented effect on amphibian biodiversity [2,3,5-7]. If naïve susceptible amphibian populations survive introduction of *Bd*, it becomes endemic with reduced mortality rates and sometimes partial recovery, suggesting selection for host resistance and/or waning pathogen virulence [8].

Morbidity and mortality rates in post-metamorphic amphibians vary greatly among species and can reach up to 100 % in susceptible captive anuran species, including the common green tree frog (*Litoria caerulea*) [1,9-12]. Fatal pathophysiological changes include epidermal degeneration, inhibited epidermal electrolyte transport, systemic electrolyte disturbances (hyponatremia and hypokalemia) [11-13], severe hypovolemia secondary to dehydration [13] and asystolic cardiac arrest [12].

Wide variation in susceptibility to chytridiomycosis exists between species, populations and individuals. Within a species or population, local environmental conditions and specific behavioural characteristics can influence disease dynamics; *Bd* is susceptible to heat and desiccation, and frogs inhabiting unfavourable habitats have improved survival [8,9,14,15]. Although recent progress has been made in understanding aspects of resistance to *Bd*, the mechanisms of immunity appear complex and much remains unknown. The post-metamorphic amphibian immune system is fundamentally similar to that of mammals, demonstrating innate and adaptive responses including specific cell-mediated and antibody responses, and immunoglobulin isotype heterogeneity [16-19]. Innate host defense mechanisms, such as antimicrobial skin peptides and symbiotic bacteria, may influence susceptibility to *Bd* infection [20,21].

To date, little evidence of an effective localized or systemic adaptive immune response in *Bd*-infected *Rana*, *Silurana* or *Litoria* species has been found [10,21-23] (Young et al., unpublished). Activation of innate and adaptive immunity has recently been suggested to be an important component of natural *Bd* resistance in *Xenopus laevis* [24]. Knowledge of amphibian

immune responses to fungal pathogens is extremely limited and the contrasting findings of the few studies available highlight the need to broaden the taxonomic focus of future immunologic studies [23].

Furthermore, there is a critical knowledge gap about why *Bd*-susceptible amphibians fail to mount an effective immune response: is it due to pathogen immune evasion, host immunosuppression or a combination of the two? Negligible cellular inflammation occurs in the skin of infected frogs, suggesting sporangia may evade host immune recognition due to their intracellular location within the superficial epidermis [25]. Recent genetic, stress hormone and *in vitro* immune function studies indirectly suggest *Bd* may actively suppress an immune response [26-29].

We used diverse methods, previously established in mammals and birds, to study immune structure and function in *Bd*-infected and control frogs. Methodology involved measuring 1) mass and cellularity of immune organs, 2) total and differential peripheral white blood cell (WBC) counts, 3) serum protein fraction concentrations via gel electrophoresis, 4) *in vivo* phytohemagglutinin (PHA) skin response, and 5) *in vivo* anti-sheep red blood cell (SRBC) hemagglutination [30]. Immunization with SRBC to evaluate humoral immunity via serum and splenic hemolytic antibody production has been previously reported in three anuran species: *Rana pipiens*, *Bufo arenarum* and *X. laevis* [31-34]. The T-cell mitogen PHA has been used to evaluate anuran splenocyte, thymocyte and lymphocyte proliferative responses *in vitro* [35-37]. The PHA skin test has not been used previously as an immune function test in amphibians, and there are no reports describing innate or adaptive immunity in *Litoria* species. Our results showed that all of the methods were useful in demonstrating a marked immune suppressive effect of *Bd* infection in *L. caerulea*. The other tree frog species (*L. infrafrenata*) failed to maintain infection with *Bd* after experimental exposure, and recovered frogs showed sub-clinical immune function effects.

### 5.2.3 *Materials and methods*

#### *Animals*

Free-ranging clinically healthy adult individuals of the common green tree frog (*L. caerulea*, n = 20) and the white-lipped tree frog (*L. infrafrenata*, n = 20) were collected from widespread residential and semi-rural areas in and around Cairns and Townsville in far northern Queensland, Australia. The two species were selected based on their large mature body size, relative ease of capture, endemism, wide distribution and stable conservation status. Each frog was placed, using a new powder-free nitrile medical examination glove (Supergloves Australia Pty Ltd, Gold Coast, QLD, Australia), into an individual plastic holding container (70 × 95 × 150 mm) for transport. Frogs were housed in individual plastic containers (230 × 230 × 350 mm) in temperature (20 – 22°C) and light (12 h light, 12 h dark) controlled quarantine facilities at James Cook University (Cairns, QLD, Australia). Aged tap water was changed daily and frogs were fed large domestic crickets (*Acheta domestica*, Pisces Enterprises Inc., Kenmore, QLD, Australia) dusted with superfine calcium carbonate (Cattlekare<sup>®</sup>, Dandenong, VIC, Australia) and multivitamin powder (Reptivite<sup>™</sup>, Zoo Med Laboratories Inc., San Luis Obispo, CA, USA), ad libitum each day.

Frogs from each species were randomly assigned equally between two experimental trials. Before the trials commenced, each frog was clinically examined by a veterinarian, body weight and snout-urostyle measurements were recorded, and a swab sample was collected from the ventral skin surfaces for determination of *Bd* zoospore equivalents by real-time polymerase chain reaction (PCR) analysis (James Cook University, Townsville, Australia) [38]. All swab samples were analyzed in triplicate and compared with James Cook University zoospore standards. All frogs were negative for *Bd* prior to commencement of the experimental trials.

All animal procedures in this study were approved by the James Cook University Animal Ethics Committee (approval number A1085) and Queensland Parks and Wildlife Service (Scientific Purposes Permit number WISP03866106).

#### ***Immune function tests in healthy uninfected tree frogs (Experiment 1)***

Experiment 1 was designed as a pilot study to validate functional immune tests in healthy frogs from the two species. At the start of Experiment 1 (day 0), *L. caerulea* (n = 10) and *L. infrafrenata* (n = 10) were anesthetized for sampling for general immunological and hematological biomarkers and for initiation of functional tests for immune competence. Anesthesia was induced by shallow immersion in 0.20% (*L. infrafrenata*) or 0.25% (*L. caerulea*) ethyl 3-aminobenzoate methanesulfonic acid solution (tricaine methanesulfonate,

Sigma-Aldrich Inc., St Louis, MO, USA) buffered with 10 mEqL<sup>-1</sup> sodium bicarbonate solution (8.4%, Pro Care Animal Health, Dandenong, VIC, Australia).

Blood samples (250 – 500 µl, < 1% body weight) were collected for hematologic, plasma biochemical and serum protein electrophoretic analysis from dorsally recumbent frogs via cardiocentesis with a 1 ml syringe and 25 g needle (Terumo Corporation, Binan, Laguna, Philippines). The PHA skin response test for T-cell mediated immunity was initiated following standard avian and mammalian procedures [30,39,40] adapted for anurans. A 0.1 ml dose of 0.5% PHA-P (Sigma-Aldrich Inc., St Louis, MO, USA) in phosphate-buffered saline (PBS) (pH 7.4, Sigma-Aldrich Inc., St Louis, MO, USA) was injected intradermally in the interdigital webbing of the left hind foot between the second and third phalanges with a 1 ml syringe and 27 g needle (Terumo Corporation, Binan, Laguna, Philippines). The same volume of PBS was injected intradermally as a control in the right hind foot interdigital webbing. The SRBC hemagglutination test was also initiated following standard avian and mammalian procedures [40,41] adapted for anurans. Each frog was injected intracelomically with 0.5 ml of a 10% suspension of SRBC (Sigma-Aldrich Inc., St Louis, MO, USA) in PBS with a 1 ml syringe and 23 g needle (Terumo Corporation, Binan, Laguna, Philippines).

On day 7, each frog was euthanized by cardiac exsanguination following induction of anesthesia as previously described. Blood samples were collected for hematologic, plasma biochemical and serum protein electrophoretic analysis and for SRBC antibody assay. Spleen, liver and kidneys were dissected, weighed and recorded as % body weight. The spleen was immediately processed for determination of total lymphocyte count, cell viability and plaque formation by antibody-producing cells.

### ***Immune function tests in Bd-infected tree frogs (Experiment 2)***

At the start of Experiment 2 (day 0), *L. caerulea* (n = 10) and *L. infrafrenata* (n = 10) were anesthetized and blood samples were collected for hematologic, plasma biochemical and serum protein electrophoretic analysis as described in Experiment 1. Skin PHA stimulation and splenic function<sup>‡</sup> tests were only performed once per frog i.e. in the final stages of the experiment. Frogs were then exposed to *Bd* via shallow immersion in a bath of dilute electrolyte solution (25 ml) inoculated with 250,000 zoospores for 24 h, after which they were returned to their holding containers with aged tap water. During the exposure period, frogs were held in small individual plastic containers (50 × 100 × 150 mm) with a lid to ensure continuous contact of the ventral skin surfaces with the inoculum.

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<sup>‡</sup> Splenic function tests required post-mortem removal of the entire spleen.

Frogs were weighed and swabs collected for PCR at 10, 20, 30, 40, 50, 60, 75 and 82 d post-exposure. On day 30 post-exposure, frogs were anaesthetised and blood samples collected for hematologic and plasma biochemical analysis as previously described. On day 75 post-exposure (corresponding to day 0 in the uninfected frogs from Experiment 1), *Bd*-exposed frogs were again anaesthetised for sampling for hematologic, plasma biochemical and serum protein electrophoretic analysis, and for initiation of functional tests for immune competence (PHA skin response and SRBC hemagglutination tests) as per the methods described in Experiment 1. One *Bd*-exposed *L. infrafrenata* failed to recover post-anaesthesia and was excluded from the trial. On day 82 post-exposure (corresponding to day 7 in the uninfected frogs from Experiment 1), each *Bd*-exposed frog (*L. caerulea* n = 10, *L. infrafrenata* n = 9) was euthanized by cardiac exsanguination following anaesthesia. Blood, spleen, liver and kidney samples were collected as per the methods described in Experiment 1.

#### ***Hematologic and Plasma Biochemical Analysis***

Blood samples from each frog were processed according to standard amphibian procedures [42,43]. Fresh blood smears were made directly from the syringe, air dried and immediately fixed with 100% methanol; 200  $\mu$ l was collected into a 0.6 ml Microtainer<sup>®</sup> pediatric lithium heparin tube (Becton and Dickinson, Franklin Lakes, New Jersey, USA); and 150 – 200  $\mu$ l was collected into a plain 1.0 ml microcentrifuge tube (Eppendorf AG, Hamburg, Germany), immediately centrifuged ( $5,590 \times g$  for 10 min) and the supernatant decanted and refrigerated at 4°C until submission for serum protein electrophoresis. Additional blood (500 – 1000  $\mu$ l) from the final sample on day 7 (healthy uninfected frogs, Experiment 1) and day 82 (*Bd*-exposed frogs, Experiment 2) was collected into a plain 1.0 ml microcentrifuge tube (Eppendorf AG, Hamburg, Germany), allowed to clot at room temperature for 1 h, then centrifuged ( $5,590 \times g$  for 10 min) and the supernatant decanted and frozen at -70°C for later SRBC antibody microtiter assay.

Total red blood cells (RBC), WBC and thrombocytes were counted manually by a veterinary pathologist in a modified Neubauer hemocytometer at 400 $\times$  magnification with Natt-Herrick's solution as the diluent [42-44]. Differential WBC and polychromatophilic RBC were counted at 1000 $\times$  magnification from Wright's-stained (Clinipure Wright's Stain and Wright's Buffer Concentrate, HD Scientific Supplies Pty Ltd, Wetherill Park, NSW, Australia) blood smears [43]. Well-mixed whole blood (5  $\mu$ l) was drawn into a pediatric microhematocrit tube (Becton and Dickinson, Franklin Lakes, New Jersey, USA) and centrifuged ( $112 \times g$  for 2 min) for packed cell volume (PCV) measurement. Hemoglobin (Hb) was assayed manually using the

cyanomethemoglobin method modified for species with nucleated RBC [45,46] and specifically for amphibians [43]. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and MCH concentration (MCHC) were calculated from Hb, PCV and RBC values using standard formulae [47].

Plasma biochemical analytes were measured from 100  $\mu$ l of whole blood using the automated bench-top VetScan<sup>®</sup> VS2 Chemistry Analyzer and VetScan<sup>®</sup> Avian/Reptilian Profile Plus rotor (Abaxis Inc., Union City, CA, USA): aspartate aminotransferase (AST), uric acid (UA), creatine kinase (CK), glucose, calcium, phosphorus, potassium and sodium.

### ***Serum protein electrophoresis***

Serum samples (n = 97) were submitted to a commercial reference laboratory (Gribbles Veterinary Pathology, Clayton, VIC, Australia) for determination of total protein and protein fraction (albumin, total globulins and  $\alpha$ -1,  $\alpha$ -2,  $\beta$  and  $\gamma$  globulin) concentrations.

Electrophoresis was conducted according to the manufacturer's recommendations using the semi-automated agarose gel electrophoresis system (Hydrasys, Sebia Inc., Norcross, GA, USA) and the split protein  $\beta$ 1 and  $\beta$ 2 gel reagent (Hydragel 30, Sebia Inc., Norcross, GA, USA). The resultant gel was fixed, stained and scanned using the same equipment. Densitometer laser tracings were used to measure protein fraction percentages using established laboratory conventions [48], and absolute values were determined on the basis of biuret total protein measurement. The albumin-globulin (A-G) ratio was calculated by dividing the albumin value by the sum of the globulin fraction values.

### ***PHA Skin Response Test***

The thickness of each intradermal injection site was measured to the nearest 0.02 mm using manual vernier callipers (Mitutoyo Corporation, Kanagawa, Japan) immediately before and then 6, 12, 24 and 48 h post-injection. The PHA stimulation response was calculated as the change in the thickness (mm) of the PHA-injected interdigital site minus the change in thickness of the PBS-injected control site.

### ***SRBC Antibody Microtiter Assay***

Total (IgM and IgY) and 2-mercaptoethanol-resistant (IgY) SRBC antibody activities were measured 7 d post-immunization using a standard microtiter method [30,49]. Saline (PBS, 50  $\mu$ l) was added to each well in 96-well round-bottomed microtiter plates (Eppendorf AG, Hamburg, Germany). Serum (50  $\mu$ l) was added to the first well of each row, and serial two-fold dilutions were performed across rows. Fifty  $\mu$ l of 0.5% SRBC suspension in PBS was then

added to each well and the plates incubated at 37°C for 3 h and then overnight at room temperature. Titers were recorded as LOG<sub>10</sub> of the reciprocal of the highest dilution showing agglutination. To measure IgY titers, serum samples were incubated for 60 min with 0.2 M 2-mercaptoethanol (Sigma-Aldrich Inc., St Louis, MO, USA) before dilution. All serum samples were assayed in duplicate and the same batch of SRBC was used for all immunizations and assays.

### ***Splenic Lymphocyte Count, Cell Viability and Plaque-forming Cells***

Each spleen was divided equally by weight and processed following standard avian and mammalian procedures [30,40,41,49,50]. One half was fixed in 10% neutral buffered formalin<sup>§</sup>, the other was homogenised into a single cell suspension in 1.0 ml Hanks balanced salt solution (HBSS) (Sigma-Aldrich Inc., St Louis, MO, USA) with a scalpel blade and immediately placed on ice. Total splenic lymphocytes in the cell suspension were counted manually in a modified Neubauer hemocytometer at 400× magnification.

Spleen cell viability was determined by incubating 0.2 ml spleen cell suspension with 0.3 ml HBSS and 0.5 ml trypan blue solution (Sigma-Aldrich Inc., St Louis, MO, USA) for 10 min. Stained (non-viable) and unstained (viable) cells were counted manually in a modified Neubauer hemocytometer at 400× magnification; cells on both sides of the grid in the five middle squares were counted and the mean count recorded. Cell viability % was calculated by dividing the mean number of unstained cells by the mean total number of stained and unstained cells.

Plaque-forming IgM-producing splenic lymphocytes were counted in two monolayer chambers following incubation of 0.04 ml spleen single cell suspension with 0.2 ml HBSS and 0.2 ml 10% SRBC suspension at 37°C for 1 h. Hemolytic plaque-forming cells (PFC) were counted manually in two microscope fields at 50× magnification and the total number of PFC calculated per spleen.

### ***Culture and harvest of Bd***

The *Bd* isolate (Melbourne-L.lesueuri-00-LB-1-p19) was originally harvested from a clinically diseased captive juvenile *L. lesueuri* and cultured on tryptone/gelatin hydrolysate/lactose (TGhL) agar with streptomycin sulfate and benzylpenicillin (Sigma Aldrich Inc., St Louis, MO, USA) [51]. Cultures were maintained in half-strength TGhL broth at 4°C. Zoospores for frog inoculation were harvested by flooding 4 d old agar plate cultures maintained at 22°C with a

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<sup>§</sup> The spleen was fixed for future histological analysis if required; analysis was not included as part of this report.

dilute electrolyte solution (mmol l<sup>-1</sup>: KH<sub>2</sub>PO<sub>4</sub> 1, CaCl<sub>2</sub>.H<sub>2</sub>O 0.2, MgCl<sub>2</sub> 0.1) and counted in a hemocytometer (Brand GMBH and CO KG, Wertheim, Germany) [10,52].

### ***Statistical Analysis***

Independent-samples *t*-tests were used to compare functional tests for immune competence in healthy frogs between the two species, between healthy and *Bd*-exposed frogs within each of the two species, and between infected *L. caerulea* with low *Bd* loads (< 1,000 zoospores) and high *Bd* loads (> 1,000 zoospores). Variables analyzed included skin PHA stimulation, serum IgY and combined serum IgM/IgY titers, total splenic cell count, splenic PFC count, splenic cell viability, ratios of kidney, liver and spleen to body weight, and various hematologic, plasma biochemical and protein electrophoretic parameters.

Paired-samples *t*-tests were used to compare various hematologic, plasma biochemical and protein electrophoretic variables pre- and post-immune stimulation within each experimental group (healthy and *Bd*-exposed) for each species.

The software package PASW<sup>®</sup> Statistics (Version 18, 2009, SPSS Inc., Chicago, IL, USA) was used for all analyses, and statistical significance was set at  $\leq 0.050$  in all cases.



#### 5.2.4 Results

##### **Bd-infected *Litoria caerulea***

At day 75 post-exposure when immune function tests were initiated, 100% of exposed *L. caerulea* (10/10) tested positive for *Bd* on PCR. Zoospore counts per sample ranged from 24 to > 10,000; six frogs had low *Bd* loads (< 1,000 zoospores) and four had high *Bd* loads (> 1,000 zoospores).\*\* One frog with > 10,000 zoospores showed mild clinical signs of disease including lethargy and cutaneous erythema; all other frogs were clinically normal.

Mean splenic total and PFC counts, splenic cell viability and liver-body weight ratio post-immune stimulation were lower in *Bd*-infected *L. caerulea* (n = 10) compared with the uninfected frogs (n = 10) (Table 5.1). Mean responses to all of the functional tests for immune competence did not differ between infected frogs with low and high *Bd* loads ( $P > 0.050$  in all cases).

Following immune stimulation of *Bd*-infected *L. caerulea*, mean thrombocyte count decreased and A-G ratio increased; all other hematologic, plasma biochemical and serum protein electrophoretic parameters did not differ significantly pre- and post-stimulation (Tables 5.2 and 5.3).

Compared with *Bd*-infected individuals, immune stimulation of healthy uninfected *L. caerulea* caused a greater magnitude of increase in total WBC (M +15.0 (SD 17.8) versus +1.7 (3.7)  $\times 10^9 \text{ l}^{-1}$ ) (Figure 5.1A), lymphocyte (M +12.2 (SD 14.5) versus +0.9 (1.8)  $\times 10^9 \text{ l}^{-1}$ ) (Figure 5.1B) and monocyte (M +1.8 (SD 1.5) versus +0.5 (0.9)  $\times 10^9 \text{ l}^{-1}$ ) counts and polychromasia (M +6.9 (SD 9.3) versus -0.7 (2.5) %), and a greater decrease in Hb concentration (M -2.6 (SD 1.2) versus -0.4 (2.9)  $\text{g dl}^{-1}$ ) (Table 5.2).

Compared with *Bd*-infected individuals, immune stimulation of healthy uninfected *L. caerulea* caused a significantly greater increase in total globulins (M +7.0 (SD 8.2) versus -3.5 (8.1)  $\text{g/L}$ ) (Figure 5.2A),  $\alpha$ -1 globulin (M +5.3 (SD 6.3) versus -0.9 (5.4)  $\text{g l}^{-1}$ ) (Figure 5.2B) and  $\gamma$  globulin (M +0.6 (SD 0.7) versus -0.5 (0.8)  $\text{g l}^{-1}$ ) (Figure 5.2C) concentrations, and a greater decrease in A-G ratio (M -0.13 (SD 0.12) versus +0.08 (0.03)) (Table 5.3).

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\*\* Zoospore loads were divided into these 'low' and 'high' categories since clinical chytridiomycosis is related to infection intensity (Voyles et al., 2009).

### ***Bd*-exposed *Litoria infrafrenata***

At day 75 post-exposure, 0% of the nine exposed *L. infrafrenata* tested positive for *Bd* on PCR. Five frogs had low *Bd* loads at either day 10 (n = 3) or 20 (n = 2) post-exposure, but all of these were negative from day 30 onwards post-exposure, and the other four frogs tested negative throughout the experiment.

Mean skin PHA response and kidney-body weight ratio were lower in exposed uninfected *L. infrafrenata* compared with healthy uninfected individuals post-immune stimulation; mean responses to the other immune function tests did not differ between the two groups (Table 5.1).

Following immune stimulation of *Bd*-exposed uninfected *L. infrafrenata*, mean Hb concentration and RBC and thrombocyte counts decreased, while glucose concentration increased; all other hematologic, plasma biochemical and serum protein electrophoretic parameters did not differ significantly (Tables 5.4 and 5.5).

Compared with *Bd*-exposed uninfected individuals, immune stimulation of healthy uninfected *L. infrafrenata* caused a greater change in total WBC (M +14.7 (SD 12.4) versus -2.7 (15.5)  $\times 10^9 \text{ l}^{-1}$ ) (Figure 5.3A) and neutrophil (M +10.5 (SD 7.9) versus -2.8 (6.0)  $\times 10^9 \text{ l}^{-1}$ ) (Figure 5.3B) counts and polychromasia (M +9.8 (SD 8.0) versus +1.8 (3.0) %), and a greater decrease in thrombocyte count (M -27.6 (SD 13.8) versus -6.9 (8.3)  $\times 10^9 \text{ l}^{-1}$ ) (Table 5.4).

Compared with *Bd*-exposed uninfected individuals, immune stimulation of healthy uninfected *L. infrafrenata* caused a significantly greater change in  $\alpha$ -1 globulin concentration (M +2.5 (SD 3.1) versus -2.3 (3.1)  $\text{g l}^{-1}$ ) (Figure 5.4), and a greater change in glucose concentration (M -0.7 (SD 0.9) versus +0.7 (0.8)  $\text{mmol l}^{-1}$ ) (Table 5.5). Changes in other variables post-immune stimulation did not differ between the two groups.

### ***Healthy uninfected tree frogs***

Functional immune competence test results for healthy uninfected *L. caerulea* and *L. infrafrenata* are presented in Table 5.1. Following immune stimulation, mean total splenic PFC ( $P = 0.006$ ) and liver to body weight ratio ( $P = 0.003$ ) were greater in *L. caerulea*, while mean spleen to body weight ratio was greater in *L. infrafrenata* ( $P = 0.001$ ). Mean responses to the other immune function tests did not differ between the two species.

Pre- and post-immune stimulation hematologic values for healthy uninfected *L. caerulea* and *L. infrafrenata* are presented in Tables 5.2 and 5.4 respectively. Following immune stimulation of *L. caerulea*, PCV, Hb, MCH and MCHC decreased, while total WBC, lymphocyte and

monocyte counts and polychromasia % increased. In *L. infrafronata*, total RBC and thrombocyte counts decreased following immune stimulation, while total WBC, neutrophil and monocyte counts, neutrophil-lymphocyte ratio, and polychromasia % increased.

Pre- and post-immune stimulation plasma biochemical and serum protein electrophoresis values for healthy uninfected *L. caerulea* and *L. infrafronata* are presented in Tables 5.3 and 5.5 respectively. Five protein fractions were defined in all samples submitted for electrophoresis. Following immune stimulation of *L. caerulea*: total globulins along with  $\alpha$ -1 and  $\gamma$  globulin fraction concentrations increased, while the A-G ratio decreased; none of the plasma biochemical values changed significantly. In *L. infrafronata*: plasma CK increased while glucose decreased post-immune stimulation; total protein, albumin, total globulins and  $\alpha$ -1 globulin concentrations, and A-G ratio, all increased.

**Table 5.1.** Functional test results for immune competence in uninfected and *Bd*-exposed *L. caerulea* and *L. infrafrenata* before and after stimulation with intradermal PHA and intracelomic SRBC.

Species	<i>Litoria caerulea</i>					<i>Litoria infrafrenata</i>				
	Uninfected (n = 10)		Infected <sup>a</sup> (n = 10)		<i>P</i> value	Uninfected (n = 10)		Exposed <sup>b</sup> (n = 9)		<i>P</i> value
Immune Parameter	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
Skin PHA Stimulation (mm)	0.14	0.46	0.04	0.08	0.495	0.25	0.39	-0.19	0.34	0.017
IgM + IgY Titre (LOG <sub>10</sub> )	4.7	3.1	3.7	1.3	0.370	4.9	2.3	4.6	2.7	0.800
IgY Titre (LOG <sub>10</sub> )	3.4 <sup>c</sup>	2.5	4.1	4.2	0.529	4.0 <sup>c</sup>	2.6	6.5	3.6	0.062
Splenic Cell Count (x 10 <sup>6</sup> )	36.4	14.9	12.7	8.6	0.000	40.6	14.4	31.4	21.0	0.277
Splenic Cell Viability (%)	69.0	11.5	35.7	14.5	0.000	70.4	11.4	62.0	6.8	0.070
Plaque-forming Cells (x 10 <sup>3</sup> )	2423	1369	898	629	0.005	855	497	755	356	0.623
Final BW <sup>d</sup> (g)	41.5	19.1	59.8	12.8	-	37.2	12.1	66.9	13.4	-
Kidney-BW Ratio	0.60	0.13	0.51	0.16	0.164	0.64	0.13	0.51	0.06	0.011
Liver-BW Ratio	5.23	1.61	2.89	0.64	0.001	3.23	0.65	3.39	1.16	0.726
Spleen-BW Ratio	0.04	0.01	0.04	0.01	0.216	0.13	0.07	0.12	0.06	0.555

<sup>a</sup> 100% of 10 exposed *L. caerulea* were infected 75 d post-exposure to *Bd*.

<sup>b</sup> 0% of 9 exposed *L. infrafrenata* were infected 75 d post-exposure to *Bd*.

<sup>c</sup> n = 9.

<sup>d</sup> BW, body weight.

**Table 5.2.** Pre- and post-immune stimulation hematologic values for uninfected and *Bd*-infected *Litoria caerulea*.

Status	Uninfected (n = 10)					Infected (n = 10)					<i>P</i> value <sup>b</sup>
	Pre (day 0)		Post (day 7)			Pre (day 0)		Post (day 7)			
Immune Stimulation Parameter	Mean	SD	Mean	SD	<i>P</i> value <sup>a</sup>	Mean	SD	Mean	SD	<i>P</i> value <sup>a</sup>	
PCV (%)	35.9	3.4	28.9	4.8	0.004	36.1	4.0	34.5	7.5	0.485	0.074
Hb (g dl <sup>-1</sup> )	8.7	1.2	6.2	1.4	0.000	9.0	2.4	8.6	1.8	0.670	0.042
RBC (x10 <sup>9</sup> l <sup>-1</sup> )	694	135	588	120	0.137	553	90	580	132	0.515	0.098
MCV (fl)	536	117	503	107	0.402	663	91	606	107	0.237	0.697
MCH (pg)	129	25	107	24	0.021	165	44	151	26	0.493	0.675
MCHC (g l <sup>-1</sup> )	245	38	214	30	0.010	247	46	252	33	0.827	0.137
Thrombocyte (x10 <sup>9</sup> l <sup>-1</sup> )	34.8	9.0	30.5	7.3	0.299	27.0	5.9	23.6	4.0	0.042	0.823
WBC (x10 <sup>9</sup> l <sup>-1</sup> )	24.6	8.9	39.7	15.4	0.026	6.8	1.9	8.5	4.0	0.175	0.044
Neutrophil (x10 <sup>9</sup> l <sup>-1</sup> )	3.9	1.8	4.9	2.2	0.303	2.0	1.0	2.3	1.8	0.576	0.513
Lymphocyte (x10 <sup>9</sup> l <sup>-1</sup> )	19.0	7.1	31.2	13.9	0.026	4.1	1.5	5.0	1.9	0.179	0.035
Neut-lymph ratio	0.22	0.10	0.18	0.09	0.224	0.54	0.32	0.46	0.24	0.376	0.694
Monocyte (x10 <sup>9</sup> l <sup>-1</sup> )	1.3	0.9	3.1	1.7	0.005	0.6	0.7	1.1	0.7	0.115	0.038
Eosinophil (x10 <sup>9</sup> l <sup>-1</sup> )	0.50	0.95	0.50	0.68	0.814	0.06	0.08	0.10	0.12	0.055	0.676
Basophil (x10 <sup>9</sup> l <sup>-1</sup> )	0.00	0.00	0.04	0.12	0.343	0.00	0.00	0.00	0.00	-	0.343
Polychromasia (%)	6.9	4.0	13.8	10.5	0.044	3.3	1.8	2.6	2.8	0.396	0.032

<sup>a</sup> Paired-samples *t*-tests between days 0 and 7 within each group (uninfected and infected).

<sup>b</sup> Independent-samples *t*-tests between the two groups for the *change* in each variable from day 0 to day 7.

**Table 5.3.** Pre- and post-immune stimulation plasma biochemical and serum protein electrophoretic values for uninfected and *Bd*-infected *Litoria caerulea*.

Status	Uninfected (n = 10)					Infected (n = 10)					<i>P</i> value <sup>b</sup>
	Pre (day 0)		Post (day 7)		<i>P</i> value <sup>a</sup>	Pre (day 0)		Post (day 7)		<i>P</i> value <sup>a</sup>	
Immune Stimulation Parameter	Mean	SD	Mean	SD			Mean	SD	Mean		SD
AST (U l <sup>-1</sup> )	69	37	73	34	0.800	68	14	97	42	0.068	0.268
CK (U l <sup>-1</sup> )	495	344	636	496	0.566	282	185	555	337	0.074	0.632
Uric Acid (μmol l <sup>-1</sup> )	37	27	40	22	0.813	35	18	37	29	0.811	0.937
Glucose (mmol l <sup>-1</sup> )	3.5	0.8	3.4	0.7	0.766	4.5	0.9	4.6	0.9	0.715	0.640
Calcium (mmol l <sup>-1</sup> )	3.03	0.46	3.00	0.54	0.861	2.75	0.40	2.72	0.40	0.697	0.936
Phosphorus (mmol l <sup>-1</sup> )	1.70	0.46	1.49	0.42	0.194	0.88	0.23	0.83	0.35	0.587	0.389
Ca-P ratio	1.89	0.58	2.16	0.59	0.193	3.29	0.82	3.78	1.42	0.260	0.639
Potassium (mmol l <sup>-1</sup> )	4.8	1.7	4.2	1.9	0.288	5.2	1.5	4.2	2.2	0.343	0.749
Sodium (mmol l <sup>-1</sup> )	111.7	6.1	111.5	4.8	0.929	109.5	6.2	108.5	4.9	0.535	0.768
Total Protein (g l <sup>-1</sup> )	57.4	6.3	62.6	6.8	0.106	57	7	54	6	0.353	0.069
A-G ratio	0.57	0.12	0.44	0.09	0.008	0.46	0.16	0.54	0.17	0.000	0.000
Albumin (g l <sup>-1</sup> )	20.5	3.4	18.7	2.2	0.130	18	5	18	4	0.604	0.106
Total globulins (g l <sup>-1</sup> )	36.9	5.2	43.9	6.6	0.024	39.2	4.9	35.8	6.6	0.207	0.010
α-1 globulin (g l <sup>-1</sup> )	22.7	4.1	28.0	4.0	0.026	21.5	3.6	20.7	5.2	0.620	0.030
α-2 globulin (g l <sup>-1</sup> )	6.9	1.2	8.2	3.1	0.302	9.2	1.5	7.9	2.0	0.092	0.072
β globulin (g l <sup>-1</sup> )	5.2	1.6	4.9	1.5	0.696	5.8	1.7	5.0	1.0	0.077	0.541
γ globulin (g l <sup>-1</sup> )	2.2	0.8	2.8	1.1	0.023	2.7	1.0	2.2	0.7	0.072	0.004

<sup>a</sup> Paired-samples *t*-tests between days 0 and 7 within each group (uninfected and infected).

<sup>b</sup> Independent-samples *t*-tests between the two groups for the *change* in each variable from day 0 to day 7.

**Table 5.4.** Pre- and post-immune stimulation hematologic values for uninfected and *Bd*-exposed but uninfected *Litoria infrafrenata*.

Status	Uninfected (n = 10)					Exposed uninfected (n = 9)					<i>P</i> value <sup>b</sup>
	Pre (day 0)		Post (day 7)			Pre (day 0)		Post (day 7)			
Immune Stimulation Parameter	Mean	SD	Mean	SD	<i>P</i> value <sup>a</sup>	Mean	SD	Mean	SD	<i>P</i> value <sup>a</sup>	
PCV (%)	26.9	7.0	25.4	4.1	0.548	35.7	6.0	32.3	4.8	0.079	0.548
Hb (g d l <sup>-1</sup> )	7.1	2.5	5.6	1.1	0.086	8.6	1.3	7.4	1.7	0.024	0.691
RBC (x10 <sup>9</sup> l <sup>-1</sup> )	736	208	630	129	0.048	783	109	662	108	0.018	0.811
MCV (fl)	383	155	421	124	0.450	459	74	500	121	0.377	0.969
MCH (pg)	107	60	93	32	0.525	111	19	116	38	0.674	0.457
MCHC (g l <sup>-1</sup> )	291	126	220	29	0.142	245	41	232	47	0.306	0.240
Thrombocyte (x10 <sup>9</sup> l <sup>-1</sup> )	54.8	12.9	27.2	7.2	0.000	33.7	10.1	26.8	7.0	0.036	0.001
WBC (x10 <sup>9</sup> l <sup>-1</sup> )	32.9	8.7	47.6	10.2	0.005	33.7	27.0	31.0	17.4	0.616	0.015
Neutrophil (x10 <sup>9</sup> l <sup>-1</sup> )	5.8	5.9	16.4	5.1	0.002	12.1	14.3	9.3	10.0	0.204	0.001
Lymphocyte (x10 <sup>9</sup> l <sup>-1</sup> )	24.3	7.7	25.5	6.2	0.726	17.8	11.1	17.3	11.1	0.860	0.700
Neut-lymph ratio	0.28	0.36	0.67	0.24	0.038	0.66	0.47	0.71	0.71	0.726	0.120
Monocyte (x10 <sup>9</sup> l <sup>-1</sup> )	2.1	1.4	5.6	3.5	0.023	3.3	3.9	4.0	3.1	0.522	0.131
Eosinophil (x10 <sup>9</sup> l <sup>-1</sup> )	0.25	0.44	0.04	0.13	0.205	0.57	0.76	0.34	0.45	0.355	0.925
Basophil (x10 <sup>9</sup> l <sup>-1</sup> )	0.47	0.79	0.00	0.00	0.095	0.00	0.00	0.00	0.00	-	0.095
Polychromasia (%)	5.5	3.6	15.3	7.9	0.004	9.3	5.1	11.1	6.7	0.108	0.013

<sup>a</sup> Paired-samples *t*-tests between days 0 and 7 within each group (uninfected and exposed).

<sup>b</sup> Independent-samples *t*-tests between the two groups for the *change* in each variable from day 0 to day 7.

**Table 5.5.** Pre- and post-immune stimulation plasma biochemical and serum protein electrophoretic values for uninfected and *Bd*-exposed but uninfected *Litoria infrafrenata*.

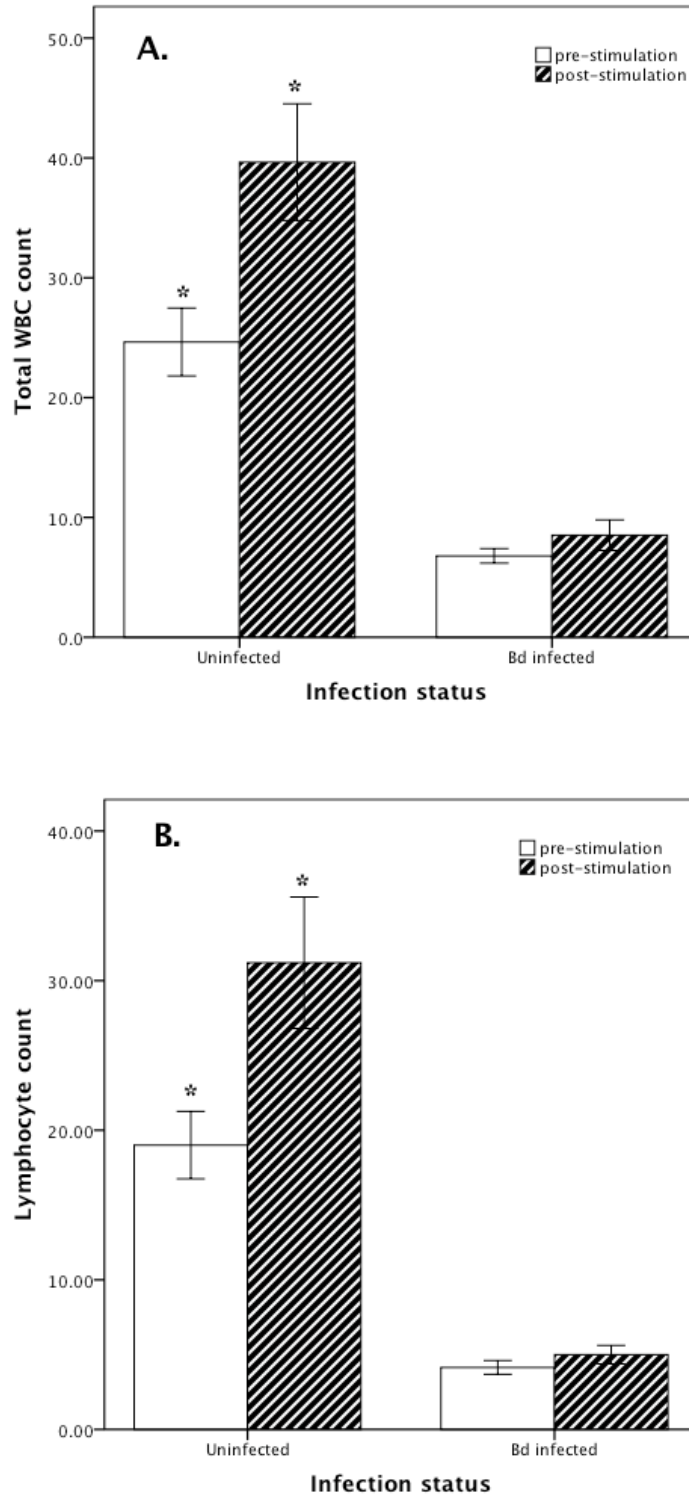
Status	Uninfected (n = 10)					Exposed uninfected (n = 9)					<i>P</i> value <sup>b</sup>
	Pre (day 0)		Post (day 7)		<i>P</i> value <sup>a</sup>	Pre (day 0)		Post (day 7)		<i>P</i> value <sup>a</sup>	
Immune Stimulation Parameter	Mean	SD	Mean	SD		Mean	SD	Mean	SD		Mean
AST (U l <sup>-1</sup> )	248	406	171	93	0.498	100	52	127	84	0.309	0.390
CK (U l <sup>-1</sup> )	556	286	1137	560	0.013	918	864	1276	929	0.218	0.539
Uric Acid (μmol l <sup>-1</sup> )	8	17	24	37	0.251	3.8	3.4	5.3	6.6	0.433	0.302
Glucose (mmol l <sup>-1</sup> )	3.6	0.6	2.9	0.8	0.036	3.4	0.4	4.2	1.0	0.020	0.002
Calcium (mmol l <sup>-1</sup> )	2.29	2.09	2.38	0.99	0.827	2.90	0.64	3.01	0.80	0.628	0.956
Phosphorus (mmol l <sup>-1</sup> )	1.54	0.63	1.73	0.76	0.404	1.59	0.43	1.52	0.32	0.412	0.279
Ca-P ratio	1.38	0.49	1.47	0.40	0.608	1.88	0.36	2.01	0.45	0.388	0.841
Potassium (mmol l <sup>-1</sup> )	3.7	0.8	3.8	0.9	0.591	4.8	2.0	4.2	1.5	0.183	0.145
Sodium (mmol l <sup>-1</sup> )	107.7	2.7	110.9	4.1	0.065	110.0	3.6	108.7	3.1	0.336	0.138
Total Protein (g l <sup>-1</sup> )	29	6	34	7	0.007	40	8	38	5	0.500	0.065
A-G ratio	0.11	0.04	0.13	0.04	0.033	0.21	0.06	0.23	0.07	0.175	0.609
Albumin (g l <sup>-1</sup> )	2.9	1.1	3.9	1.4	0.013	7.0	2.7	6.9	1.0	0.866	0.123
Total globulins (g l <sup>-1</sup> )	26.1	6.1	30.5	5.8	0.016	33.2	5.9	31.0	5.0	0.470	0.070
α-1 globulin (g l <sup>-1</sup> )	9.2	2.1	11.6	4.2	0.041	11.6	2.0	9.3	3.0	0.057	0.004
α-2 globulin (g l <sup>-1</sup> )	5.3	1.7	6.8	2.9	0.418	6.1	2.6	5.7	2.3	0.654	0.079
β globulin (g l <sup>-1</sup> )	10.1	5.5	11.0	4.0	0.192	13.7	4.2	14.6	2.7	0.665	0.968
γ globulin (g l <sup>-1</sup> )	1.4	0.8	1.2	0.3	0.271	1.3	0.4	1.4	0.3	0.883	0.317

<sup>a</sup> Paired-samples *t*-tests between days 0 and 7 within each group (uninfected and exposed).

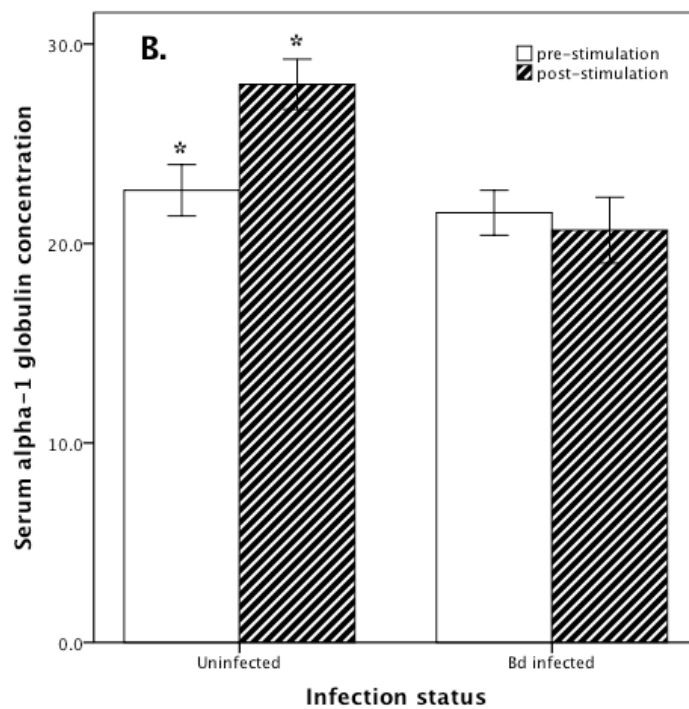
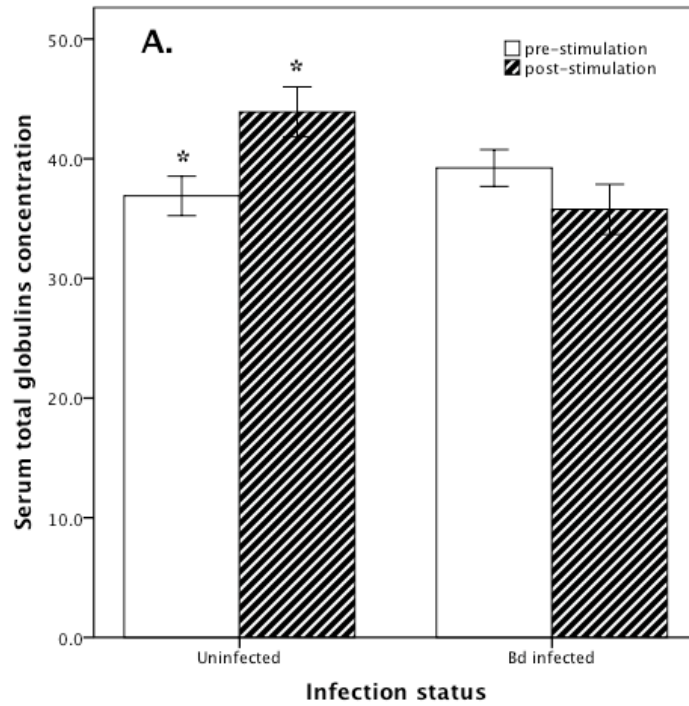
<sup>b</sup> Independent-samples *t*-tests between the two groups for the *change* in each variable from day 0 to day 7.

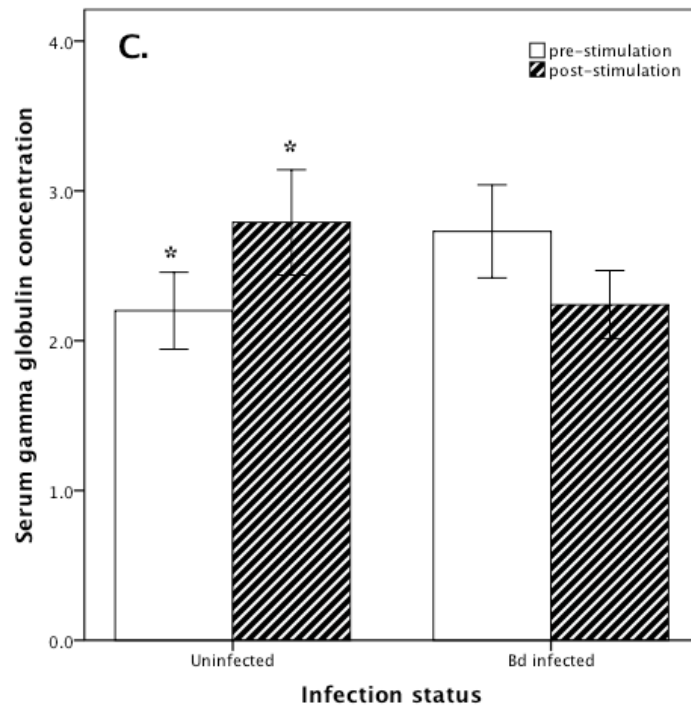


**Figure 5.1.** Total WBC (A) and lymphocyte (B) counts ( $\times 10^9 \text{ l}^{-1}$ ) pre- and post-immune stimulation in healthy uninfected ( $n = 10$ ) and *Bd*-infected ( $n = 10$ ) common green tree frogs (*Litoria caerulea*). Bars are mean  $\pm$  SEM. \*  $P < 0.050$  within each group.

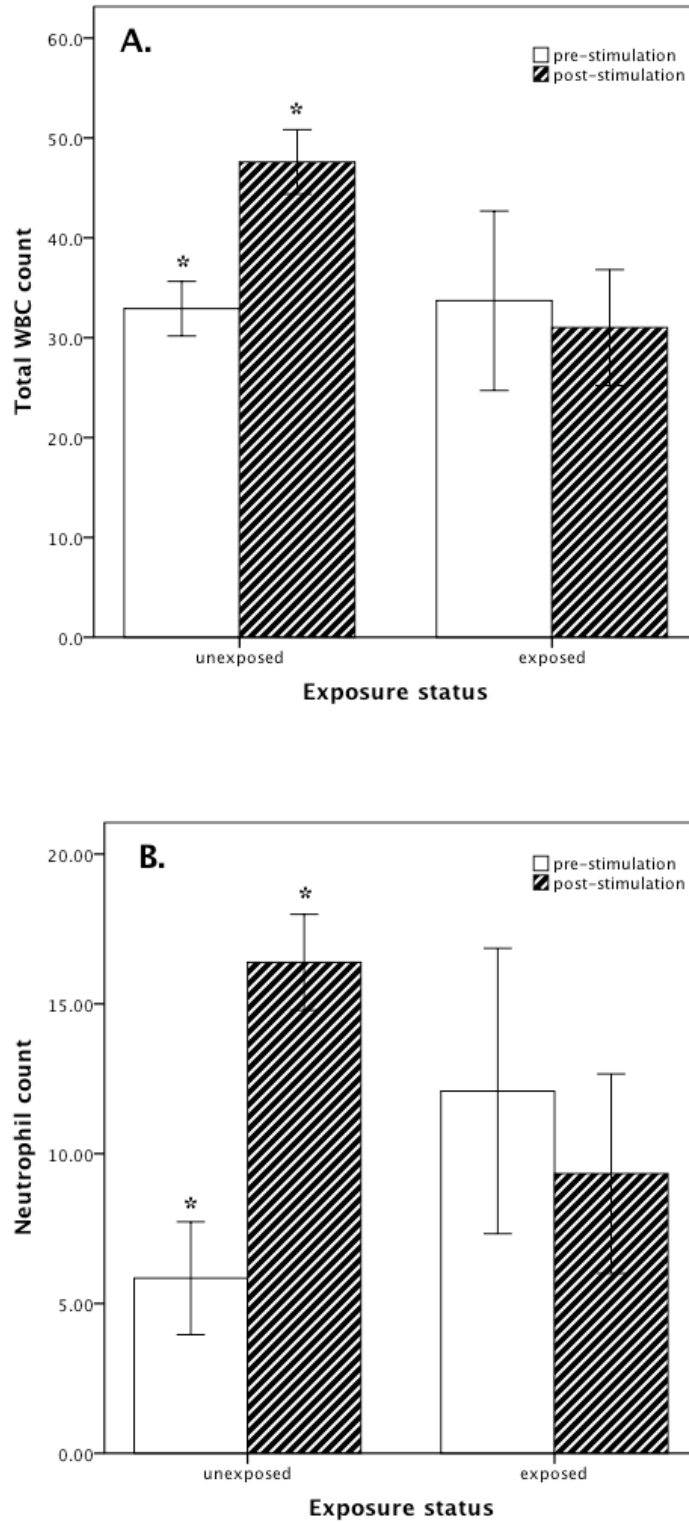


**Figure 5.2.** Serum total globulins (A),  $\alpha$ -1 globulin (B) and  $\gamma$  globulin (C) concentrations ( $\text{g l}^{-1}$ ) pre- and post-immune stimulation in healthy uninfected ( $n = 10$ ) and *Bd*-infected ( $n = 10$ ) common green tree frogs (*Litoria caerulea*). Bars are mean  $\pm$  SEM. \*  $P < 0.050$  within each group.

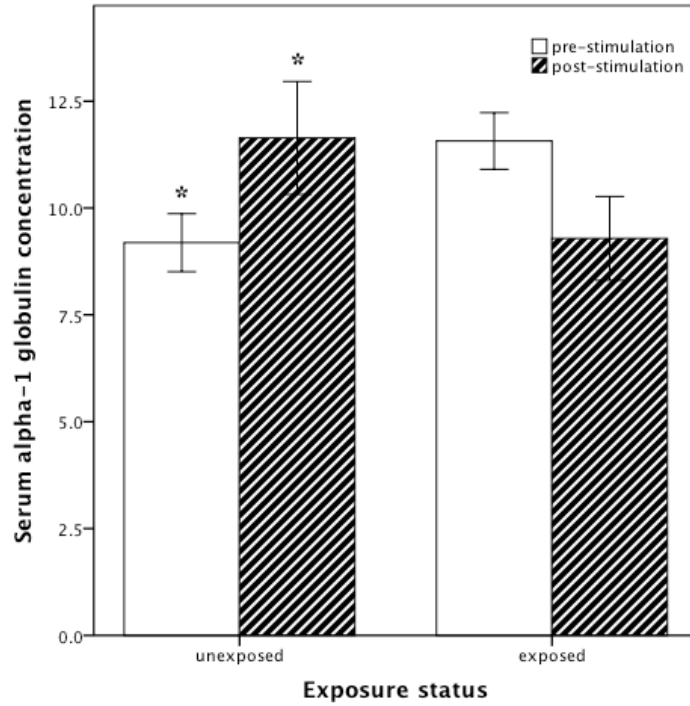




**Figure 5.3.** Total WBC (A) and neutrophil (B) counts ( $\times 10^9 \text{ l}^{-1}$ ) pre- and post-immune stimulation in healthy unexposed ( $n = 10$ ) and *Bd*-exposed but uninfected ( $n = 9$ ) white-lipped tree frogs (*Litoria infrafrenata*). Bars are mean  $\pm$  SEM. \*  $P < 0.050$  within each group.



**Figure 5.4.** Serum  $\alpha$ -1 globulin concentration ( $\text{g l}^{-1}$ ) pre- and post-immune stimulation in healthy unexposed ( $n = 10$ ) and *Bd*-exposed but uninfected ( $n = 9$ ) white-lipped tree frogs (*Litoria infrafrenata*). Bars are mean  $\pm$  SEM. \*  $P < 0.050$  within each group.



### 5.2.5 Discussion

Our results provide the first direct evidence that *Bd* actively suppresses a systemic immune response when infecting the amphibian host. Our experimental design was flawed in that the trial in healthy frogs commenced as a pilot study to validate methodology prior to application in the trial with *Bd*-infected frogs. Insufficient specimens were available to include a negative control group in the infection trial. This was largely compensated for by 1) standardizing timing of sample collection relative to initiation of immune tests, 2) a single experienced operator (SY) performing all of the experimental procedures, 3) using identical analytic equipment, reagents and methodology, 4) maintaining identical experimental laboratory and husbandry conditions (including staff, housing, ambient temperature, humidity, water and nutrition) and 5) regular assessment of frogs by a veterinarian (SY). Additionally, a large parallel reinfection experiment in our laboratory found that, with the exception of a mild decrease in WBC and lymphocyte counts on day 30, no hematologic immune biomarkers varied significantly 60 or 125 days into the trial compared with pre-trial values in a negative control group of *L. caerulea* (Young et al., unpublished). This group received identical treatment to the *Bd* exposed frogs but without the *Bd* inoculum, and blood was collected from all frogs on three occasions during the trial.

Our results are a major step forward in following up on previous genetic and *in vitro* studies. In *Silurana tropicalis*, *Bd* infection appeared to cause down-regulation of some immune genes including those associated with Toll-like receptors, complement pathways, and B- and T-lymphocytes [26,27]. Other studies found that soluble factors in *Bd* culture supernatant inhibited *in vitro* lymphocyte proliferation assays (J. Ramsey et al., unpublished in [28]). A recent field study showed higher urinary corticosterone metabolites in *L. wilcoxii* infected with *Bd*, indicating a physiological stress response [29].

Stress hormones are known to alter normal WBC distribution, and peripheral leukocyte profiles consistent with a classical mammalian stress-related response include a relative neutrophilia, lymphopenia and eosinopenia [53]. Peripheral neutrophilic and eosinopenic responses have previously been reported in *Bd*-infected larval anurans (*Rana catesbeiana*), although lymphocyte concentration did not change [54]. Conflicting findings have been reported in post-metamorphic anurans: juvenile *L. chloris* showed relative peripheral neutropenic, eosinopenic and basophilic responses to *Bd* infection [20], while *L. caerulea* showed a lymphopenic response [55]. The amphibian leukocyte response to stress varies according to species and many other intrinsic and extrinsic factors, including season and gender [43,56,57], making interpretation of leukocyte profiles alone difficult.

We successfully used a range of hematologic, plasma biochemical and protein electrophoresis biomarkers, along with various functional tests, to assess immune competence in two species of healthy uninfected tree frogs, in *Bd*-infected *L. caerulea* and in *Bd*-exposed *L. infrafrenata*. Antigenic stimulation of healthy uninfected frogs resulted in quantifiable splenic, WBC, serum protein and immunoglobulin responses in both species, but this response was minimal in *Bd*-infected *L. caerulea*. Compared with uninfected frogs, *Bd* infection caused a significant reduction in splenic, WBC, serum protein and immunoglobulin responses. Our findings of systemic host immune suppression by *Bd* could be a key factor in its pathogenicity. Further, changes in immune response of *Bd*-exposed *L. infrafrenata* suggest direct sub-clinical immunologic effects associated with pathogen exposure.

### **Litoria caerulea**

Following stimulation of healthy uninfected *L. caerulea*, immune organ activation resulted in circulating WBC and serum protein responses, total (IgM and IgY) and IgY-specific serum antibody production, and splenic lymphocytic IgM-specific antibody production. These indicate active cellular and humoral responses consistent with those seen in other taxonomic groups [30,49]. Skin PHA stimulation was minimal and variable in contrast to other taxa studied. The change in RBC indices (reduced PCV, Hb, MCH and MCHC concurrent with increased polychromasia) were not unexpected due to repeated blood sample collection within a short period of time, and indicate an adequate bone marrow regenerative response to relative anemia [47]. This range of data, combined with additional splenic cell measurements, provides comprehensive baseline immune function data for this species.

The 10 *L. caerulea* exposed to *Bd* all became infected, but only four were considered to have high zoospore burdens 75 d post-exposure when immune function tests were initiated, and only one individual was clinically affected. Stimulation of *Bd*-infected *L. caerulea* caused little activation of the various immune biomarkers measured, with the exception of a decrease in mean thrombocyte count and an A-G ratio increase. Since none of the five protein fractions increased after immune stimulation, the significance of the ratio change alone remains unclear but may indicate a subtle serum protein response. The decreased mean thrombocyte count is most likely a response to recent blood collection and relative anemia, but may also represent depressed bone marrow function. Mean thrombocyte count also decreased in the uninfected frogs but this was not statistically significant. The multiple distinct hematologic and protein electrophoresis responses to antigenic stimulation in healthy uninfected *L. caerulea* were absent in *Bd*-infected frogs.

Compared with healthy uninfected *L. caerulea*, *Bd* infection caused a significant reduction in total spleen cell concentration, splenic cell viability, splenic lymphocyte antibody production and liver-body weight ratio. The WBC, lymphocyte, monocyte and serum protein responses to immune stimulation were also reduced. All of these changes indicate a significantly impaired ability of *Bd*-infected frogs to respond adequately to antigenic stimulation. Infected frogs had a lower Hb concentration decrease and polychromatophilic RBC response following immune stimulation. Although these two changes are likely to be a direct regenerative response to recent blood collection, the lower magnitude of these responses in the *Bd*-infected frogs may indicate suppression of organ response to physiologic stimulation.

### **Litoria infrafrenata**

All *Bd*-exposed *L. infrafrenata* failed to maintain infection despite identical experimental conditions to those for *L. caerulea*, and five of the nine frogs that tested positive early in the trial self-cured. Previous laboratory and field studies show that *L. caerulea* is highly susceptible to *Bd* [11,12,58], but little data is available for *L. infrafrenata*. Our results indicate that *L. infrafrenata* is a naturally resistant host. The exposure experiment and sampling protocols were completed despite the loss of infection, and results show sub-clinical immunologic effects in response to *Bd* exposure and clearance in this species.

Pre-exposure data collected from *L. infrafrenata* provides comprehensive baseline immune function data for this species. Parameters did not differ greatly from *L. caerulea*, hence our results do not suggest a clear mechanism for greater resistance in *L. infrafrenata*. Active cellular, humoral and splenic responses in *L. infrafrenata* were generally consistent with those seen in our study in *L. caerulea*, and in other taxonomic groups [30,49]. Intradermal PHA stimulation caused a slightly greater response in *L. infrafrenata* compared with *L. caerulea*, but this was still minimal compared with other species [30,49] and quite variable. The skin peptide profiles of *L. infrafrenata* and *L. caerulea* differ greatly [59] and this could partly explain inter-species differences in host resistance, although *L. infrafrenata* produces no known major antibiotic peptides compared with at least five that have been identified in *L. caerulea* [59]. Research to further evaluate species-specific skin peptides and their role in local host immune response would be valuable.

Notable inter-species differences in our results include the neutrophilic versus lymphocytic WBC response and the hyperglycemia and elevated CK post-immune stimulation in *L. infrafrenata*. Neutrophil reference values in *L. infrafrenata* vary with season, and are significantly higher compared with *L. caerulea* [43]. Furthermore, the two species differ in temperament, with *L. caerulea* generally calm and tolerant of handling, and *L. infrafrenata*



often exhibiting clinical signs of stress associated with handling and confinement. This is likely to account for the neutrophilic WBC response and the increased glucose and CK values in *L. infrafrenata*. The reduced RBC count concurrent with increased polychromatophilic response again was not unexpected due to repeated blood sample collection, and indicate an adequate bone marrow regenerative response.

With the exception of a reduced skin PHA response and kidney-body weight ratio in exposed frogs that cleared infection, responses to all of the other functional immune tests did not differ between healthy uninfected and exposed uninfected *L. infrafrenata*. Variations in RBC indices and plasma glucose were not unexpected as previously discussed. However, the post-immune stimulation WBC, neutrophil, polychromatophilic RBC, thrombocyte and  $\alpha$ -1 globulin responses were of lower magnitude in the exposed frogs. This indicates a direct sub-clinical effect of *Bd*-exposure and subsequent clearance on immune system function in frogs challenged post-metamorphosis. Larval common toads (*Bufo bufo*) experimentally exposed to low *Bd* doses usually died at or soon after metamorphosis without detectable infections, suggesting fitness costs attributable to exposure, control and clearance in the absence of extensive pathogen proliferation [60].

Our baseline immune function data provide a valuable tool for progressing future immunologic and *Bd* pathogenicity studies in amphibians. General mechanisms of fungal suppression include inducing anti-inflammatory cytokines, decreasing pro-inflammatory cytokines and complement evasion [61]. Further work on understanding the mechanisms of *Bd* immune suppression is needed and may involve exposing frogs directly to identified pathogen-secreted factors and assessing immune function using the *in vivo* methods that we have shown here to be sensitive indicators.

Our comparison of immune function between uninfected and *Bd*-infected *L. caerulea* has identified active systemic host immune suppression as an important contributing factor to susceptibility to *Bd* infection in amphibians. Our results may explain the lack of an adaptive immune response to infection [22] (Young et al., unpublished; Cashins et al., unpublished), and suggest that if vaccine development or other immune modulation is attempted in future trials, it will be necessary to better understand the mechanisms of immune suppression in order to overcome it.

### **Conflict of interest statement**

The authors declare that they have no competing interests.

## **Acknowledgments**

We thank Helen Martin and Deb Buckett at Gribbles Veterinary Pathology for assistance with protein electrophoresis, Ammar Aziz from James Cook University for assistance with microtiter assays, and the Australian Government Department of the Environment and Heritage for provision of funding for this project.

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## CHAPTER 6

### Host resistance against chytridiomycosis

#### 6.1 Introduction

There are few safe and effective treatment protocols for chytridiomycosis and limitations associated with current regimes include long treatment durations, high labour input, heat intolerance of many amphibians and inability to treat wild amphibians *in situ*. These have prompted researchers to investigate mechanisms of host resistance against *B. dendrobatidis* and immune modulation in the management of this disease. Despite amphibian immunity in general being well described in the literature, very little is known about specific mechanisms of defense against fungal pathogens such as *B. dendrobatidis*. Furthermore, the few available studies provide conflicting evidence about the potential role of adaptive immunity in host resistance against infection with this fungal pathogen.

This chapter consists of one manuscript submitted for publication detailing investigations into resistance against reinfection with *B. dendrobatidis* in tree frogs. Our results showed not only that prior infection has no significant effect on re-infection rate compared with naïve frogs exposed for the first time, but that prior infection also appears to have a long-term adverse effect on host resistance.

#### 6.2 Host resistance against reinfection

This manuscript represents my original research and writing and has been submitted for publication to the peer-reviewed journal *Research in Veterinary Science*. I received study design advice from Rick Speare, Lee Berger and Lee Skerratt. Paul Whitehorn assisted with all husbandry and many experimental procedures. Paul Whitehorn and Jamie Voyles assisted with field collection of frogs. Stephen Garland ran the PCR analyses. I performed all of the statistical analyses with advice from Lee Skerratt. Rick Speare, Jamie Voyles, Lee Skerratt and Lee Berger assisted with interpretation of results. Lee Berger and Lee Skerratt gave substantial editorial input to the final written paper.

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The following manuscript is an exact, word-for-word copy of the manuscript submitted for publication, with the exception of minor amendments recommended by thesis reviewers. Section, table and figure numbering has been reformatted for this thesis. Since the journal uses American English, spelling follows this convention.

## **Chytridiomycosis makes frogs more susceptible to reinfection**

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### 6.2.1 Abstract

The amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) (*Bd*) has caused mass mortality leading to global amphibian population declines and extinctions. Little is known about specific mechanisms of amphibian defense against fungal pathogens, and recent studies provide conflicting evidence about the role of adaptive immunity in host resistance to *Bd*. We aimed generally to further investigate the role of host immunity in chytridiomycosis, and specifically to determine whether frogs develop long-term resistance against reinfection. We experimentally infected common green tree frogs (*Litoria caerulea*) with *Bd*, treated them with chloramphenicol to clear infection at 90 d post-exposure, and then re-exposed them to the pathogen at 383 d. More re-exposed frogs (78%) remained infected with *Bd* compared with naïve frogs (28%), and infected re-exposed frogs (including those that cleared infection) had a higher infection intensity that increased at a greater rate compared with infected naïve frogs. Also, more infected naïve frogs (59%) self-cured compared with the infected re-exposed group (0%), indicating naïve frogs controlled and cleared infection better. Within 30 d of the first exposure, *Bd*-infected frogs had reduced total white blood cell, neutrophil, lymphocyte and monocyte counts, and higher plasma glucose concentrations, compared with pre-exposure levels. Neutrophil counts were lower, and plasma glucose concentrations higher, in infected frogs compared with uninfected controls. Hence, prior infection may adversely affect long-term host resistance and this has widespread implications for the future management of chytridiomycosis in amphibian populations.

**Key Words:** *Batrachochytrium dendrobatidis*, chytridiomycosis, *Litoria caerulea*, reinfection, resistance, tree frog

### 6.2.2. Introduction

The recent global spread of the emerging infectious disease chytridiomycosis has caused declines and extinctions of many amphibian species and contributed to devastating vertebrate biodiversity losses (Berger et al., 1998; Lips et al., 2006; Skerratt et al., 2007). The fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*), can spread rapidly through host populations (Lips et al., 2006; Skerratt et al., 2007), causes high mortality rates in many species (Daszak et al., 2003; Lips et al., 2006; Schloegel et al., 2006), and persists at low host densities (Woodhams and Alford, 2005; Skerratt et al., 2007). Sporangia are confined to the superficial layers of the epidermis and the infectious zoospore stage is water-borne (Berger et al., 2005a). The common green tree frog (*Litoria caerulea*) is highly susceptible to *Bd* and most experimentally infected individuals have progressed to death (Voyles et al., 2007, 2009). Fatal pathophysiological changes include epidermal degeneration, inhibited epidermal electrolyte transport, systemic electrolyte disturbances (hyponatremia and hypokalemia) (Voyles et al., 2007, 2009; Young et al., 2012), severe hypovolemia secondary to dehydration (Young et al., 2012) and asystolic cardiac arrest (Voyles et al., 2009).

There are few reports of safe and effective treatment protocols for chytridiomycosis (Retallick and Miera, 2007; Bishop et al., 2009; Bowerman et al., 2010; Martel et al., 2010; Young et al., 2012), and only one protocol has cured terminally ill anurans with severe disease (Young et al., 2012). Limitations associated with current effective treatment regimes include long treatment durations, high labour input, heat intolerance of many amphibians and inability to treat wild amphibians *in situ*. These have prompted researchers to investigate mechanisms of resistance against *Bd* and immune modulation in the management of this formidable disease.

The post-metamorphic amphibian immune system is of similar complexity to other vertebrates, with innate and adaptive responses including pathogen-specific cell-mediated and antibody responses, and immunoglobulin isotype heterogeneity (Blomberg et al., 1980; Du Pasquier et al., 1989, 2000; Whittington and Speare, 1996). Despite amphibian immunity in general being well described in the literature (Du Pasquier et al., 1989, 2000; Robert and Ohta, 2009), very little is known about specific mechanisms of defense against fungal pathogens such as *Bd*. To date, little evidence of an effective localized or systemic adaptive immune response in *Bd*-infected *Rana*, *Silurana* or *Litoria* species has been found (Berger et al., 2005b; Woodhams et al., 2007a; Stice and Briggs, 2010; Rosenblum et al., 2012). Innate host defense mechanisms, such as antimicrobial skin peptides and symbiotic bacteria, may influence host susceptibility to *Bd* infection (Woodhams et al., 2007a,b). However, negligible cellular inflammation occurs in the skin of infected frogs (Berger et al., 1999; Nichols et al., 2001) and other innate host defense

mechanisms have not been shown to consistently and effectively protect susceptible host species against infection (Rollins-Smith et al., 2006).

Recent studies provide conflicting evidence about the potential role of adaptive immunity in chytridiomycosis. Inoculation of mountain yellow-legged frogs (*Rana muscosa*) with formalin-killed *Bd* had no significant effect on infection prevalence or intensity, overall mortality or time to infection (Stice and Briggs, 2010). Boreal toads (*Bufo boreas*) that received a heat-killed vaccine did not have improved survival over non-immunized toads exposed to *Bd* (Rollins-Smith et al., 2011). Conversely, prior *Bd* exposure was found to have a beneficial effect on survival time in boreal toads, but only when they could select dry microenvironments (Murphy et al., 2011). Immunization of naturally resistant *Xenopus laevis* with heat-killed *Bd* stimulated specific IgM and IgY serum antibody production (Ramsey et al., 2010), but the role of serum antibodies in host defense against chytridiomycosis is unknown. Host-pathogen-environmental interactions are clearly complex and critical in determining the outcome of infection with *Bd*. In combination with the wide variation in host species susceptibility and the contrasting findings of the few studies available, these factors highlight the need to broaden the focus of future research.

We experimentally infected common green tree frogs (*Litoria caerulea*) with *Bd*, treated them with chloramphenicol to clear infection, and then re-exposed them to the pathogen. Our results showed not only that prior infection had no significant effect on infection rate compared with naïve frogs exposed for the first time, but also that prior infection appears to have a long-term adverse effect on resistance to re-infection with *Bd*.

### 6.2.3 *Materials and methods*

All animal procedures in this study were approved by the James Cook University Animal Ethics Committee (approval number A1085) and Queensland Parks and Wildlife Service (Scientific Purposes Permit number WISP03866106).

#### *Experimental Animals*

Free-ranging clinically healthy adult individuals of the common green tree frog (*Litoria caerulea*, n = 55) were collected from widespread residential and semi-rural areas in and around Cairns and Townsville in far northern Queensland, Australia. The species was selected based on large mature body size, relative ease of capture, endemism, wide distribution and stable conservation status. Each frog was placed, using a new powder-free nitrile medical examination glove (Supergloves Australia Pty Ltd, Gold Coast, QLD 4217, Australia), into an individual plastic holding container (70 × 95 × 150 mm) for transport. Frogs were housed in individual plastic containers (230 × 230 × 350 mm) in temperature (20 – 22°C) and light (12 h light, 12 h dark) controlled quarantine facilities at James Cook University, Cairns, Australia. Aged tap water was changed daily and frogs were fed large domestic crickets (*Acheta domestica*, Pisces Enterprises Inc., Kenmore, QLD 4069, Australia) dusted with superfine calcium carbonate (Cattlekare<sup>®</sup>, Dandenong, VIC 3770, Australia) and multivitamin powder (Reptivite<sup>™</sup>, Zoo Med Laboratories Inc., San Luis Obispo, CA 93401, USA), *ad libitum* each day.

Before the experimental trials commenced, each frog was thoroughly examined by a veterinarian, body weight and snout-urostyle measurements were recorded, and a swab sample was collected from the ventral skin surfaces for determination of *Bd* zoospore equivalents by real-time polymerase chain reaction (PCR) analysis (James Cook University, Townsville 4811, Australia) (Boyle et al., 2004). All swab samples were analyzed in triplicate and compared with James Cook University zoospore standards. All frogs were negative for *Bd* prior to commencement of the experimental trials.

#### *Culture and harvest of Batrachochytrium dendrobatidis*

The *Bd* isolate (Melbourne-L.lesueuri-00-LB-1-p19) was originally harvested from a clinically diseased captive juvenile *Litoria lesueuri* and cultured on tryptone/gelatin hydrolysate/lactose (TGhL) agar with streptomycin sulfate and benzylpenicillin (S9137 and P7794 respectively, Sigma Aldrich Inc., St Louis, Missouri 63103, USA) (Longcore et al., 1999). Cultures were maintained in half-strength TGhL broth at 4°C (Boyle et al., 2003; Berger et al., 2005a). Zoospores for frog inoculation were harvested by flooding 4 d old agar plate cultures

maintained at 22°C with a dilute electrolyte solution (mMol/L: KH<sub>2</sub>PO<sub>4</sub> 1.0, CaCl<sub>2</sub>.H<sub>2</sub>O 0.2, MgCl<sub>2</sub> 0.1) and counted in a hemocytometer (Brand GMBH and CO KG, Wertheim 97877, Germany) (Boyle et al., 2003; Berger et al., 2005a,b).

### ***Exposure Experiment Phase 1***

Frogs (n = 29) were randomly assigned to negative control (n = 10) and exposure (n = 19) groups. Prior to the start of the experiment, each frog was anesthetized by shallow immersion in 0.25 % ethyl 3-aminobenzoate methanesulfonic acid solution (tricaine methanesulfonate, Sigma-Aldrich Inc., St Louis, MO 63103, USA) buffered with 10 mEq/L sodium bicarbonate solution (8.4 %, Pro Care Animal Health, Dandenong, VIC 3175, Australia). Blood samples (150 – 200 µL, < 1 % body weight) were collected for hematologic and plasma biochemical analysis from dorsally recumbent frogs via cardiocentesis with a 1 mL syringe and 25 gauge needle (Terumo Corporation, Binan, Laguna 4024, Philippines). Mean body weight was higher in the exposure group (49.2 g) compared with the negative controls (35.9 g) at the start of the trial.

On day 0, frogs (n = 19) were exposed to *Bd* via shallow immersion in a bath of the dilute electrolyte solution (25 mL) inoculated with 250,000 zoospores for 24 h. Negative control frogs (n = 10) were held in a bath of the dilute electrolyte solution (25 mL) including solution collected from agar plates without *Bd* cultures. After 24 h frogs were returned to their holding containers with aged tap water. During the exposure period, frogs were held in small individual plastic containers (50 × 100 × 150 mm) with a lid to ensure continuous contact of the ventral skin surfaces with the inoculum. Frogs were weighed and swabs collected for PCR every 10 d until 90 d post-exposure. On days 30 and 60 post-exposure, all frogs were anesthetized and blood samples collected for hematologic and plasma biochemical analysis as described previously.

Prior to commencement of the treatment phase of the experiment on day 90, two negative control frogs were euthanized (54 and 56 d post-exposure) due to acute clinical signs consistent with bacterial septicemia from severe extensive rostral trauma. Both frogs tested negative for *Bd* before euthanasia. On day 90, one *Bd*-exposed frog became terminally ill from severe chytridiomycosis (*Bd* load: 9,130 zoospore equivalents) and was euthanized.

At 90 d post-exposure 15/18 of the *Bd*-exposed frogs tested positive for *Bd* and treatment was commenced. Negative control frogs (n = 8) were all uninfected. Frogs (n = 26) were continuously immersed (23/24 h) in a shallow (100 mL) 20 mg/L chloramphenicol bath for 28 d; chloramphenicol cures *Bd*-infected *L. caerulea* within 21 d following continuous immersion

at this concentration (Young et al., 2012). Fresh treatment solution was made up daily, and frogs were removed from the bath for 1 h daily during which time they were rinsed for 5 min in warm tap water (30 – 32°C) and then placed in a dry container with food. Swabs were collected for PCR analysis and frogs were weighed every 7 d for 42 d after treatment commenced (from days 90 – 132). Infection was cleared in all *Bd*-positive frogs by day 118; frogs were considered cured from *Bd* infection following three consecutive negative PCR results (Hyatt et al., 2007; Skerratt et al., 2011). On day 125 post-exposure, all frogs were anesthetized and blood samples collected for hematologic and plasma biochemical analysis as described previously.

### ***Exposure Experiment Phase 2***

On day 142 after commencement of the experimental trial, frogs from the original exposure group (n = 18) were re-exposed, and a separate group of positive control frogs (n = 9) were exposed for the first time, to *Bd* via shallow immersion in a bath of the dilute electrolyte solution (25 mL) inoculated with 250,000 zoospores for 24 h. Negative control frogs from the original group (n = 8) were held in a bath of the dilute electrolyte solution (25 mL) including solution collected from agar plates without *Bd* cultures. After 24 h frogs were returned to their holding containers with aged tap water.

Frogs were weighed and swabs collected for PCR immediately prior to Phase 2 and then every 10 d for 50 d following Phase 2 exposure (until 192 d after commencement of the experimental trial). With the exception of a single low *Bd* load (2 zoospore equivalents) in one positive control frog 10 d after exposure, swabs from all of the re-exposed, positive control and negative control frogs were negative on PCR at days 142, 152, 162, 172, 182 and 192. Phase 2 exposure was hence considered unsuccessful, for unknown reasons, and infection was re-attempted in Phase 3.

### ***Exposure Experiment Phase 3***

On day 383 after commencement of the trial, Phase 3 commenced with the following experimental groups: original negative control frogs (n = 8), original *Bd*-exposed and treated frogs (n = 18), positive control frogs from Phase 2 (n = 8; the ninth frog with the single positive PCR result from Phase 2 was excluded), and a new group of recently collected naïve positive control frogs (n = 17). Frogs from the original exposure group (n = 18) and all of the positive control frogs (n = 25) were exposed to *Bd* via shallow immersion in a bath of the dilute electrolyte solution (25 mL) inoculated with 250,000 zoospores for 24 h. Negative control frogs (n = 8) were held in a bath of the dilute electrolyte solution (25 mL) including solution collected from agar plates without *Bd* cultures. After 24 h frogs were returned to their holding containers



with aged tap water. Frogs were weighed and swabs collected for PCR immediately prior to Phase 3 and then every 10 d for 120 d following Phase 3 exposure (until 503 d after commencement of the experimental trial).

### ***Hematologic and Plasma Biochemical Analysis***

Blood samples from each frog were processed according to standard amphibian procedures (Wright, 2001; Young et al., in press). Fresh blood smears were made directly from the syringe, air dried and immediately fixed with 100 % methanol; 150 – 200  $\mu$ L whole blood was collected into a 0.2 mL Microtainer<sup>®</sup> pediatric lithium heparin tube (Becton and Dickinson, Franklin Lakes, New Jersey 07417, USA).

Total red blood cells (RBC), white blood cells (WBC) and thrombocytes were counted manually by a veterinary pathologist in a modified Neubauer hemocytometer at 400 $\times$  magnification with Natt-Herrick's solution as the diluent (Natt and Herrick, 1952; Young et al., in press). Differential WBC and polychromatophilic RBC were counted at 1000 $\times$  magnification from Wright's-stained blood smears (Clinipure Wright's Stain and Wright's Buffer Concentrate, HD Scientific Supplies Pty Ltd, Wetherill Park, NSW 1851, Australia). Well-mixed whole blood (5  $\mu$ L) was drawn into a pediatric microhematocrit tube (Becton and Dickinson, Franklin Lakes, New Jersey 07417, USA) and centrifuged (112  $\times$  g for 2 min) for packed cell volume (PCV) measurement. Hemoglobin (Hb) was assayed manually using the cyanomethemoglobin method modified for species with nucleated RBC (Drabkin, 1945; Melrose et al., 1995) and specifically for amphibians (Young et al., in press).

Plasma biochemical analytes were measured from 100  $\mu$ L of whole blood using the automated bench-top VetScan<sup>®</sup> VS2 Chemistry Analyzer and VetScan<sup>®</sup> Avian/Reptilian Profile Plus rotor (Abaxis Inc., Union City, CA 94587, USA): aspartate aminotransferase (AST), uric acid (UA), creatine kinase (CK), glucose, calcium, phosphorus, potassium, sodium and total protein (TP).

### ***Statistical Analysis***

Chi-Square tests for independence were performed to analyse the effect of previous *Bd* infection on infection frequency and infection clearance between *Bd*-exposed groups in Phase 3.

Independent-samples *t*-tests were used to compare the following variables between groups: absolute body weight, body weight change, blood values (0, 30, 60, 90 and 125 d post-exposure, Phase 1) and *Bd* load (LOG<sub>10</sub> zoospore count, every 10 d for 120 d post-exposure, Phase 3).

One-way analyses of variance were used to compare absolute body weight and body weight change among the three groups from Phase 3 (every 10 d for 120 d post-exposure).

Mixed between-within subjects analyses of variance were conducted to compare repeated measures of absolute body weight and blood values over time between the two experimental groups from Phase 1, and *Bd* load (LOG<sub>10</sub> zoospore count) and absolute body weight over time between the three experimental groups from Phase 3.

Positively skewed variables (*Bd* zoospore equivalents) were logarithmically transformed before analysis to meet the assumptions of normal distribution and homogeneity of variances for parametric statistics. The software package PASW<sup>®</sup> Statistics (Version 18, 2009, SPSS Inc., Chicago, IL, 660606, USA) was used for all analyses, and statistical significance was set at  $\leq 0.050$  in all cases.

#### 6.2.4. Results

##### *Exposure Experiment Phase 1*

###### *Infection frequency*

In experimental Phase 1, 84 % (16/19) of the exposed frogs and 0 % of the negative controls (8/8) were infected with *Bd* 90 d post-exposure. Infection levels ranged from 2 to 2329 zoospore equivalents per sample, and mean infection intensity continued to increase over the 90 d. Chloramphenicol cured 100 % of infected frogs that entered the treatment phase (n = 15) within 21 d of starting treatment. Seven d after treatment commenced, 100 % of frogs (15 sub-clinically infected *Bd*-exposed, 3 uninfected *Bd*-exposed and 8 uninfected negative control frogs) tested negative for *Bd* with PCR (reported in Young et al., 2012). All 26 frogs had negative PCR results 14, 21, 28, 35 d post-treatment, and all remained clinically normal for the treatment duration.

###### *Body weight*

Both experimental groups gained weight ( $P < 0.001$ ) over the course of Phase 1. Exposed infected frogs gained 16 % and negative controls 36 % of original body weight over 125 d. Final mean absolute body weight did not differ between the exposed infected (57.4 g, n = 15) and negative control (48.8 g, n = 8) groups ( $P = 0.073$ ). There was no difference in the rate of body weight increase between the two experimental groups ( $P = 0.373$ ).

###### *Blood values*

Within the infected frogs from the *Bd*-exposed (BD) group (Table 6.1), over the first 30 d RBC, WBC, neutrophil, lymphocyte and monocyte counts, and plasma AST, calcium, phosphorus and potassium decreased, while plasma UA and glucose increased. Between days 0 and 60, thrombocyte count and plasma glucose, calcium and phosphorus decreased, while plasma UA increased. From 60 to 125 d, Hb and lymphocyte count increased while plasma glucose and sodium decreased.

Within the uninfected negative control (NC) group (Table 6.2), over the first 30 d thrombocyte and WBC counts, and plasma UA, calcium and potassium concentrations decreased, while lymphocyte count increased. Between days 0 and 60, Hb decreased, while plasma UA and TP increased. From 60 to 125 d, thrombocyte count decreased, while PCV and plasma UA and calcium increased.

On day 0, the following mean absolute blood values differed between the BD (n = 16) and the NC (n = 10) groups: PCV (%: BD 38.8, NC 33.3;  $P = 0.030$ ), Hb (g/dL: BD 9.8, NC 8.0;  $P =$

0.027), monocyte count ( $\times 10^9/L$ : BD 1.2, NC 2.5;  $P = 0.025$ ), calcium (mmol/L: BD 3.28, NC 2.75;  $P = 0.040$ ), phosphorus (mmol/L: BD 1.59, NC 1.09;  $P < 0.001$ ) and sodium (mmol/L: BD 112, NC 106;  $P = 0.009$ ).

On day 30, the following mean absolute blood values differed between the BD ( $n = 16$ ) and the NC ( $n = 10$ ) groups: thrombocyte count ( $\times 10^9/L$ : BD 31.6, NC 26.3;  $P = 0.036$ ), neutrophil count ( $\times 10^9/L$ : BD 2.1, NC 4.7;  $P = 0.016$ ), calcium (mmol/L: BD 2.89, NC 2.28;  $P = 0.003$ ) and potassium (mmol/L: BD 5.7, NC 4.3;  $P = 0.048$ ).

On day 60, the following mean absolute blood values differed between the BD ( $n = 16$ ) and the NC ( $n = 8$ ) groups: Hb (g/dL: BD 9.7, NC 7.2;  $P = 0.003$ ), thrombocyte count ( $\times 10^9/L$ : BD 23.9, NC 31.5;  $P = 0.009$ ) and glucose (mmol/L: BD 3.7, NC 3.0;  $P = 0.037$ ).

On day 125, the following mean absolute blood values differed between the BD ( $n = 15$ ) and the NC ( $n = 8$ ) groups: Hb (g/dL: BD 11.0, NC 7.4;  $P < 0.001$ ), thrombocyte count ( $\times 10^9/L$ : BD 20.4, NC 25.6;  $P = 0.022$ ) and glucose (mmol/L: BD 2.6, NC 3.1;  $P = 0.044$ ).

There was a significant difference in the rate of change in absolute thrombocyte and plasma phosphorus concentrations between the two experimental groups for repeated measures at days 0, 30 and 60. Thrombocyte count increased then decreased in the BD group, compared with the NC group in which the opposite effect occurred ( $P = 0.001$ ). Plasma phosphorus decreased in both groups ( $P = 0.002$ ), but the rate of decrease was more rapid in the BD group: ( $P = 0.015$ ). Treatment effects for the other hematologic and plasma biochemical variables were not significant (time-group interaction  $P > 0.050$  in all cases).

### ***Exposure Experiment Phase 3***

#### ***Infection frequency***

At the end of experimental Phase 3, 120 d post-exposure, a greater number of the treated re-exposed (BD-RE) frogs (78%, 14/18) remained infected with *Bd* compared with the naïve<sup>§</sup> exposed (BD-N) frogs (28%, 7/25) ( $X^2(1, n = 43) = 8.481, P = 0.004$ ). Overall, a similar proportion of frogs in the two exposure groups became infected during Phase 3, indicating equally successful exposures: BD-RE 78% (14/18), BD-N 68% (17/25) ( $X^2(1, n = 43) = 0.130, P = 0.718$ ). The difference between the groups was due to a greater proportion of BD-N frogs (59%, 10/17) clearing infection by 120 d post-exposure compared with the BD-RE frogs (0%,

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<sup>§</sup> 'Naïve' refers to frogs with no past evidence of infection.

0/14) ( $\chi^2(2, n = 43) = 12.868, P = 0.002$ ). All NC frogs (8/8) remained uninfected during the trial.

#### *Infection intensity*

Infected frogs in the BD-RE group had a higher mean maximum zoospore count ( $M 2,278 \pm SD 4,207; n = 14$ ) during the trial compared with the BD-N frogs ( $M 1,186 \pm SD 3,317; n = 17$ ) ( $P = 0.013$ ). Intensity of infection increased over time in both groups ( $P = 0.005$ ), but the rate of increase was significantly greater in the BD-RE group ( $P = 0.046$ ) (Figure 6.1).

Due to the heterogeneity in response of the BD-N group, the data was further explored by excluding the BD-N subset that self-cured ( $n = 10$ ). Excluding these frogs, there was no difference in mean maximum zoospore count in the BD-RE group ( $M 2,657 \pm SD 4,452; n = 14$ ) compared with the BD-N group ( $M 2,859 \pm SD 4,877; n = 7$ ) ( $P = 0.853$ ) at the end of the trial. Intensity of infection increased over time in both groups ( $P = 0.002$ ), and the rate of increase was similar in both groups ( $P = 0.494$ ) (Figure 6.2).

#### *Body weight*

There were no differences in absolute body weight between infected frogs in the BD-RE group ( $n = 14$ ), infected frogs in the BD-N group ( $n = 17$ ), and the uninfected NC group ( $n = 8$ ) at any of the time points post-exposure during Phase 3 ( $P > 0.050$  in all cases). Mean absolute body weight increased over time in all groups ( $P < 0.001$ ) and there was no difference in the rate of body weight increase between the three experimental groups: ( $P = 0.210$ ). The BD-RE group gained 24 %, the BD-N group 17% and the NC group 21 % of original body weight over 120 d. Final absolute mean body weight did not differ between the BD-RE (65.6 g), BD-N (66.6 g) and NC (55.8 g) groups ( $P = 0.317$ ).

Excluding the BD-N frogs that self-cured ( $n = 10$ ), there was no difference in mean absolute body weight at the start of Phase 3 between the BD-RE (53.1 g,  $n = 14$ ), the BD-N (61.2 g,  $n = 7$ ) and the NC (45.9 g,  $n = 8$ ) groups ( $P = 0.119$ ). Similarly, final mean absolute body weight 120 d post-exposure did not differ between the BD-RE (65.6 g), the BD-N (65.6 g) and the NC (55.8 g) groups ( $P = 0.377$ ). Absolute body weight increased over time in all of the three groups ( $P < 0.001$ ), but the rate of increase differed between the groups: ( $P = 0.026$ ) (Figure 6.3).

**Table 6.1.** Hematologic and plasma biochemical values (mean (M) +/- SD) for *Batrachochytrium dendrobatidis*-infected common green tree frogs. Frogs were infected on day 0, and then treated to clear infection on day 90; all frogs tested negative on PCR before final sample collection on day 125.

Parameter	Day 0		Day 30		$P^a$	Day 60		$P^b$	Day 125		$P^c$
	M	SD	M	SD		M	SD		M	SD	
PCV (%)	38.8	5.4	39.0	7.6	0.916	38.8	6.9	0.973	40.9	7.6	0.250
Hb (g/dL)	9.8	1.7	10.3	2.1	0.438	9.7	1.6	0.921	11.0	1.7	0.023
RBC ( $\times 10^9/L$ )	780	135	646	147	0.008	713	120	0.154	690	153	0.711
Thrombocyte ( $\times 10^9/L$ )	29.7	7.2	31.6	4.5	0.302	24.0	5.7	0.012	20.4	4.8	0.053
WBC ( $\times 10^9/L$ )	16.2	4.8	8.8	3.4	0.000	15.5	8.0	0.736	19.1	8.2	0.121
Neutrophil ( $\times 10^9/L$ )	3.8	1.9	2.1	1.3	0.001	3.9	3.4	0.866	2.8	1.6	0.196
Lymphocyte ( $\times 10^9/L$ )	10.8	4.0	5.8	2.8	0.000	10.0	5.4	0.601	14.1	6.9	0.036
Monocyte ( $\times 10^9/L$ )	1.2	0.6	0.7	0.5	0.001	1.1	0.9	0.602	1.6	1.8	0.317
Eosinophil ( $\times 10^9/L$ )	0.3	0.3	0.2	0.2	0.533	0.5	0.6	0.256	0.6	0.7	0.233
Basophil ( $\times 10^9/L$ )	0.1	0.3	0.0	0.0	0.209	0.0	0.0	0.209	0.0	0.0	0.334
Polychromasia (%)	5.5	5.5	4.8	2.3	0.613	3.6	2.3	0.153	2.7	1.7	0.235
AST (U/L)	118	62	81	51	0.015	83	41	0.077	88	66	0.738
CK (U/L)	745	723	326	271	0.046	408	365	0.148	566	418	0.250
UA ( $\mu\text{mol/L}$ )	26	25	35	25	0.024	44	29	0.013	41	22	0.619
Glucose (mmol/L)	4.3	1.1	5.1	1.0	0.000	3.7	0.7	0.048	2.6	0.5	0.000
Calcium (mmol/L)	3.28	0.64	2.89	0.51	0.014	2.83	0.56	0.019	2.80	0.86	0.819
Phosphorus (mmol/L)	1.59	0.30	1.15	0.25	0.000	0.89	0.37	0.000	0.84	0.34	0.560
Potassium (mmol/L)	6.9	1.7	5.7	2.2	0.033	5.9	1.5	0.060	5.2	1.7	0.195
Sodium (mmol/L)	112	6	112	5	0.573	114	5	0.069	109	5	0.001
Total Protein (g/L)	60.6	10.7	60.6	9.9	1.000	65.7	9.5	0.113	67.0	11.6	0.682

<sup>a</sup> Paired-samples *t*-tests between days 0 and 30 (n = 16).

<sup>b</sup> Paired-samples *t*-tests between days 0 and 60 (n = 16).

<sup>c</sup> Paired-samples *t*-tests between days 60 and 125 (n = 15).

**Table 6.2.** Hematologic and plasma biochemical values (mean (M) +/- SD) for the uninfected negative control common green tree frog group from Experiment 1. All frogs tested negative on PCR for *Batrachochytrium dendrobatidis* throughout the experiment.

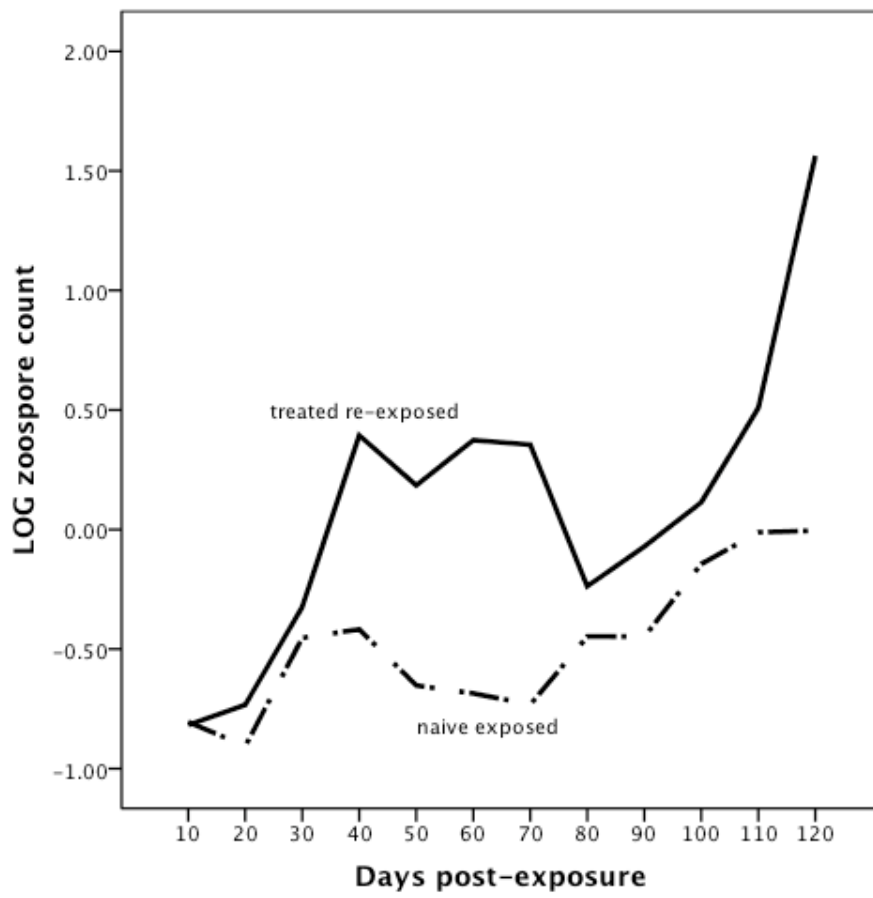
Parameter	Day 0		Day 30		$P^a$	Day 60		$P^b$	Day 125		$P^c$
	M	SD	M	SD		M	SD		M	SD	
PCV (%)	33.3	6.6	32.1	9.5	0.625	33.0	7.2	0.760	36.9	6.6	0.015
Hb (g/dL)	8.0	2.1	8.6	2.5	0.343	7.2	1.9	0.041	7.4	2.1	0.798
RBC ( $\times 10^9/L$ )	707	247	621	189	0.203	684	177	0.584	739	106	0.458
Thrombocyte ( $\times 10^9/L$ )	33.5	8.8	26.3	7.8	0.019	31.5	6.6	0.724	25.6	4.8	0.034
WBC ( $\times 10^9/L$ )	20.2	6.3	13.9	7.3	0.024	20.1	10.8	0.816	19.8	8.9	0.911
Neutrophil ( $\times 10^9/L$ )	5.5	2.3	4.7	2.7	0.516	3.6	2.2	0.289	2.6	1.4	0.272
Lymphocyte ( $\times 10^9/L$ )	4.5	1.4	7.6	4.0	0.018	13.6	8.1	0.508	15.2	8.4	0.374
Monocyte ( $\times 10^9/L$ )	2.5	1.5	1.4	1.0	0.065	2.4	2.1	0.788	1.6	1.0	0.252
Eosinophil ( $\times 10^9/L$ )	0.4	0.4	0.2	0.2	0.156	0.4	0.5	0.441	0.4	0.4	0.777
Basophil ( $\times 10^9/L$ )	0.0	0.0	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-
Polychromasia (%)	3.9	1.6	5.4	2.6	0.071	5.8	3.1	0.080	4.8	3.7	0.316
AST (U/L)	133	108	137	104	0.654	185	137	0.440	123	99	0.061
CK (U/L)	530	389	451	294	0.535	643	294	0.377	697	183	0.722
UA ( $\mu\text{mol/L}$ )	27	24	19	16	0.039	31	14	0.048	37	16	0.041
Glucose (mmol/L)	3.7	1.3	4.4	1.4	0.099	3.0	1.0	0.495	3.1	0.6	0.661
Calcium (mmol/L)	2.75	0.58	2.28	0.37	0.019	2.55	0.20	0.559	2.83	0.16	0.001
Phosphorus (mmol/L)	1.09	0.29	1.05	0.29	0.737	0.99	0.27	0.576	0.97	0.18	0.756
Potassium (mmol/L)	5.6	1.6	4.3	1.1	0.035	5.6	1.0	1.000	5.9	1.9	0.647
Sodium (mmol/L)	106	4	108	6	0.344	110	5	0.172	110	3	0.879
Total Protein (g/L)	55.6	14.2	56.8	12.1	0.750	64.8	7.9	0.047	68.5	4.4	0.126

<sup>a</sup> Paired-samples *t*-tests between days 0 and 30 (n = 10).

<sup>b</sup> Paired-samples *t*-tests between days 0 and 60 (n = 8).

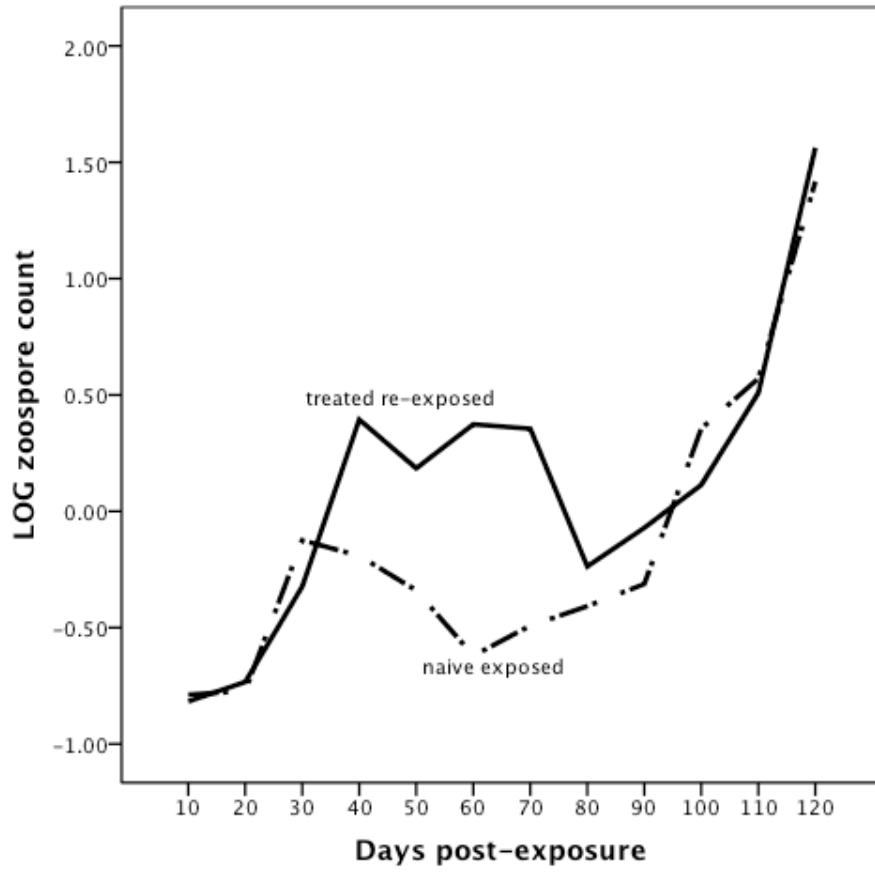
<sup>c</sup> Paired-samples *t*-tests between days 60 and 125 (n = 8).

**Figure 6.1.** *Batrachochytrium dendrobatidis* zoospore load over time in treated re-exposed (n = 14) and naïve exposed (n = 17) common green tree frogs.

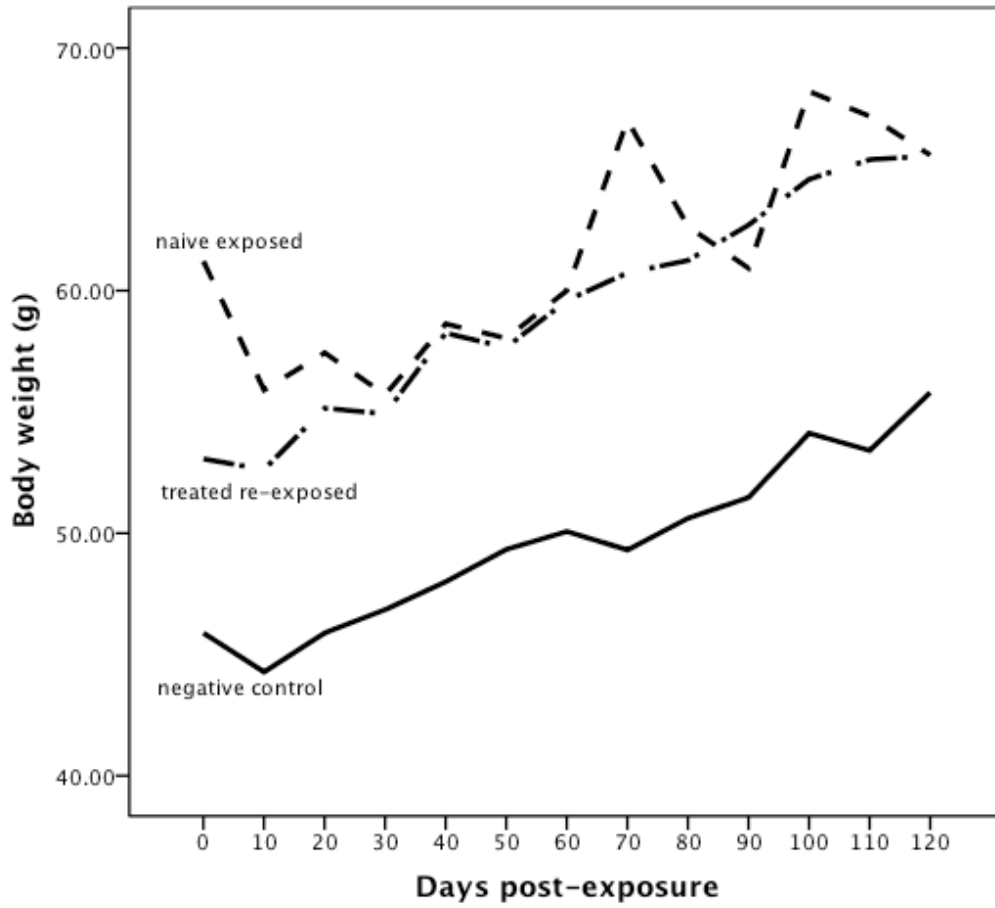




**Figure 6.2.** *Batrachochytrium dendrobatidis* zoospore load over time in common green tree frogs that remained infected from the treated re-exposed (n = 14) and naïve exposed (n = 7) groups.



**Figure 6.3.** Body weight over time in common green tree frogs from the negative control group (n = 8), and in frogs that remained infected with *Batrachochytrium dendrobatidis* from the treated re-exposed (n = 14) and naïve exposed (n = 7) groups.



### 6.2.5. Discussion

We found no long-term beneficial effect of prior pathogen exposure on the outcome of *Bd* infection in common green tree frogs. Conversely, a greater proportion of previously exposed frogs remained infected with *Bd* post-exposure, and infected re-exposed frogs had a higher infection intensity that increased at a greater rate, compared with infected naïve frogs. Additionally, a greater proportion of infected naïve frogs self-cured compared with the infected re-exposed group. Hence, we found that naïve frogs were better able to control and clear infection compared with exposed frogs, suggesting suppression of the host immune response associated with prior pathogen exposure. Overall our findings suggest that previous pathogen exposure and infection may have a long-term adverse effect on host resistance to *Bd*.

Limitations of our study include the delay in collection of the Phase 3 positive control frogs and the unknown history of *Bd* exposure prior to collection of frogs from the wild. The Phase 3 positive controls potentially may not have been of the same genetic stock or from identical microclimates as the Phase 1 frogs, both of which may have influenced immune response to *Bd* challenge. This was compensated for as much as possible by collecting frogs from the same geographic locations for each phase of the trial, and standardizing all of the experimental procedures and conditions post-collection. The “naïve” frogs in this study represent individuals with no past evidence of infection, but not necessarily with no past exposure to *Bd*. If any of these frogs had previously been exposed in the wild and self-cured, immune response to repeat exposure may have differed from unexposed frogs, potentially confounding the results. Insufficient captive-bred frogs were available for experimental use for these trials, making collection from the wild unavoidable, and all frogs tested negative for *Bd* before commencing the exposure trials. However, it would be useful to repeat these trials using truly naïve captive-bred frogs to remove any confounding factors.

The *Bd*-infected naïve frogs that self-cured are of particular interest given that *L. caerulea* is a highly susceptible host species. Fewer significant differences were found between re-exposed and naïve exposed frogs when individuals that cleared infection were excluded from the analyses. If these frogs were not previously exposed to *Bd* in the wild prior to collection, this may indicate an important role of innate immune mechanisms in susceptibility to chytridiomycosis. However, within the re-exposure group, none of the uninfected exposed frogs from Phase 1 ( $n = 3$ ) were the same as those individuals that cleared infection during Phase 3 ( $n = 4$ ). Further research to more clearly define immune responses in individuals that spontaneously clear infection is warranted.

Recent genetic, stress hormone and *in vitro* immune function studies indirectly suggest *Bd* may actively suppress an immune response (Ribas et al., 2009; Rosenblum et al., 2009; J. Ramsey et al., unpublished in Rollins-Smith et al., 2011; Kindermann et al., 2012). Results from a parallel study in our laboratory provide the first direct evidence that *Bd* actively suppresses systemic adaptive immunity in infected *L. caerulea* (Young et al., unpublished). We also found changes in the immune response of *Bd*-exposed but uninfected white-lipped tree frogs (*L. infrafrenata*), indicating short- to medium-term sub-clinical immunologic effects associated with pathogen exposure and subsequent clearance (Young et al., unpublished). Larval common toads (*Bufo bufo*) experimentally exposed to low *Bd* doses usually died at or soon after metamorphosis without detectable infections, suggesting fitness costs attributable to exposure, control and clearance in the absence of extensive pathogen proliferation (Garner et al., 2009; Skerratt et al., 2009). Our findings here further support the assertions that host suppression by the pathogen may be a key factor in the pathogenicity of *Bd*, and that there may be negative host effects related to pathogen exposure and infection.

Within 30 d of the first exposure, *Bd*-infected frogs had reduced total WBC, neutrophil, lymphocyte and monocyte counts compared with pre-exposure levels, and lower neutrophil counts than the uninfected control group. These cellular changes may further support suppression of immune organ activation by the pathogen early in the course of infection; this time period may warrant more comprehensive future investigations. Total WBC count also decreased in the uninfected control group between days 0 and 30, albeit to a lesser degree than the infected frogs, potentially indicating a component of physiological stress during initial adaptation to captivity in both groups. Although interpretation of plasma glucose fluctuations can be difficult, we controlled external factors by standardizing experimental procedures and conditions, along with time between feeding and sample collection. Plasma glucose concentration increased significantly during infection, then decreased following infection clearance, in *Bd*-infected frogs. Mean plasma glucose concentration was also higher in infected frogs compared with uninfected controls, but only at day 60 post-exposure. These findings together support a physiological stress response associated with *Bd* infection. A recent field study showed higher urinary corticosterone metabolites in Stony Creek frogs (*L. wilcoxii*) infected with *Bd*, indicating physiological stress (Kindermann et al., 2012). Stress hormones are well known to impact immune responses across multiple taxonomic groups, including alterations in normal WBC distribution (Dhabar et al., 1996). Multiple other plasma electrolyte concentrations decreased in the infected frogs but these were mirrored in the uninfected controls. The RBC, Hb and thrombocyte changes over time in both groups were variable and not unexpected due to repeated blood sample collection, indicating an adequate bone marrow regenerative response to relative anemia (Campbell and Ellis, 2007).

We were unable to determine the effect of prior pathogen exposure on short-term host resistance due to exposure failure in Phase 2 of our experimental trial. \*\* Intra-peritoneal injection of heat-killed *Bd* in *X. laevis* stimulated pathogen-specific IgM and IgY serum antibody production that peaked 14 d after the final immunization, and was still present at 28 d but at lower levels (Ramsey et al., 2010). Titers were not measured more than 28 d after the final booster injection so the duration of antibody persistence in *X. laevis* was undetermined. It is possible in our study that an initial adaptive immune response was stimulated in *L. caerulea*, but the presence of protective antibodies was only short-lived and they had waned before re-exposure. However, *L. caerulea* is a highly susceptible host (Berger et al., 2004; Voyles et al., 2007, 2009) compared with *X. laevis*, a species that is naturally resistant to the lethal effects of *Bd* and that is only distantly related to most species impacted by *Bd* (Ramsey et al., 2010; Soto-Azat et al., 2010). Two highly susceptible ranid species (*R. mucosa* and *R. sierrae*) appear to lack a robust localized or systemic immuno-genetic response to *Bd* (Rosenblum et al., 2012), and *Bd*-infected *L. caerulea* have impaired serum and splenic immunoglobulin production (Young et al., unpublished), suggesting that stimulation of a protective adaptive immune response against *Bd* is an unlikely outcome of prior exposure to the pathogen.

We did not find any evidence of long-term protective immunity in *L. caerulea* following recovery from *Bd* infection, and importantly we also found that prior pathogen exposure may adversely impact long-term host resistance. Future research is needed to confirm whether short-term pathogen-specific adaptive immune responses can occur in susceptible species, and if so, whether these responses can be potentiated to stimulate long-lasting resistance. Persistence of a protective adaptive immune response would be a key factor in determining the success of control programmes using immunization. If susceptible host species lack the ability to mount protective adaptive responses, then the focus of future research needs to shift beyond this to identification of pathogen-secreted factors and other mechanisms of host immune suppression by the pathogen, along with alternative methods of host immune modulation.

**Conflict of interest statement:** The authors declare that they have no competing interests.

### Acknowledgments

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\*\* Long-term antibody persistence was ruled out based on the progressive zoospore load in the frogs during Phase 1 prior to treatment. Chloramphenicol contamination was ruled out due to strict laboratory protocols and equipment sterilisation between *Bd* exposures. The most likely explanation for exposure failure was primary culture failure.

## 6.2.6. References

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## CHAPTER 7

### Pathogenesis of amphibian chytridiomycosis

#### 7.1 Introduction

The amphibian chytrid fungus *Batrachochytrium dendrobatidis* is lethal to many species of amphibians but the mechanism by which it causes death is unknown. The organism is confined to the superficial layers of the epidermis and causes no consistent systemic pathology. The two main hypotheses as to the cause of mortality in infected amphibians are that the pathogen 1) disrupts osmoregulation in the skin, and/or 2) produces a systemically active toxin. Determining how *B. dendrobatidis* kills amphibians is essential to understanding the biology of chytridiomycosis. Furthermore, understanding the pathophysiological effects will provide critical information for researchers, wildlife managers and veterinarians, and enable treatment of infected frogs and facilitation of population recovery.

This chapter consists of two published peer-reviewed papers detailing research conducted by the James Cook University Amphibian Disease Ecology Group aiming to determine the pathogenesis of chytridiomycosis. We describe results from the first stage of this research suggesting *B. dendrobatidis* kills amphibians by disrupting normal epidermal functioning, leading to osmotic imbalance through loss of electrolytes. In the second stage of this research, we find a mechanism for amphibian mortalities: *B. dendrobatidis* causes fatal pathophysiological changes including epidermal degeneration, inhibited epidermal electrolyte transport, systemic electrolyte disturbances (hyponatraemia and hypokalaemia) and asystolic cardiac arrest.

#### 7.2 Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis

This published peer-reviewed paper represents original research led by Jamie Voyles and Lee Berger. My role in the paper included provision of veterinary technical expertise for methodology and results interpretation, and substantial written contributions to the methods, results and discussion sections.

Voyles, J., Berger, L., Young, S., Speare, R., Webb, R., Warner, J., Rudd, D., Campbell, R. & Skerratt, L. F. (2007). Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis. *Diseases of Aquatic Organisms* 77: 113-118.

Since the journal uses American English, spelling follows this convention.

# Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis

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**ABSTRACT:** Mounting evidence implicates the disease chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis*, in global amphibian declines and extinctions. While the virulence of this disease has been clearly demonstrated, there is, as yet, no mechanistic explanation for how *B. dendrobatidis* kills amphibians. To investigate the pathology of chytridiomycosis, blood samples were collected from uninfected, acclinically infected and clinically diseased amphibians and analyzed for a wide range of biochemical and hematological parameters. Here, we show that green tree frogs *Litoria caerulea* with severe chytridiomycosis had reduced plasma osmolality, sodium, potassium, magnesium and chloride concentrations. Stable plasma albumin, hematocrit and urea levels indicated that hydration status was unaffected, signifying depletion of electrolytes from circulation rather than dilution due to increased water uptake. We suggest that *B. dendrobatidis* kills amphibians by disrupting normal epidermal functioning, leading to osmotic imbalance through loss of electrolytes. Determining how *B. dendrobatidis* kills amphibians is fundamental to understanding the host–pathogen relationship and thus the population declines attributed to *B. dendrobatidis*. Understanding the mechanisms of mortality may also explain interspecific variation in susceptibility to chytridiomycosis.

**KEY WORDS:** Amphibian declines · Chytridiomycosis · *Batrachochytrium dendrobatidis* · Pathogenesis · Mortality · Osmoregulation

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## INTRODUCTION

Amphibians are currently undergoing the fastest rate of extinction of any vertebrate group (Stuart et al. 2004). While habitat destruction or overexploitation are obvious primary causes in some declines, determining why amphibians are experiencing catastrophic declines in protected areas has been more challenging. Amphibian mass-mortalities and declines have coincided with the appearance of the fungal pathogen *Batrachochytrium dendrobatidis* in wild amphibian communities (Lips et al. 2006, Berger et al. 2007). This fungus is lethal to many species of amphibians (Berger et al. 1998, 2005a, 2007, Longcore et al. 1999, Nichols et al. 2001, Lips et al. 2006), yet the

mechanism by which it causes death is unknown (Berger et al. 1998, 2007). *B. dendrobatidis* is confined to the superficial layers of the epidermis and causes no consistent pathological changes in internal organs (Berger et al. 1998, 2007, Pessier et al. 1999). Two hypotheses as to the cause of mortality in amphibians infected with *B. dendrobatidis* have been suggested. The first is that *B. dendrobatidis* disrupts osmoregulation in the skin of infected amphibians, and the second is that *B. dendrobatidis* produces a toxin that affects organs (Berger et al. 1998, 2007, Blaustein et al. 2005). These hypotheses are not necessarily mutually exclusive. However, no study has provided data to determine if either or both hypotheses are correct.

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Amphibian skin is well studied due to its unique functions (Deyrup 1964, Heatwole & Barthalmus 1994, Jorgensen 1997). The integument is a site of regulated transport for water, ions (electrolytes) and respiratory gases (Deyrup 1964, Heatwole & Barthalmus 1994, Jorgensen 1997). Permeability of frog skin varies over the body surface of an individual and also among species (Deyrup 1964, Heatwole & Barthalmus 1994). In some species osmotic permeability is greatest in an area of ventral integument commonly referred to as the pelvic patch (Czopek 1965, Baldwin 1974, Word & Hillman 2005), where there is dense cutaneous vasculature (Czopek 1965). Concomitantly, *Batrachochytrium dendrobatidis* occurs more commonly and at higher density in the ventral integument of infected frogs (Berger et al. 2005b, Puschendorf & Bolaños 2006). *B. dendrobatidis* grows within the keratinized cells of the superficial epidermis and causes irregular skin sloughing, hyperplasia and hyperkeratosis (Berger et al. 1998, 2005b, 2007, Pessier et al. 1999). Other pathological changes including cytoplasmic degeneration and vacuolation in scattered cells have been observed by light and electron microscopy, but these changes are not usually severe (Berger et al. 2007). Thus, it is unclear how a superficial skin infection kills frogs.

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

## MATERIALS AND METHODS

Blood samples were collected twice over the course of infection during an outbreak in captive-bred *Litoria caerulea*. This outbreak was monitored by swabbing frogs every 10 d for 73 d for *Batrachochytrium dendrobatidis* zoospore equivalents, determined by real-time PCR analysis (Boyle et al. 2004). Together with clinical signs of disease, most of which are apparent only in the few days before death (Berger et al. 2007), these PCR results were used as indicators of severity of infection (Fig. 1). Blood was collected for hematology and plasma biochemistry before and after frogs became diseased.

Frogs were housed individually at 18 to 23°C and fed vitamin-dusted crickets. Ten *Litoria caerulea* became

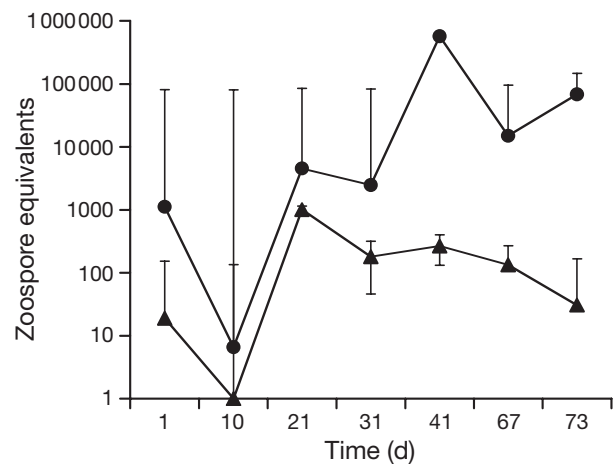


Fig. 1. *Litoria caerulea* infected with *Batrachochytrium dendrobatidis*. Zoospore equivalents over time in severely diseased (●) (n = 7) and acclinical (▲) (n = 3) frogs. Uninfected amphibians (with zero zoospores) are not shown. Results are from real-time PCR on skin swabs

infected, and 2 were uninfected. Within 16 d after suspected exposure, blood (<1% frog weight) was collected with a heparinized syringe and needle via the ventral abdominal vein or heart after anesthesia by shallow immersion in a solution bath of 0.1% MS-222 (tricaine methanesulfonate, Sigma Chemical). MS-222 does not kill *Batrachochytrium dendrobatidis* (Webb et al. 2005). A sample of whole blood was analyzed (Bayer 865 rapidlab blood gas analyzer) for electrolyte concentrations, pH and carbon dioxide. Hemoglobin was measured using the modified Drabkin's method. Total red cells were counted using a modified Neubauer hemocytometer with Nat-Herrick's solution as the diluent. Plasma osmolality was measured using a Knauer automatic osmometer with 400 mmol l<sup>-1</sup> standards.

*Litoria caerulea* were monitored daily for clinical signs of chytridiomycosis including lethargy, inappetance, decreased respiration rate, cutaneous erythema, irregular skin sloughing and abnormal posture (legs splayed out posteriorly). A second blood sample was collected immediately before euthanasia for 7 infected *L. caerulea* when they showed obvious clinical signs between 53 and 73 d post-exposure (Days 53, 56, 59, 59, 59, 60, 72) and for 3 infected and 2 uninfected frogs that were clinically normal (Days 72 to 74). Samples were tested by blood gas analysis as previously described. A large range of blood biochemical parameters was measured to evaluate functioning of, or damage to, organs including liver, kidney muscle and pancreas. For this, plasma was analyzed for 16 biochemical parameters (amylase, lactate, aspartate, aminotransferase, creatine kinase, glutamate dehydrogenase, hemoglobin, platelets, hematocrit, albumin,

bile acids, cholesterol, glucose, globulin, total protein and urea) (Olympus AU400 at 37C at IDEXX Laboratories, Brisbane).

These methods were performed with James Cook University Animal Ethics Committee approval (Permit No. A593). All data were analyzed using SPSS statistics, Version 12.0. Data sets were tested for normality and homogeneity of variance (homoscedasticity), and non-parametric instead of parametric tests were used when violations of assumptions occurred.

### RESULTS

Skin swab samples collected on the date of death in diseased frogs (n = 7) and at the termination of the experiment in acclinically infected amphibians (n = 3) indicated *Batrachochytrium dendrobatidis* zoospore equivalents were greater in severely diseased amphibians (Mann-Whitney test, p = 0.017). Swabs from

uninfected amphibians (n = 2) were negative. There were no significant differences in blood test results between frogs that were uninfected and frogs that were infected but acclinical. However, amphibians with severe clinical signs of disease had significantly reduced plasma osmolality compared with both uninfected and acclinical groups combined (Table 1). Further, severely diseased frogs had significant reductions in plasma sodium, potassium, chloride and magnesium concentrations (Table 1). None of the other 16 biochemical parameters (listed in 'Materials and methods') varied significantly (Table 1).

In addition to comparing results among groups of amphibians with different severities of disease, we evaluated the changes in a range of biochemical parameters within individual *Litoria caerulea* as infections progressed. Significant biochemical changes occurred solely in amphibians that developed severe disease. Plasma concentrations of sodium, potassium, chloride and blood pH in severely diseased amphibians were

Table 1. *Litoria caerulea* infected with *Batrachochytrium dendrobatidis*. Biochemical and hematological parameters. Designation of acclinically infected/uninfected or severely infected was determined by clinical signs and by measuring the number of zoospor equivalents by real-time PCR. Uninfected frogs were grouped with the acclinical frogs because there were no significant differences between the 2 groups. AST: aspartate aminotransferase; GLDH: glutamate dehydrogenase; RBC: red blood cell; PCV: packed cell volume. \*Significant at p < 0.05

Measured variables	Infection status						p-value
	Uninfected/acclinical			Clinical			
	Mean	SD	N	Mean	SD	N	
Mass (g)	50.52	16.1	5	35.7	5.3	7	0.109
<b>Electrolyte concentrations</b>							
Osmolality (mmol l <sup>-1</sup> )	217.2	28.5	5	153.5	46.6	6	0.026*
Sodium (mmol l <sup>-1</sup> )	102.8	7.9	5	74.4	9.5	7	0.001*
Potassium (mmol l <sup>-1</sup> )	2.74	0.3	5	1.5	0.8	7	0.016*
Magnesium (mmol l <sup>-1</sup> )	0.98	0.4	5	0.3	0.2	7	0.027*
Chloride (mmol l <sup>-1</sup> )	79	7.5	5	47.3	9.4	7	0.001*
Sodium:potassium ratio	32.52	3.5	5	30.2	6.6	6	0.491
<b>Blood gases</b>							
CO <sub>2</sub> (pCO <sub>2</sub> mmHg)	35.54	12.2	5	25.3	9.7	6	0.153
pH	7.31	0.1	5	7.2	0.2	7	0.369
<b>Enzymes/Markers</b>							
Amylase (IU l <sup>-1</sup> )	3100.6	948	5	3482.4	1517.5	5	0.646
AST (IU l <sup>-1</sup> )	368	194.5	5	1003.3	895.2	7	0.154
GLDH (IU l <sup>-1</sup> )	12	4.8	5	31.7	30	7	0.181
Creatine kinase (IU l <sup>-1</sup> )	1627.8	895.6	5	2782.4	2524.1	7	0.356
<b>Hematology</b>							
RBC (×10 <sup>12</sup> l <sup>-1</sup> )	0.42	0.96	5	0.52	0.17	6	0.291
Hemoglobin (g l <sup>-1</sup> )	0.163	0	5	0.2	0.1	6	0.704
Platelets	8680.2	5220.2	5	6523	3158.7	6	0.418
PCV (%)	22.8	5.6	5	26.5	7.1	6	0.371
<b>Other blood constituents</b>							
Albumin (g l <sup>-1</sup> )	26.6	5.4	5	30.1	3.9	7	0.212
Bile acids (µmol l <sup>-1</sup> )	7	8.5	5	35.7	41.1	7	0.160
Cholesterol (mmol l <sup>-1</sup> )	1.4	0.9	5	2	2.3	5	0.630
Glucose (mmol l <sup>-1</sup> )	3.62	1.1	5	3.6	2.1	3	0.964
Globulin (g l <sup>-1</sup> )	17.2	3.7	5	26.7	9.8	7	0.068
Total protein (g l <sup>-1</sup> )	43.8	8.7	5	56.9	12.9	7	0.079
Urea (mmol l <sup>-1</sup> )	9.02	2.4	5	11.5	8.1	7	0.531

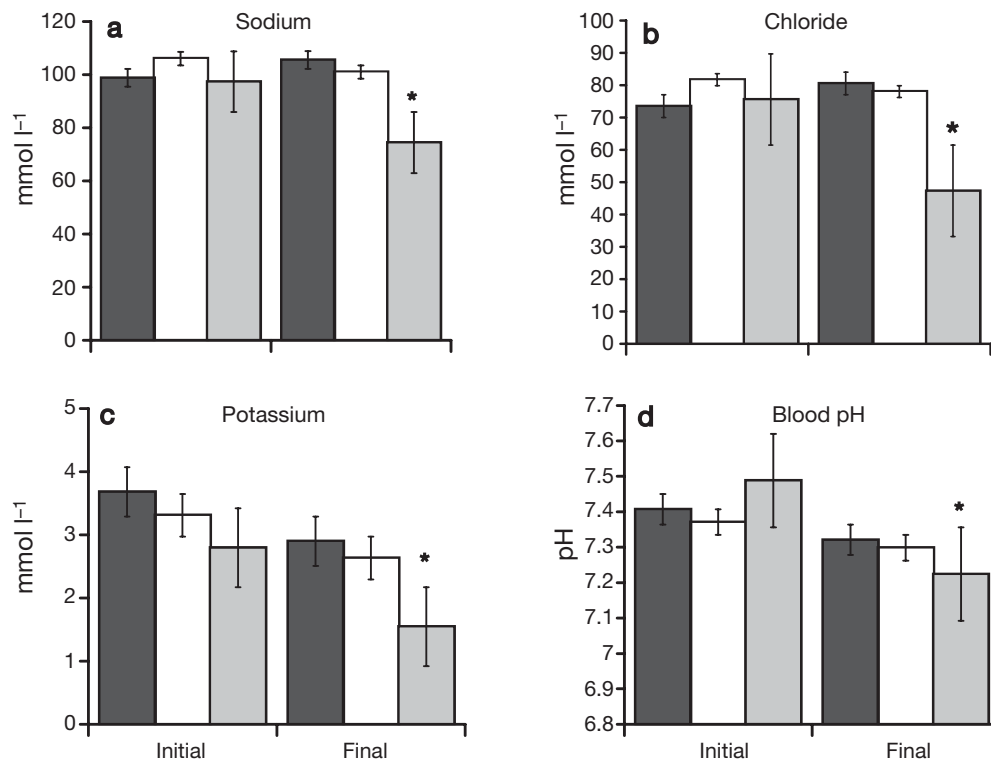


Fig. 2. *Litoria caerulea* infected with *Batrachochytrium dendrobatidis*. Changes in (a–c) electrolyte concentrations and (d) blood pH between initial and final blood samples of frogs. Bar graphs show mean values ( $\pm$ SEM). Uninfected individuals are separated from acclinically infected individuals. (a–c) Plasma electrolyte concentrations or (d) blood pH were reduced in final blood samples from severely diseased (light grey bars, n = 7), but not in acclinically infected (white bars, n = 3) or uninfected (dark grey bars, n = 2) frogs (paired *t*-test). \*Significant at (a,b)  $p < 0.001$ ; (c)  $p = 0.036$ ; (d)  $p = 0.006$

significantly decreased in final blood samples compared with initial blood samples (Fig. 2). None of these changes were detected in acclinically infected or uninfected amphibians (Fig. 2). Furthermore, no other measured parameter (carbon dioxide, glucose, red cell count, hematocrit and hemoglobin) varied significantly in any group over time.

## DISCUSSION

We provide evidence that severe chytridiomycosis causes decreases in blood pH, plasma osmolality and plasma sodium, potassium, magnesium and chloride concentrations. Reduction in electrolyte concentrations could occur if electrolytes were diluted due to increased water uptake or if there was an overall loss from the blood. We found no significant change from initial to final measurements in body mass, hematocrit, albumin, or urea, indicating that hydration status is probably unaffected in severely diseased frogs. Therefore, it appears that the reductions in electrolyte concentrations were due to depletion from circulation rather than water uptake. The disproportionate loss of

electrolytes compared with water signifies an imbalance in osmotic homeostasis.

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

Pathogens are known to compromise function in other epithelia such as mammalian trachea and intestine (Berkes et al. 2003, Kunzelmann & McMorran 2006). Electrolyte transport is disrupted by pathogen adhesion to protein-based receptors (Cameron & Douglas 1996, Berkes et al. 2003, Kunzelmann & McMorran 2006) or by pathogen-secreted toxins that alter cell function (Berkes et al. 2003, Kunzelmann & McMorran 2006). For example, sodium transport and fluid balance were disrupted when a viral pathogen was introduced to mouse tracheal epithelium (Kunzelmann et al. 2000). This resulted from a down-regulation of amiloride-sensitive sodium channels in the apical membranes of the epithelium (Kunzelmann et al. 2000). It is possible that *Batrachochytrium dendrobatidis* disrupts sodium channels by a comparable mechanism. The selective barrier properties of frog skin are primarily determined by electrolyte transport in the mitochondrial-rich cells of the stratum granulosum (Ehrenfeld & Klein 1997), where *B. dendrobatidis* is found in severe infections (Berger et al. 1998). This hypothesis is further supported by Ussing chamber tests (Ussing & Zerahn 1951) using skin samples from infected *Bufo woodhousii*, which showed that active sodium transport was reduced in diseased toads under short-circuit conditions (Voyles et al. 2005). However, these results did not clarify if the loss of sodium and other electrolytes could occur via the skin or other excretion pathways. More specific tests investigating disruption of electrolyte transport are underway.

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

The 3 *Litoria caerulea* that appeared healthy despite infection had no significant changes in plasma osmolality or electrolyte concentrations. These results suggest that electrolyte reductions occur only in terminal stages of infection and may account for the neurological signs such as muscle tetany that precede death (Berger et al. 2007). While most amphibians can tolerate changes in plasma electrolyte levels (Deyrup 1964), the observed decrease of approximately 30% plasma sodium and 50% plasma potassium concentrations in diseased frogs may be too extreme. Additional research is needed to resolve whether these conditions lead to death through cardiac arrhythmia, myocardial failure, organ failure, or a combination of these and other factors.

Determining how *Batrachochytrium dendrobatidis* kills amphibians is essential to understanding the biology of chytridiomycosis. Additionally, research on the mechanisms of pathogenesis may explain why there is interspecific variation in susceptibility to the disease, a key question for amphibian conservation. Resolving the pathophysiological effects will provide crucial information for researchers, wildlife managers and veterinary clinicians in treating captive frogs and facilitating the recovery of wild frog populations that are currently affected by the disease.

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### **7.3 Pathogenesis of chytridiomycosis**

This published peer-reviewed paper represents original research led by Jamie Voyles. My role in the paper included: surgical implantation of the cardiac bio-transmitters in experimental frogs, blood and urine biochemistry and sediment sample processing, provision of veterinary technical expertise for methodology and results interpretation, and substantial written contributions to the methods, results and discussion sections.

Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W. F., Dinudom, A., Cook, D., Webb, R., Alford, R. A., Skerratt, L. F. & Speare, R. (2009). Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science* 326: 582-585.

Since the journal uses American English, spelling follows this convention.

Appendix 2 contains supporting online material referred to in the paper.

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Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W. F., Dinudom, A., Cook, D., Webb, R., Alford, R. A., Skerratt, L. F. & Speare, R. (2009). Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science* 326: 582-585.







## CHAPTER 8

### Captive management and treatment of chytridiomycosis

#### 8.1 Introduction

The chytrid fungus *Batrachochytrium dendrobatidis* has caused dramatic declines and extinctions of amphibian species worldwide over the last three decades. This virulent water-borne pathogen has caused epidemic waves of high mortality as it spread through susceptible wild populations in Australia, North, Central and South America and New Zealand, and is now endemic in surviving populations in these continents and in Europe and Africa. Prevalence of chytridiomycosis in the international amphibian trade is high and importation of infected frogs into zoos has caused disease epidemics in established collections. Management of disease spread requires effective national and international quarantine and control strategies. In the first published peer-reviewed paper in this chapter, we identified key management and conservation strategies for zoological institutions in preventing pathogen spread, and in disease surveillance, captive-breeding and re-introduction programmes, to limit the impact of this formidable disease on wild amphibian populations.

Development of standardised effective treatment protocols to cure *Bd* is critical for future global amphibian conservation efforts and to help prevent future species extinctions. There have been few reports of successful treatment of chytridiomycosis, and prior to this study there were no known reports of curing amphibians with severe disease. In the second published peer-reviewed paper in this chapter, we effectively and consistently cured both sub-clinical and terminally ill *Bd*-infected green tree frogs. This is the first known treatment protocol described for curing terminally ill amphibians with chytridiomycosis.

This chapter consists of two published peer-reviewed papers: 1) strategies for management of chytridiomycosis and amphibian conservation, and 2) treatment of amphibian chytridiomycosis.

#### 8.2 Strategies for captive management and conservation

I drafted the original management and conservation review paper and subsequently received significant editorial contributions from Lee Berger and Rick Speare, along with specific management advice from Rob Puschendorf.

Young, S., Berger, L. & Speare, R. (2007). Amphibian chytridiomycosis: strategies for captive management and conservation. *International Zoo Yearbook* 41: 85-95.

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Young, S., Berger, L. & Speare, R. (2007). Amphibian chytridiomycosis: strategies for captive management and conservation. *International Zoo Yearbook* 41: 85-95.























### **8.3 Treatment of amphibian chytridiomycosis**

This published peer-reviewed paper represents my original research and writing, with substantial study design advice from Rick Speare and editorial contributions from Lee Berger and Lee Skerratt.

Young, S., Speare, R., Berger, L. & Skerratt, L. F. (2012b). Chloramphenicol with fluid and electrolyte therapy cures terminally ill green tree frogs (*Litoria caerulea*) with chytridiomycosis. *Journal of Zoo and Wildlife Medicine* 43: 330-337.

Since the journal uses American English, spelling follows this convention.

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Young, S., Speare, R., Berger, L. & Skerratt, L. F. (2012).  
Chloramphenicol with fluid and electrolyte therapy cures  
terminally ill green tree frogs (*Litoria caerulea*) with  
chytridiomycosis. *Journal of Zoo and Wildlife Medicine* 43:  
330-337.

















## CHAPTER 9

### Conclusions and recommendations

This thesis represents extensive and practically important work that contributes significantly to the field of wildlife and ecosystem health. Amphibian disease surveillance techniques, amphibian diagnostic testing, general knowledge about amphibian immunity, specific knowledge about host immune response to chytridiomycosis, and recommendations for the treatment and management of this formidable amphibian disease, have all been advanced by the findings in this thesis. Working with free-ranging wildlife species and the talented and passionate people at the human-wildlife interface has both many rewards and challenges. Political and funding constraints encountered early on were largely responsible for the evolution of my research objectives away from specific community wildlife group collaboration towards laboratory-based amphibian diagnostic and disease investigations in collaboration with other scientific organisations and colleagues. Significant limitations associated with the use of free-ranging wildlife in controlled experimental trials were encountered, creating the logistic and experimental design challenges described in Chapters 5 and 6. Despite this, there were many tangible and practical outcomes from this research, culminating in clinical treatment and management recommendations for chytridiomycosis that will be broadly applicable to amphibian conservation medicine and wildlife health management for many years to come.

#### 9.1 Amphibian disease surveillance

Analysis of amphibian disease surveillance data and techniques enabled the identification and description of key benefits and limitations associated with passive syndromic surveillance. The syndrome manifesting as irreversible emaciation in *L. infrafrenata* was discovered through professional epidemiological analysis and interpretation of passive syndromic surveillance data, in combination with diagnostic expertise. The surveillance data had been accumulating on paper without analysis for many years and it is highly doubtful that the syndrome would have been formally documented in a useful form without this collaborative input.

Community wildlife groups can play a valuable role in disease surveillance and contribute economically to this crucial but under-resourced sector. However, important limitations associated with passive syndromic surveillance used alone as a primary tool in wildlife disease investigation and management include: inherent sample bias, data inaccessibility and non-uniformity, and lack of epidemiological data including prevalence data (Harden et al. 2006).

Our research also shows that the system of community groups carrying out passive syndromic surveillance without adequate diagnostic pathology is fundamentally flawed since accurate definitive diagnoses are not possible. When this system of surveillance is combined with professional diagnostic expertise and active disease surveillance, useful investigations can be initiated to determine whether disease syndromes identified by community groups are novel and if their incidence is increasing. This was clearly demonstrated in the case of the irreversible emaciation syndrome in *L. infrafronata*. Our professional investigations identified a novel endemic disease strongly associated with sparganosis; this was originally incorrectly classified by the community group as an emerging disease of unknown aetiology, but with the prediction of a viral aetiology based on inadequate diagnostic pathology and personnel training and experience, along with operator bias.

It is recommended that trained professionals should be involved in all wildlife disease investigations to establish case definitions, and for diagnostic pathology, complementary active disease surveillance, and data analysis and interpretation. The limitations identified highlight the need for concurrent active surveillance and the involvement of pathologists, epidemiologists and other experts during disease investigations. In addition to professionals providing accurate clinical and pathological diagnoses, the quality of disease surveillance data collected by community wildlife groups could be greatly improved by the use of real-time computerised reporting systems, with standardised terminology and established syndromic case definitions, integrated with a national database such as that maintained by the Australian Wildlife Health Network.

Future investigations are needed to confirm the aetiology and epidemiology of the irreversible emaciation syndrome in *L. infrafronata*, in particular the role of *Spirometra erinacei* infection. The broader impact of this cestode as a primary pathogen on frog populations also needs to be determined by future research. An active surveillance system should be designed to determine distribution and prevalence of *S. erinacei* in amphibians and other intermediate vertebrate and invertebrate host species, along with the definitive carnivore hosts. Surveillance should also be designed to identify risk factors for the amphibian host e.g. age, host population density, domestic animal population density. Results from active surveillance should be compared with passive surveillance data to further assess the benefits and limitations of both methods, and the significance of the disease to amphibian populations in northern Australia. The emaciation syndrome could affect the abundance of *L. infrafronata*, and further investigations will contribute to the broader understanding of the role of emerging and endemic diseases in the decline of amphibians.

## 9.2 Diagnostic testing in amphibians

Amphibian disease investigations can be severely restricted by the lack of sensitive and specific diagnostic tests available and validated for use in amphibians. Few baseline and reference values for diagnostic tests exist, making assessment of health status and identification of abnormal health extremely challenging. Wide variation between amphibian species, and within species due to many intrinsic and extrinsic factors, further complicates diagnostic investigations. This research has contributed substantially to the validation of diagnostic tests and the establishment of reference values for amphibian health monitoring and disease investigations.

Reference intervals were established for a wide range of haematologic, plasma biochemical and protein electrophoretic parameters in two Australian frog species. Various diagnostic tests were developed and validated for use in these species, including comprehensive leucocyte morphological descriptions, total and differential blood cell counts, a modified manual haemoglobin assay, and measurement of plasma biochemistry analytes using an automated bench-top chemistry analyser. Concordance of three analytic methods for measurement of total protein, albumin and globulins was also assessed.

The biuret method for total protein determination is the most accurate laboratory method available (Campbell, 2006), and electrophoretic techniques for measuring serum or plasma protein concentrations are the reference standard (Kaneko, 1997). Since biuret and refractometry total protein values in this study were relatively well correlated, refractometry can be recommended for rapid in-house total protein determination in *L. caerulea* and *L. infrafrenata*, particularly if sample volume is small and/or there are cost constraints. In both species, albumin concentrations measured with the bench-top chemistry analyser, using the bromocresol green dye-binding method, were only weakly correlated with the electrophoretic method, and resulted in falsely low albumin and falsely high globulin concentrations. The bench-top chemistry analyser used in this study cannot be recommended for albumin or globulin determination in *L. caerulea* or *L. infrafrenata*, and protein electrophoresis should be used instead. However, the chemistry analyser was found to be a valuable in-house tool for rapid measurement of the other biochemical parameters in this study and can be recommended for future use for this purpose.

The reference intervals established in this study will be useful for future disease screening and potentially for the early detection of emerging diseases within Australian tree frog populations. Significant inter- and intra-species differences were detected for a number of haematologic and

biochemical parameters in *L. caerulea* and *L. infrafrenata*, highlighting the importance of establishing species-specific and, within species, season-specific reference intervals. There was little variation in values for either species between years, indicating that the populations studied are stable in terms of disease; the reference intervals are true baseline values and will be invaluable for future diagnostic investigations and early detection of disease emergence in both species. Established reference intervals are applicable to the specific analytic methods used in this study and should ideally be validated before future use with other frog populations.

Intra-erythrocytic haemogregarines were identified in 19 % of *L. infrafrenata* samples collected during this project. Although infected frogs were clinically normal, they had significant haematologic abnormalities compared with the reference population, indicating important sub-clinical impacts on health. Low burdens of haemoparasites are generally thought to be unimportant (Graczyk et al., 1996; Wright, 2001a; Campbell & Ellis, 2007), but moderate to high burdens may be of clinical significance, particularly if coupled with indicators of anaemia (Wright, 2001a, 2006). Further investigations are needed to classify and determine the clinical significance of the haemogregarines, and whether the parasite is endemic or emerging in Australian *L. infrafrenata* populations.

### **9.3 Host immune response to chytridiomycosis**

Diverse methods were developed, validated and implemented to study immune function in *B. dendrobatidis*-infected and exposed frogs, and in healthy uninfected frogs (*L. caerulea* and *L. infrafrenata*). All of the methods used demonstrated a marked immune suppressive effect of *B. dendrobatidis* infection in *L. caerulea*. *Litoria infrafrenata* failed to maintain infection after experimental exposure, and recovered frogs showed sub-clinical immune function effects. Results from this study provide the first direct evidence that the amphibian chytrid fungus actively suppresses a systemic immune response when infecting the host. This is a major step forward in following up on previous genetic and *in vitro* studies. Host immune suppression is likely a key factor enabling *B. dendrobatidis* to be a formidable disease with unprecedented effects on biodiversity.

This host immune suppression, found here to be an important contributing factor to *B. dendrobatidis* susceptibility in amphibians, may explain the lack of an adaptive immune response to infection (Stice & Briggs, 2010; Young et al., submitted (Chapter 6); Cashins et al., unpublished). If vaccine development or other immune modulation is attempted in future trials, it will be necessary to better understand the mechanisms of immune suppression in order to overcome it. Baseline immune function data resulting from this research will provide a valuable



tool for progressing future immunologic and *B. dendrobatidis* pathogenicity studies in amphibians. General mechanisms of fungal suppression include inducing anti-inflammatory cytokines, decreasing pro-inflammatory cytokines and complement evasion (Chai et al., 2009). Further work on understanding the mechanisms of *B. dendrobatidis* immune suppression is needed. Future studies should aim to further characterise the pathogen, including identification and isolation of specific pathogen-secreted immunosuppressive factors. Experimental trials could then be designed to expose frogs directly to these pathogen-secreted and other pathogen-associated factors, and the host immune response assessed using the sensitive diagnostic methods described in this project. Studying the *in vitro* effects of isolated pathogen-associated factors on host lymphoid cells, along with host cytokine production and activity, could also provide valuable information to direct future research efforts.

Results from the large re-exposure trial reported in this study indicated no long-term beneficial effect of prior pathogen exposure on the outcome of *B. dendrobatidis* infection in *L. caerulea*. Conversely, a greater proportion of previously exposed frogs became infected, and infected re-exposed frogs had a higher infection intensity that increased at a greater rate, compared with infected naïve frogs. Additionally, a greater proportion of infected naïve frogs self-cured compared with the infected re-exposed group. This indicates that naïve frogs are better able to control and clear infection, and may indirectly indicate suppression of the host immune response associated with prior pathogen exposure. Overall these findings suggest that previous pathogen exposure and infection may have a long-term adverse effect on host resistance to *B. dendrobatidis*. This further supports host suppression by the pathogen being a key factor in the pathogenicity of *B. dendrobatidis* (Young et al., submitted; Chapter 5), along with negative host fitness effects related to exposure and infection (Garner et al., 2009; Skerratt et al., 2009). Excluding self-cured frogs, there were fewer differences between the re-exposed and naïve exposed groups, and this may also indicate an important role of innate immunity in disease resistance.

Future research is needed to confirm whether short-term pathogen-specific adaptive immune responses can occur in susceptible species, and if so, whether these responses can be potentiated to stimulate long-lasting resistance against *B. dendrobatidis*. Persistence of a protective adaptive immune response would be a key factor in determining the success of control programmes using immunisation, due to seasonal environmental fluctuations influencing host behaviour and pathogen growth and reproduction. If susceptible host species lack the ability to mount protective adaptive responses, then the focus of future research needs to shift beyond this to identification of pathogen-associated factors and other mechanisms of host immune suppression by the pathogen, along with alternative methods of host immune modulation such as cytokine-

and cell-based immune activation, enhancement of the role of symbiotic organisms and manipulation of non-specific extrinsic factors.

#### **9.4 Pathogenesis of chytridiomycosis**

The James Cook University Amphibian Disease Ecology Group undertook extensive experimental investigations to determine the pathogenesis of chytridiomycosis, in parallel and overlapping with my research project. We found that *L. caerulea* with severe disease had reduced plasma osmolality and multiple electrolyte abnormalities due to circulatory electrolyte depletion. These results suggested that *B. dendrobatidis* kills amphibians by disrupting normal epidermal functioning, leading to osmotic imbalance through loss of electrolytes (Voyles et al., 2007).

We then proceeded to discover a mechanism for amphibian mortalities. In experimentally infected *L. caerulea*, *B. dendrobatidis* caused fatal pathophysiological changes including epidermal degeneration, inhibited epidermal electrolyte transport, systemic electrolyte disturbances and asystolic cardiac arrest. Resolving the pathogenesis of this disease is a key step forward in understanding this unparalleled amphibian pandemic (Voyles et al., 2009).

Future research in this area needs to focus on determining common pathophysiology among closely and distantly related amphibian species, and broadening research trials to incorporate field studies with naturally infected susceptible host species, tolerant reservoir species and naturally resistant species.

#### **9.5 Captive management of chytridiomycosis**

‘Infection of amphibians with chytrid fungus resulting in chytridiomycosis’ was listed as a ‘key threatening process’ in Australia in July 2002 under the *Environment Protection and Biodiversity Protection Act*. This led to the development of a Threat Abatement Plan, which aims to minimise the impact of chytridiomycosis on amphibian populations through prevention of pathogen spread, recovery of at-risk threatened species, control of infection, education and coordination of management activities (Australian Government Department of the Environment and Heritage, 2006). Effective management of chytridiomycosis requires national surveillance to determine accurately disease distribution, protection of disease-free populations, rapid detection of and response to new outbreaks, restriction of amphibian movements, implementation of national hygiene and quarantine protocols, and routine monitoring of stock for chytridiomycosis.

Recommendations for captive management strategies for amphibian chytridiomycosis were reviewed and updated during this project (Young et al., 2007). Disease prevalence in the international amphibian trade is high and importation of infected frogs into zoos has caused disease epidemics in established amphibian collections (Nichols et al., 1998, 2001; Pessier et al., 1999; Banks & McCracken, 2002; Schloegel et al., 2006). Effective disinfection, hygiene, treatment, quarantine and other control protocols must be implemented both nationally and internationally to ensure success of captive-breeding programmes for threatened species, and to reduce risks associated with amphibian translocations between field sites, captive collections and countries.

Recommendations to improve the future management of chytridiomycosis include dedicating global resources to zoological and other institutions for captive breeding of threatened species, and for emergency response to species and population declines. To date, few captive-breeding programmes for threatened species have been successful owing to a lack of critical knowledge about the species. Research into the natural history and biology of these species must be prioritised to enable captive-breeding success. Captive-breeding programmes must be well-planned and implemented in advance of the threat to survival of a species, and there must be extensive collaboration between participating institutions worldwide. Surveillance of amphibian populations must be coordinated nationally and internationally to determine accurately the distribution of chytridiomycosis and to rapidly detect and respond to outbreaks (Australian Government Department of the Environment and Heritage, 2006). Increased efforts to educate the public should be undertaken to reduce disease transmission through human activity and accidental translocation of infected amphibians and other materials. Zoological institutions can play an important role in supporting these activities, both internally and externally.

While zoos can play a key role in contributing to amphibian conservation, maintaining threatened species in captivity must be recognised as only a short-term solution because of space limitations and the inability to maintain genetically viable populations indefinitely. Resources must be made available immediately to preserve and restore the natural habitats of amphibians. Removing other significant threats to amphibians such as habitat loss and degradation will maximise population sizes and may assist in survival and recovery from the impact of chytridiomycosis. Habitat protection will ultimately preserve whole ecosystems, not just individual threatened species, and should be a priority in the global response to chytridiomycosis.

## 9.6 Treatment of chytridiomycosis

This research project describes the first known clinical treatment protocol for curing terminally ill *B. dendrobatidis*-infected frogs. Development of standardised effective treatment protocols to cure infected frogs is critical for future global amphibian conservation efforts and to help prevent future species extinctions. Clinically severe chytridiomycosis causes fatal pathophysiological changes in *L. caerulea*, including epidermal degeneration, inhibited epidermal electrolyte transport, systemic electrolyte disturbances (hyponatraemia and hypokalaemia), hypovolaemia, dehydration and asystolic cardiac arrest (Voyles et al., 2007, 2009; Young et al., 2012b). Terminally ill frogs were cured by a combination of treating the pathophysiological effects of *B. dendrobatidis* with aggressive parenteral fluid and electrolyte therapy (thereby correcting severe dehydration and electrolyte abnormalities), killing the pathogen with chloramphenicol, and heat, without adverse drug effects. Sub-clinically infected frogs were consistently cured with chloramphenicol treatment alone, without adverse drug effects.

Further studies are needed to determine whether heat is a concurrent requirement with chloramphenicol to kill *B. dendrobatidis* and effect cure in terminally ill frogs, and whether electrolyte supplementation via bath solution is an effective form of treatment. Additionally, experimental trials with sub-clinically infected frogs should be repeated with infected but untreated positive control frogs to compare fungicidal treatment success versus spontaneous clearance in this species. Advantages of chloramphenicol include availability (in developed countries), low cost, high amphibian safety margin, ability to treat large numbers of amphibians, and high efficacy rate. The main disadvantages are operator safety concerns and the long duration of treatment required, although no drug tested to date effectively clears *B. dendrobatidis* after a single application. Alternate formulations for chloramphenicol administration such as topical ointments may be more suitable for amphibians less tolerant of continuous immersion, have fewer operator safety risks, and warrant further inter-species clinical efficacy and safety trials. Further studies are also needed to evaluate the efficacy, safety and pharmacokinetics of chloramphenicol in aquatic versus semi-aquatic and terrestrial amphibians, but preliminary evidence for wide clinical application is promising.

Safe and effective treatments for chytridiomycosis are fundamental for improving captive management of the disease and reducing its impact on wild amphibian populations. Several amphibian species are currently only secure in captivity and captive breeding programs are preventing their extinction (Mendelson et al., 2006; Gagliardo et al., 2008). Effective treatment of *B. dendrobatidis*-infected frogs would enable cure of critical captive individuals and of wild

individuals for use in captive survival assurance colonies (Gagliardo et al., 2008), and could prevent extinctions of populations and entire species. Wild individuals from areas where threatened populations are rapidly declining from disease epidemics, and from endemic areas during periods of increased pathogen virulence, could be treated to assure continued survival. The last remaining group of sharp-snouted day frogs (*Taudactylus acutirostris*) died from *B. dendrobatidis* infection in captivity in 1995; extinction of this species may have been prevented had an effective treatment been available (Banks & McCracken, 2002; Schloegel et al., 2006). While broader investigations are needed to evaluate inter-species efficacy, the development of successful standardized treatment protocols for curing sub-clinical and terminally ill *B. dendrobatidis*-infected frogs has enormous implications for amphibian conservation medicine and preventing future declines and extinctions.

## **9.7 Conclusion**

An enormous amount of research is still needed to improve amphibian diagnostic capabilities and to further unravel the complexities of the host-pathogen-environment interactions that make amphibian chytridiomycosis such a formidable disease. Extensive species and population surveys, active disease surveillance, *in situ* and captive disease response plans, and dedicated resources to facilitate progress in these areas are high priorities. Concurrent future research to improve our knowledge about host immunity, immunomodulation and pathogen factors to mitigate the impact of chytridiomycosis on wild amphibian populations remains a high priority. Improved national, international and inter-disciplinary collaboration is needed urgently, along with better and timely resource and output sharing. No contribution to the field of wildlife and ecosystem health is too little as we face a century of accelerating and devastating impacts on this planet resulting directly from its overpopulation with a single species, *Homo sapiens*.

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## APPENDIX 1

### Summary of case details and diagnostic findings

The following table from the final funding body report (Young et al., 2010) summarises the case details and diagnostic findings in frog specimens collected through James Cook University as part of the disease surveillance component of my research.

Case ID	Date	Species	Age/Sex	Origin	Diagnostic Findings
FDRQ 001	04/01/06	<i>Litoria infrafrenata</i>	Mature adult female	Cooktown	Emaciation <i>Spirometra erinacei</i> infection
FDRQ 002	04/01/06	<i>Litoria infrafrenata</i>	Mature adult female	Edge Hill Cairns	Injury
FDRQ 003	04/02/06	<i>Litoria infrafrenata</i>	Mature adult female	Holloways Beach Cairns	Injury Ascites
FDRQ 004	05/02/06	<i>Litoria infrafrenata</i>	Mature adult female	Trinity Beach Cairns	Emaciation <i>Spirometra erinacei</i> infection
FDRQ 005	06/02/06	<i>Litoria infrafrenata</i>	Mature adult female	Edmonton Cairns	Injury – abdominal hernia Hyperbilirverdinaemia
FDRQ 006	06/02/06	<i>Litoria infrafrenata</i>	Mature adult	Trinity Bay Cairns	Emaciation
FDRQ 007	06/02/06	<i>Litoria infrafrenata</i>	Mature adult	Brinsmead Cairns	Emaciation
FDRQ 008	06/02/06	<i>Litoria infrafrenata</i>	Mature adult	Edmonton Cairns	Emaciation
FDRQ 009	06/02/06	<i>Litoria infrafrenata</i>	Mature adult	Trinity Beach Cairns	Emaciation <i>Spirometra erinacei</i> infection
FDRQ 010	06/02/06	<i>Litoria infrafrenata</i>	Young adult	Brinsmead Cairns	Emaciation
FDRQ 011	06/02/06	<i>Litoria infrafrenata</i>	Mature adult	Yarrabah	Emaciation <i>Spirometra erinacei</i> infection
FDRQ 012	06/02/06	<i>Litoria infrafrenata</i>	Mature adult	Edmonton Cairns	Emaciation <i>Spirometra erinacei</i> infection
FDRQ 013	06/02/06	<i>Litoria infrafrenata</i>	Mature adult	Manunda Cairns	Emaciation <i>Spirometra erinacei</i> infection
FDRQ 014	15/02/06	<i>Litoria caerulea</i>	Mature adult male	Mooroobool Cairns	Skin condition
FDRQ 015	15/02/06	<i>Litoria infrafrenata</i>	Mature adult male	Manoora Cairns	Anasarca (blue-tinged fluid) Fair body condition
FDRQ 016	15/02/06	<i>Litoria caerulea</i>	Young adult male	Edmonton Cairns	Gastrointestinal parasitism

FDRQ 017	15/02/06	<i>Litoria infrafrenata</i>	Mature adult female	Mooroobool Cairns	Mild anasarca (blue-tinged fluid)
FDRQ 018	15/02/06	<i>Litoria infrafrenata</i>	Mature adult female	Mooroobool Cairns	Mild anasarca (blue-tinged fluid)
FDRQ 019	15/02/06	<i>Litoria infrafrenata</i>	Mature adult female	Mooroobool Cairns	Mild anasarca (blue-tinged fluid)
FDRQ 020	15/02/06	<i>Litoria infrafrenata</i>	Mature adult male	Mooroobool Cairns	<i>Spirometra erinacei</i> infection
FDRQ 021	20/02/06	<i>Litoria infrafrenata</i>	Mature adult	Gordonvale Cairns	Emaciation <i>Spirometra erinacei</i> infection
FDRQ 022	20/02/06	<i>Litoria infrafrenata</i>	Mature adult	Edge Hill Cairns	Skin condition
FDRQ 023	20/02/06	<i>Litoria infrafrenata</i>	Mature adult	Machans Beach Cairns	<i>Spirometra erinacei</i> infection Coelomic mass
FDRQ 024	20/02/06	<i>Litoria infrafrenata</i>	Mature adult	Cairns	<i>Spirometra erinacei</i> infection Hepatic mass
FDRQ 025	20/02/06	<i>Bufo marinus</i>	Mature adult	Edmonton Cairns	No significant histological findings
FDRQ 026	20/02/06	<i>Litoria infrafrenata</i>	Mature adult	Edge Hill Cairns	Emaciation
FDRQ 027	20/02/06	<i>Litoria infrafrenata</i>	Mature adult	Cairns	Injury
FDRQ 028	22/02/06	<i>Litoria infrafrenata</i>	Mature adult female	Cooktown	Poor body condition after prolonged captivity
FDRQ 029	22/02/06	<i>Litoria infrafrenata</i>	Mature adult male	Stratford Cairns	Poor body condition after prolonged captivity
FDRQ 030	22/02/06	<i>Litoria infrafrenata</i>	Mature adult female	Mooroobool Cairns	Emaciation <i>Spirometra erinacei</i> infection
FDRQ 031	22/02/06	<i>Litoria infrafrenata</i>	Mature adult male	Mooroobool Cairns	Anasarca (blue-tinged fluid) Hyperbilirverdinaemia
FDRQ 032	22/02/06	<i>Litoria infrafrenata</i>	Mature adult male	Portsmith Cairns	Anasarca (blue-tinged fluid) Poor body condition (captive)
FDRQ 033	22/02/06	<i>Litoria infrafrenata</i>	Mature adult female	Mooroobool Cairns	Hyperbilirverdinaemia
FDRQ 034	23/02/06	<i>Litoria infrafrenata</i>	Mature adult female	Bayview Heights Cairns	Poor body condition (captive) Renal parasitism
FDRQ 035 – 048	02/03/06	<i>Cyclorana novaehollandiae</i>	Metamorphs x 11 Tadpoles x 3	Mount Carbine	Hepatic necrosis Skeletal abnormalities Ascites, Lipaemia Suspect toxic or nutritional aetiology
FDRQ 049	02/03/06	<i>Litoria infrafrenata</i>	Mature adult female	White Rock Cairns	Injury Hyperbilirverdinaemia Renal parasitism

FDRQ 050	15/03/06	<i>Litoria infrafrenata</i>	Mature adult female	Edge Hill Cairns	Injury Coelomic nematodes
FDRQ 051	11/04/06	<i>Litoria infrafrenata</i>	Mature adult female	Mooroobool Cairns	Injury Hyperbiliriverdinaemia
FDRQ 052	01/06/06	<i>Litoria infrafrenata</i>	Mature adult female	Manoora Cairns	Emaciation <i>Spirometra erinacei</i> infection
FDRQ 053	03/06/06	<i>Litoria caerulea</i>	Mature adult female	Peachester SE Qld via QPWS	Chytridiomycosis
FDRQ 054	03/06/06	<i>Litoria caerulea</i>	Mature adult female	SE Qld via QPWS	Systemic microsporidiosis
FDRQ 055	03/06/06	<i>Litoria caerulea</i>	Mature adult male	Brighton SE Qld via QPWS	Injury
FDRQ 056	03/06/06	<i>Litoria infrafrenata</i>	Mature adult female	Mooroobool Cairns	Emaciation <i>Spirometra erinacei</i> infection Hyperbiliriverdinaemia
FDRQ 057	28/06/06	<i>Litoria infrafrenata</i>	Mature adult female	Yorkeys Knob Cairns	Hepatic and renal cysts Encysted nematodes - bladder
FDRQ 058	30/06/06	<i>Litoria infrafrenata</i>	Mature adult male	Manunda Cairns	Emaciation <i>Spirometra erinacei</i> infection
FDRQ 059	30/06/06	<i>Limnodynastes terraereginae</i>	Mature adult female	Calliope SE Qld via QPWS	Cause of death unknown
FDRQ 060	30/06/06	<i>Litoria caerulea</i>	Mature adult male	Redland Bay SE Qld via QPWS	Dermatitis Renal disease
FDRQ 061	30/06/06	<i>Litoria caerulea</i>	Subadult male	Sunshine Coast via QPWS	Suspect chytridiomycosis
FDRQ 062	30/06/06	<i>Litoria caerulea</i>	Subadult male	Brighton SE Qld via QPWS	Injury Focal hepatitis and necrosis
FDRQ 063	30/06/06	<i>Bufo marinus</i>	Mature adult male	Nth Maleny SE Qld via QPWS	Hepatic disease Pulmonary <i>Rhabdias</i> infection
FDRQ 064	03/07/06	<i>Adelotus brevis</i>	Mature adult female	Greenslopes SE Qld via QPWS	Chytridiomycosis
FDRQ 065	03/07/06	<i>Litoria peronii</i>	Mature adult male	Peachester SE Qld via QPWS	Chytridiomycosis
FDRQ 066	03/07/06	<i>Litoria nasuta</i>	Mature adult female	SE Qld via QPWS	Chytridiomycosis
FDRQ 067	03/07/06	<i>Litoria nasuta</i>	Mature adult male	SE Qld via QPWS	Injury Bilateral blindness
FDRQ 068	03/07/06	<i>Litoria nasuta</i>	Mature adult female	Fernvale SE Qld via QPWS	Injury
FDRQ 069	03/07/06	<i>Litoria rubella</i>	Mature adult	Sunshine Coast via QPWS	Cause of death unknown
FDRQ 070	04/07/06	<i>Litoria nasuta</i>	Mature adult male	Fernvale SE Qld via QPWS	Injury



FDRQ 071	05/07/06	<i>Litoria infrafrenata</i>	Mature adult female	Clifton Beach Cairns	Emaciation <i>Spirometra erinacei</i> infection Pulmonary <i>Rhabdias</i> infection
FDRQ 072	14/07/06	<i>Litoria caerulea</i>	Mature adult female	Redlynch Cairns	Coelomic mass
FDRQ 073	14/07/06	<i>Litoria caerulea</i>	Mature adult male	Gordonvale Cairns	Poor body condition after prolonged captivity
FDRQ 074	14/07/06	<i>Litoria infrafrenata</i>	Mature adult female	Machans Beach Cairns	Injury
FDRQ 075	03/08/06	<i>Litoria infrafrenata</i>	Mature adult male	Manoora Cairns	Emaciation <i>Spirometra erinacei</i> infection Pulmonary <i>Rhabdias</i> infection Gastrointestinal nematodes <i>Hepatozoon</i> sp. infection
FDRQ 076	13/08/06	<i>Litoria nasuta</i>	Mature adult male	Kuranda	Cause of death unknown
FDRQ 077	19/09/06	<i>Litoria infrafrenata</i>	Mature adult female	Manoora Cairns	Emaciation <i>Spirometra erinacei</i> infection Hyperbiliverdinaemia <i>Hepatozoon</i> sp. infection
FDRQ 078	11/10/06	<i>Litoria infrafrenata</i>	Mature adult male	Machans Beach Cairns	Otic chondroma
FDRQ 079	12/10/06	<i>Litoria infrafrenata</i>	Mature adult female	Kuranda	Emaciation <i>Spirometra erinacei</i> infection Pulmonary <i>Rhabdias</i> infection Encysted coelomic nematodes
FDRQ 080	13/10/06	<i>Litoria infrafrenata</i>	Mature adult male	Edge Hill Cairns	Injury <i>Hepatozoon</i> sp. infection Microfilaria infection
FDRQ 081	18/10/06	<i>Litoria caerulea</i>	Mature adult female	Mooroobool Cairns	<i>Spirometra erinacei</i> infection
FDRQ 082	18/10/06	<i>Litoria infrafrenata</i>	Mature adult female	Mooroobool Cairns	Emaciation <i>Spirometra erinacei</i> infection
FDRQ 083	26/10/06	<i>Litoria infrafrenata</i>	Mature adult male	Cooktown	Massive cloacal prolapse <i>Hepatozoon</i> sp. infection
FDRQ 084	28/11/06	<i>Litoria infrafrenata</i>	Mature adult male	Brinsmead Cairns	Injury <i>Hepatozoon</i> sp. infection
FDRQ 085	02/12/06	<i>Litoria infrafrenata</i>	Mature adult female	Clifton Beach Cairns	Dermal squamous papilloma <i>Spirometra erinacei</i> infection <i>Hepatozoon</i> sp. infection
FDRQ 086	08/12/06	<i>Litoria lesueuri</i>	Mature adult female	Kuranda	Injury Gastrointestinal nematodes
FDRQ 087	04/01/07	<i>Litoria infrafrenata</i>	Mature adult female	Holloways Beach Cairns	Mandibular osteomyelitis Pulmonary <i>Rhabdias</i> infection Encysted coelomic nematodes

FDRQ 088	05/01/07	<i>Litoria infrafrenata</i>	Mature adult female	Holloways Beach Cairns	Injury Gastrointestinal nematodes
FDRQ 089	05/01/07	<i>Litoria infrafrenata</i>	Mature adult female	Edmonton Cairns	Emaciation <i>Spirometra erinacei</i> infection
FDRQ 090	15/01/07	<i>Litoria infrafrenata</i>	Subadult	Wonga Beach	Severe skeletal deformities after prolonged captivity
FDRQ 091	15/01/07	<i>Litoria infrafrenata</i>	Subadult	Aeroglen Cairns	Severe skeletal deformities after prolonged captivity
FDRQ 092	16/01/07	<i>Litoria caerulea</i>	Mature adult male	Edmonton Cairns	Emaciation <i>Spirometra erinacei</i> infection Pulmonary <i>Rhabdias</i> infection Gastrointestinal nematodes
FDRQ 093	31/01/07	<i>Litoria infrafrenata</i>	Mature adult male	Freshwater Cairns	Injury
FDRQ 094	31/01/07	<i>Litoria infrafrenata</i>	Mature adult male	Edge Hill Cairns	Pulmonary <i>Rhabdias</i> infection Encysted coelomic nematodes
FDRQ 095	31/01/07	<i>Litoria caerulea</i>	Subadult male	Mossman	Injury Gastrointestinal nematodes
FDRQ 096	01/02/07	<i>Litoria infrafrenata</i>	Mature adult female	Manunda Cairns	Emaciation <i>Spirometra erinacei</i> infection <i>Hepatozoon</i> sp. infection Gastrointestinal nematodes Hyperbiliriverdinaemia
FDRQ 097	01/02/07	<i>Litoria infrafrenata</i>	Mature adult male	Smithfield Cairns	Injury
FDRQ 098	02/02/07	<i>Litoria infrafrenata</i>	Mature adult female	Machans Beach Cairns	Emaciation <i>Spirometra erinacei</i> infection Pulmonary <i>Rhabdias</i> infection <i>Hepatozoon</i> sp. infection
FDRQ 099	26/02/07	<i>Litoria infrafrenata</i>	Mature adult	Forest Gdns Cairns	Injury
FDRQ 100	26/02/07	<i>Litoria infrafrenata</i>	Young adult	Brinsmead Cairns	Injury
FDRQ 101	26/02/07	<i>Litoria infrafrenata</i>	Mature adult	Manunda Cairns	Urinary tract nematodes Myopathy
FDRQ 102	26/02/07	<i>Litoria infrafrenata</i>	Mature adult	Trinity Beach Cairns	Emaciation <i>Spirometra erinacei</i> infection
FDRQ 103	02/03/07	<i>Litoria caerulea</i>	Young adult male	Redlynch Cairns	Injury
FDRQ 104	04/03/07	<i>Litoria splendida</i>	Subadult	Kuranda (captive)	Chytridiomycosis
FDRQ 105	05/03/07	<i>Litoria splendida</i>	Subadult	Kuranda (captive)	Chytridiomycosis
FDRQ 106	07/03/07	<i>Litoria splendida</i>	Subadult	Kuranda (captive)	Chytridiomycosis

FDRQ 107	08/03/07	<i>Litoria splendida</i>	Subadult	Kuranda (captive)	Chytridiomycosis
FDRQ 108	08/03/07	<i>Litoria splendida</i>	Subadult	Kuranda (captive)	Chytridiomycosis
FDRQ 109	11/03/07	<i>Litoria splendida</i>	Subadult	Kuranda (captive)	Chytridiomycosis
FDRQ 110	12/03/07	<i>Litoria splendida</i>	Subadult	Kuranda (captive)	Chytridiomycosis
FDRQ 111	16/03/07	<i>Litoria wilcoxii</i>	Mature adult male	Eungella National Park via QPWS	Renal parasitism Cause of death unknown
FDRQ 112	21/03/07	<i>Litoria wilcoxii</i>	Mature adult male	Eungella National Park via QPWS	Injury
FDRQ 113 A B C D	22/03/07	<i>Taudactylus eungellensis</i>	Tadpoles x 4	Eungella National Park via QPWS	Cause of death unknown; no significant histological findings
FDRQ 114	29/03/07	<i>Litoria infrafrenata</i>	Mature adult female	Aeroglen Cairns	Bacterial septicaemia
FDRQ 115	17/04/07	<i>Litoria xanthomera</i>	Mature adult	Wondecla via QPWS	Injury
FDRQ 116	17/04/07	<i>Litoria caerulea</i>	Mature adult female	Townsville	Severe generalised bacterial abscessation
FDRQ 117	27/04/07	<i>Bufo marinus</i>	Mature adult male	Mount Tambourine	Severe bacterial skin abscessation
FDRQ 118	08/06/07	<i>Litoria caerulea</i>	Young adult female	Townsville	Anasarca, anaemia Pulmonary <i>Rhabdias</i> infection
FDRQ 119	19/06/07	<i>Litoria infrafrenata</i>	Mature adult	Aeroglen Cairns	Systemic organ dysfunction, aetiology unknown
FDRQ 120	19/06/07	<i>Litoria infrafrenata</i>	Mature adult female	Freshwater Cairns	Injury
FDRQ 121	23/06/07	<i>Litoria infrafrenata</i>	Mature adult female	Cooktown	Emaciation <i>Spirometra erinacei</i> infection
FDRQ 122	03/07/07	<i>Litoria caerulea</i>	Juvenile	Townsville (captive)	Severe generalised metabolic bone disease
FDRQ 123	03/07/07	<i>Litoria caerulea</i>	Juvenile	Townsville (captive)	Severe generalised metabolic bone disease
FDRQ 124	03/07/07	<i>Litoria caerulea</i>	Juvenile	Townsville (captive)	Severe generalised metabolic bone disease
FDRQ 125	03/07/07	<i>Litoria caerulea</i>	Juvenile	Townsville (captive)	Severe generalised metabolic bone disease
FDRQ 126	03/08/07	<i>Litoria splendida</i>	Mature adult male	Gordonvale Cairns	Emaciation <i>Spirometra erinacei</i> infection

FDRQ 127	08/08/07	<i>Litoria nannotis</i>	Subadult	Mount Spurgeon	Chytridiomycosis
FDRQ 128	08/08/07	<i>Litoria genimaculata</i>	Subadult	Mount Spurgeon	Chytridiomycosis
FDRQ 129	08/08/07	<i>Litoria genimaculata</i>	Subadult	Kirrama	Chytridiomycosis
FDRQ 130	25/08/07	<i>Litoria infrafrenata</i>	Mature adult	Cairns	Emaciation <i>Spirometra erinacei</i> infection
FDRQ 131	12/09/07	<i>Litoria infrafrenata</i>	Mature adult	Cairns	Severe bacterial dermatopathy Nephropathy
FDRQ 132	21/09/07	<i>Litoria caerulea</i>	Mature adult	Townsville	Severe bacterial dermatopathy Pulmonary <i>Rhabdias</i> infection
FDRQ 133	25/09/07	<i>Litoria rheocola</i>	Juvenile male	Tully Gorge National Park	Suspect chytridiomycosis
FDRQ 134	25/09/07	<i>Litoria nasuta</i>	Mature adult female	Tully Gorge National Park	Injury
FDRQ 135	12/12/07	<i>Litoria caerulea</i>	Mature adult female	Townsville	Emaciation
FDRQ 136	05/02/08	<i>Litoria nannotis</i>	Juvenile male	Windin	Cause of death unknown; no significant histological findings
FDRQ 137	15/02/08	<i>Litoria caerulea</i>	Subadult female	Trinity Beach	Injury
FDRQ 138	19/02/08	<i>Litoria caerulea</i>	Mature adult male	Townsville	Hepatopathy, cause unknown
FDRQ 139 A B C D	27/02/08	<i>Litoria infrafrenata</i>	Tadpoles x 4	Cairns	Platyhelminth infection
FDRQ 140	30/03/08	<i>Litoria caerulea</i>	Mature adult female	Cairns (captive)	Suspect acute bacterial septicaemia
FDRQ 141	18/04/08	<i>Physignathus lesueurii</i>	Mature adult female	Clifton Beach	Emaciation <i>Spirometra erinacei</i> infection
FDRQ 142	23/04/08	<i>Litoria caerulea</i>	Juvenile	Clifton Beach	Injury
FDRQ 143	01/05/08	<i>Litoria caerulea</i>	Mature adult male	Cairns (captive)	Bacterial pneumonia
FDRQ 144	16/05/08	<i>Litoria caerulea</i>	Mature adult male	Cairns (captive)	Intracoelomic haemorrhage
FDRQ 145	16/05/08	<i>Litoria caerulea</i>	Mature adult male	Cairns (captive)	No significant histological findings
FDRQ 146	23/05/08	<i>Litoria nannotis</i>	Juvenile	Mount Spurgeon	Chytridiomycosis
FDRQ 147	23/05/08	<i>Litoria genimaculata</i>	Mature adult male	Mount Spurgeon	Chytridiomycosis
FDRQ 148	24/05/08	<i>Litoria caerulea</i>	Mature adult male	Cairns (captive)	Bacterial septicaemia

FDRQ 149	26/05/08	<i>Litoria caerulea</i>	Mature adult male	Cairns (captive)	Chytridiomycosis (mild) Leukopaenia (severe)
FDRQ 150	26/05/08	<i>Litoria caerulea</i>	Mature adult male	Cairns (captive)	Nephropathy, cause unknown
FDRQ 151	27/06/08	<i>Litoria nannotis</i>	Immature male	Mount Spurgeon	Chytridiomycosis
FDRQ 152	10/07/08	<i>Litoria caerulea</i>	Mature adult female	Cairns (captive)	Chytridiomycosis
FDRQ 153	14/07/08	<i>Litoria infrafrenata</i>	Mature adult female	Tully	Injury
FDRQ 154	23/07/08	<i>Litoria infrafrenata</i>	Mature adult female	Tully	Injury
FDRQ 155	18/03/09	<i>Litoria caerulea</i>	Mature adult female	Cairns	Bacterial septicaemia
FDRQ 156	18/03/09	<i>Mixophyes fleayi</i>	Mature adult male	Mount Cougal National Park	Hepatopathy, cause unknown
FDRQ 157 A B C D E	18/03/09	<i>Litoria spenceri</i>	Tadpoles x 5	Currumbin (captive)	Cause of death unknown, suspect toxic or nutritional
FDRQ 158	13/05/09	<i>Mixophyes fasciolatus</i>	Mature adult female	Currumbin (captive)	Cause of death unknown
FDRQ 159	14/05/09	<i>Bufo marinus</i>	Mature adult male	Currumbin	No significant histological findings
FDRQ 160	20/06/09	<i>Litoria caerulea</i>	Mature adult male	Kuranda	Injury

## **APPENDIX 2**

### **Supporting online material**

Appendix 2 contains supporting online material for the published peer-reviewed paper in section 7.3:

Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W. F., Dinudom, A., Cook, D., Webb, R., Alford, R. A., Skerratt, L. F. & Speare, R. (2009). Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science* 326: 582-585.

Since the journal uses American English, spelling follows this convention.

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Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W.  
F., Dinudom, A., Cook, D., Webb, R., Alford, R. A., Skerratt,  
L. F. & Speare, R. (2009). Pathogenesis of chytridiomycosis,  
a cause of catastrophic amphibian declines. *Science* 326:  
582-585.































## **APPENDIX 3**

### **Consent statements from co-authors**

## **Co-author Consent to Include Jointly Authored Papers in the Thesis**

**Thesis Title:** *Emerging Amphibian Diseases in Queensland and Host Immune Response to Disease*

**Name of Candidate:** Samantha Young

### **CHAPTER 3 PAPER 1 / APPENDIX 1**

#### **Publication Details**

Young, S., Speare, R., Berger, L., Skerratt, L. F. & Mendez, D. (2010). Emerging Amphibian Diseases and Disease Surveillance in Queensland – Stage 2 (February 2007 – April 2010). Final report for the project Emerging Amphibian Diseases and Disease Surveillance in Queensland – Stage 2 (February 2007 – April 2010) to the Australian Government Department of the Environment, Water, Heritage and the Arts.

#### **Nature and Extent of the Intellectual Input of Each Author**

This published report is the original writing of Samantha Young, the primary investigator, with minor editorial contributions from Rick Speare, Lee Berger, Lee Skerratt and Diana Mendez. Samantha Young performed the majority of the surveillance work and specimen collection, and all of the clinical examinations, physical data collection, post mortem examinations and diagnostic sample collection. Rick Speare, Lee Berger and Lee Skerratt gave advice on study design and results interpretation throughout the research. Rick Speare, Lee Berger and Diana Mendez assisted with histological analysis.

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**I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis.**

**Name:** Rick Speare

**Signature:**

**Name:** Lee Berger

**Signature**

**Name:** Lee Skerratt

**Signature:**

**Name:** Diana Mendez

**Signature:**

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## **Co-author Consent to Include Jointly Authored Papers in the Thesis**

**Thesis Title:** *Emerging Amphibian Diseases in Queensland and Host Immune Response to Disease*

**Name of Candidate:** Samantha Young

### **CHAPTER 3 – PAPER 2**

#### **Publication Details**

Young, S., Skerratt, L. F., Mendez, D., Speare, R., Berger, L. & Steele, M. (2012). Using community surveillance data to differentiate between emerging and endemic amphibian diseases. *Diseases of Aquatic Organisms* 98: 1-10.

#### **Nature and Extent of the Intellectual Input of Each Author**

This published paper represents the original data organisation, classification, analysis and writing of Samantha Young, the primary investigator. Rick Speare, Lee Skerratt and Lee Berger assisted with data interpretation. Diana Mendez performed the histological analysis. Mike Steele performed the original statistical analysis and Samantha Young performed additional revised analyses with advice from Lee Skerratt. Lee Berger, Lee Skerratt, Diana Mendez and Rick Speare provided substantial editorial input, Diana Mendez wrote the histology results section, and Mike Steele wrote the majority of the statistical methods section.

---

**I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis.**

**Name:** Lee Skerratt

**Signature:**

**Name:** Diana Mendez

**Signature:**

**Name:** Rick Speare

**Signature:**

**Name:** Lee Berger

**Signature:**

**Name:** Mike Steele

**Signature:**

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## Co-author Consent to Include Jointly Authored Papers in the Thesis

**Thesis Title:** *Emerging Amphibian Diseases in Queensland and Host Immune Response to Disease*

**Name of Candidate:** Samantha Young

### CHAPTER 3 – PAPER 2

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Young, S., Skerratt, L. F., Mendez, D., Speare, R., Berger, L. & Steele, M. (2012). Using community surveillance data to differentiate between emerging and endemic amphibian diseases. *Diseases of Aquatic Organisms* 98: 1-10.

#### **Nature and Extent of the Intellectual Input of Each Author**

This published paper represents the original data organisation, classification, analysis and writing of Samantha Young, the primary investigator. Rick Speare, Lee Skerratt and Lee Berger assisted with data interpretation. Diana Mendez performed the histological analysis. Mike Steele performed the original statistical analysis and Samantha Young performed additional revised analyses with advice from Lee Skerratt. Lee Berger, Lee Skerratt, Diana Mendez and Rick Speare provided substantial editorial input, Diana Mendez wrote the histology results section, and Mike Steele wrote the majority of the statistical methods section.

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**I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis.**

**Name:** Lee Skerratt                      **Signature:**

**Name:** Diana Mendez                      **Signature:**

**Name:** Rick Speare                      **Signature:**

**Name:** Lee Berger                      **Signature:**

**Name:** Mike Steele                      **Signature:**

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**Thesis Title:** *Emerging Amphibian Diseases in Queensland and Host Immune Response to Disease*

**Name of Candidate:** Samantha Young

### **CHAPTER 3 – PAPER 3**

#### **Publication Details**

Phillot, A. D. & Young, S. (2009). Occurrence of cloacal prolapse in wild hylids in the Wet Tropics, Australia. *Diseases of Aquatic Organisms* 86: 77-80.

#### **Nature and Extent of the Intellectual Input of Each Author**

This published paper represents original surveillance data collection, analysis and writing by Andrea Phillott, the primary investigator. Samantha Young provided veterinary technical expertise for results interpretation and substantial written contributions to the introduction and discussion sections.

---

**I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis.**

**Name:** Andrea Phillott

**Signature:**

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## Co-author Consent to Include Jointly Authored Papers in the Thesis

**Thesis Title:** *Emerging Amphibian Diseases in Queensland and Host Immune Response to Disease*

**Name of Candidate:** Samantha Young

### CHAPTER 4 – PAPER 1

#### **Publication Details**

Young, S., Warner, J., Speare, R., Berger, L., Skerratt, L. F. & Muller, R. Hematologic and plasma biochemical reference intervals for health monitoring of wild Australian tree frogs. *Veterinary Clinical Pathology* (In press).

#### **Nature and Extent of the Intellectual Input of Each Author**

This paper represents the original research and writing of Samantha Young, the primary investigator. Rick Speare, Lee Berger and Lee Skerratt provided study design advice, Jeff Warner provided technical methodology advice and cell identification assistance, and Reinhold Muller performed all of the statistical analyses. Jeff Warner, Lee Berger and Lee Skerratt provided substantial editorial contributions, and Reinhold Muller assisted with writing the statistical analysis sections.

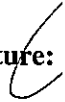
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**I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis.**

**Name:** Jeff Warner

**Signature:**

**Name:** Rick Speare

**Signature:** 

**Name:** Lee Berger

**Signature:**

**Name:** Lee Skerratt

**Signature:**

**Name:** Reinhold Muller

**Signature:**

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### **CHAPTER 4 – PAPER 1**

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**Name:** Jeff Warner                      **Signature:**

**Name:** Rick Speare                      **Signature:**

**Name:** Lee Berger                      **Signature:**

**Name:** Lee Skerratt                      **Signature:**

**Name:** Reinhold Muller                      **Signature:**

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#### **Nature and Extent of the Intellectual Input of Each Author**

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**I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis.**

**Name:** Jeff Warner                      **Signature:**

**Name:** Rick Speare                      **Signature:**

**Name:** Lee Berger                      **Signature**

**Name:** Lee Skerratt                      **Signature:**

**Name:** Reinhold Muller                      **Signature:**

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## Co-author Consent to Include Jointly Authored Papers in the Thesis

**Thesis Title:** *Emerging Amphibian Diseases in Queensland and Host Immune Response to Disease*

**Name of Candidate:** Samantha Young

### CHAPTER 5 – PAPER 1

#### **Publication Details**

Young, S., Whitehorn, P., Berger, L., Skerratt, L. F., Speare, R., Garland, S. & Webb, R. *Batrachochytrium dendrobatidis* suppresses immune function in tree frogs. *Diseases of Aquatic Organisms* (submitted).

#### **Nature and Extent of the Intellectual Input of Each Author**

This paper represents the original research and writing of Samantha Young, the primary investigator. Rick Speare, Lee Berger and Lee Skerratt provided study design advice. Paul Whitehorn assisted with field collection of frogs, along with all husbandry and some experimental procedures. Stephen Garland ran the PCR analyses and Rebecca Webb processed the tissue samples for histology. Samantha Young performed all of the statistical analyses, with advice from Lee Skerratt. Lee Berger and Lee Skerratt provided substantial editorial input.

---

**I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis.**

**Name:** Paul Whitehorn                      **Signature**

**Name:** Lee Berger                              **Signature:**

**Name:** Lee Skerratt                              **Signature:**

**Name:** Rick Speare                              **Signature:**

**Name:** Stephen Garland                              **Signature:**

**Name:** Rebecca Webb                              **Signature:**

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## Co-author Consent to Include Jointly Authored Papers in the Thesis

**Thesis Title:** *Emerging Amphibian Diseases in Queensland and Host Immune Response to Disease*

**Name of Candidate:** Samantha Young

### CHAPTER 6 – PAPER 1

#### **Publication Details**

Young, S., Whitehorn, P., Garland, S., Speare, R., Skerratt, L. F., Berger, L. & Voyles, J. Chytridiomycosis makes frogs more susceptible to reinfection. *Research in Veterinary Science* (submitted).

#### **Nature and Extent of the Intellectual Input of Each Author**

This paper represents the original research and writing of Samantha Young, the primary investigator. Rick Speare, Lee Berger and Lee Skerratt provided study design advice. Paul Whitehorn assisted with all husbandry and many experimental procedures. Paul Whitehorn and Jamie Voyles assisted with field collection of frogs. Stephen Garland ran the PCR analyses. Samantha Young performed all of the statistical analyses, with advice from Lee Skerratt. Rick Speare, Jamie Voyles, Lee Skerratt and Lee Berger assisted with interpretation of results. Lee Berger and Lee Skerratt provided substantial editorial input.

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**I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis.**

**Name:** Paul Whitehorn      **Signature:**

**Name:** Stephen Garland      **Signature:**

**Name:** Rick Speare      **Signature:**

**Name:** Lee Berger      **Signature:**

**Name:** Lee Skerratt      **Signature:**

**Name:** Jamie Voyles      **Signature:**

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## Co-author Consent to Include Jointly Authored Papers in the Thesis

**Thesis Title:** *Emerging Amphibian Diseases in Queensland and Host Immune Response to Disease*

**Name of Candidate:** Samantha Young

### CHAPTER 7 – PAPER 1

#### **Publication Details**

Voyles, J., Berger, L., Young, S., Speare, R., Webb, R., Warner, J., Rudd, D., Campbell, R. & Skerratt, L. F. (2007). Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis. *Diseases of Aquatic Organisms* 77: 113-118.

#### **Nature and Extent of the Intellectual Input of Each Author**

This published paper represents the original research and writing of Jamie Voyles and Lee Berger, the primary investigators. Samantha Young provided veterinary technical expertise for methodology and results interpretation, and substantial intellectual and editorial contributions to the methods, results and discussion sections.

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**I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis.**

**Name:** Jamie Voyles                      **Signature:**

**Name:** Lee Berger                        **Signature**

**Name:** Rick Speare                      **Signature:**

**Name:** Rebecca Webb                **Signature:**

**Name:** Jeff Warner                      **Signature:**

**Name:** Donna Rudd                    **Signature:**

**Name:** Ruth Campbell                **Signature:**

**Name:** Lee Skerratt                    **Signature:**

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**Name:** Jamie Voyles                      **Signature:**

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**Thesis Title:** *Emerging Amphibian Diseases in Queensland and Host Immune Response to Disease*

**Name of Candidate:** Samantha Young

### **CHAPTER 7 – PAPER 2**

#### **Publication Details**

Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W. F., Dinudom, A., Cook, D., Webb, R., Alford, R. A., Skerratt, L. F. & Speare, R. (2009). Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science* 326: 582-585.

#### **Nature and Extent of the Intellectual Input of Each Author**

This published paper represents the original research and writing of Jamie Voyles, the primary investigator. The role of Samantha Young in the paper included: surgical implantation of the cardiac bio-transmitters in experimental frogs, blood biochemistry and urine sediment sample processing, provision of veterinary technical expertise for methodology and results interpretation, and substantial intellectual and editorial contributions to the methods, results and discussion sections.

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**Name:** Anuwat Dinudom                      **Signature:**

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**Name:** Jamie Voyles                      **Signature:**

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**Name:** Rick Speare                      **Signature:**

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## Co-author Consent to Include Jointly Authored Papers in the Thesis

**Thesis Title:** *Emerging Amphibian Diseases in Queensland and Host Immune Response to Disease*

**Name of Candidate:** Samantha Young

### CHAPTER 8 – PAPER 1

#### **Publication Details**

Young, S., Berger, L. & Speare, R. (2007). Amphibian chytridiomycosis: strategies for captive management and conservation. *International Zoo Yearbook* 41: 85-95.

#### **Nature and Extent of the Intellectual Input of Each Author**

This paper is the original writing of Samantha Young. Lee Berger and Rick Speare provided substantial intellectual and editorial contributions.

---

**I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis.**

**Name:** Lee Berger

**Signature** 

**Name:** Rick Speare

**Signature:**

---

## Co-author Consent to Include Jointly Authored Papers in the Thesis

**Thesis Title:** *Emerging Amphibian Diseases in Queensland and Host Immune Response to Disease*

**Name of Candidate:** Samantha Young

### CHAPTER 8 – PAPER 2

#### **Publication Details**

Young, S., Speare, R., Berger, L. & Skerratt, L. F. (2012). Chloramphenicol with fluid and electrolyte therapy cures terminally ill green tree frogs (*Litoria caerulea*) with chytridiomycosis. *Journal of Zoo and Wildlife Medicine* 43: 330-337.

#### **Nature and Extent of the Intellectual Input of Each Author**

This published paper is the original research and writing of Samantha Young, the primary investigator. Rick Speare provided substantial study design advice, and Lee Berger and Lee Skerratt provided substantial editorial contributions.

---

**I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis.**

**Name:** Rick Speare

**Signature:**

**Name:** Lee Berger

**Signature**

**Name:** Lee Skerratt

**Signature**

---