# Diseases in Australian Frogs

Thesis submitted by

Lee BERGER

BVSc (*Uni Melb*)

in October 2001

for the degree of Doctor of Philosophy

in the School of Public Health and Tropical Medicine

James Cook University

# STATEMENT OF ACCESS

I, the undersigned author of this work, understand that James Cook University will make this thesis available for use within the University Library and, via the Australian Digital Theses network, for use elsewhere.

I understand that, as an unpublished work, a thesis has significant protection under the Copyright Act and;

I do not wish to place any further restriction on access to this work

_____ _____

Signature Date

## STATEMENT ON SOURCES

## ***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 Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

…….. ……

(Signature) (Date)

## ELECTRONIC COPY

I, the undersigned, the author of this work, declare that the electronic copy of this thesis provided to the James Cook University Library, is an accurate copy of the print thesis submitted, within the limits of the technology available.

_____ _____

Signature Date

# ABSTRACT

The aims of this project were to investigate the diseases present in Australian amphibians and to determine the cause of the mass mortalities in wild frogs in Queensland that had resulted in population declines.

Initially diagnostic pathology methods were used to examine endangered frogs collected from a mass die-off in north Queensland, as well as to survey other frogs for disease. The survey was achieved using a collaborative approach involving herpetologists, ecologists and other scientists around Australia who submitted sick and dead frogs for testing. Epidermal infections of a new species of chytrid fungus, *Batrachochytrium dendrobatidis*, were consistently found in frogs during mass mortalities in the wild and in captivity, including some of the last individuals of *Taudactylus acutirostris*. Detailed investigations of this pathogen included experimental transmissions, pathology, studies on the distribution in adults and tadpoles, epidemiology, and examination of the morphology and lifecycle. Further work was aimed at developing diagnostic tests, and treatment and disinfection protocols.

A wide range of diseases were diagnosed in frogs submitted for the disease survey, including those caused by *Mucor amphibiorum*, *Spirometra erinacei*, *Aphanomyces* sp., *Chlamydia pneumoniae*, and neoplasms. Vacuolating and ulcerative dermatoses also occurred but the aetiology was not determined. However, the only disease that was common and had an obvious impact on the abundance of frogs was chytridiomycosis, which accounted for the deaths of 56.5% of frogs submitted.

*B. dendrobatidis* was found to be widespread across Australia with a broad host range, and caused disease in 35 native Myobatrachid and Hylid species as well as the Bufonid, *Bufo marinus*. The incidence of chytridiomycosis showed a distinct seasonal effect with most frogs dying in winter. Most infected frogs were submitted from Queensland and New South Wales, with less from Victoria, South Australia and Western Australia. Large outbreaks occurred regularly in *Litoria caerulea* around Brisbane. Diseased frogs were from a variety of habitats at high and low altitudes.

Sporangia of *B. dendrobatidis* grow within cells of the *stratum granulosum* and *stratum corneum*. As the fungus matures it is carried outwards with the epidermal cell. The entire contents of sporangia cleave into flagellated zoospores that are released from discharge tubes which protrude through the skin surface. Chytridiomycosis occurs as an extensive infection of the ventral skin and feet resulting in hyperkeratosis, hyperplasia, erosions, focal necrosis, and occasional ulceration of the epidermis, with minimal inflammation in the skin. Ultrastructural pathology demonstrated that infection of the epidermis stimulated an increased turnover of epidermal cells leading to hyperplasia.

Initially disease was transmitted experimentally by exposing frogs to infected skin scrapings, and then once pure cultures of *B. dendrobatidis* were established, frogs were exposed to zoospores. Experimental infections in *Mixophyes fasciolatus* and *L. caerulea* at between 17°C and 24°C resulted in 100% mortality. Deaths (or terminal illness requiring euthanasia) occurred 9 - 76 days after exposure to the fungus, with most frogs dying between 18 and 48 days. Doses as low as 100 zoospores resulted in the death of 3/3 *M. fasciolatus*, while 3 frogs each given 10 zoospores did not succumb. At 27°C, 4/8 experimentally infected *M. fasciolatus* died while the rest remained healthy. Infection was confirmed in 3 of these, but was eliminated by 98 days. Attempts to infect *Bufo marinus* and *Limnodynastes peronii* were not successful.

Healthy tadpoles could apparently carry infections in their mouthparts from soon after hatching until metamorphic climax when sporangia were rapidly redistributed to the skin of the body as the beaks were shed. The infected metamorphs died at about 2 - 3 weeks old. The distribution of sporangia in tadpoles and metamorphs during development followed the changes in the distribution of keratin.

Accurate testing of sick frogs was achieved using histology or examination of skin scrapings. Histological examination of toe-clips was useful for ante mortem testing of healthy frogs, but was found to have low sensitivity (52.7%). Polyclonal antibodies were generated to *B. dendrobatidis* by inoculating rabbits and sheep. Although these antibodies cross-reacted with other chytridiomycetes, they were used in an

immunoperoxidase test that increased sensitivity of diagnosis (61.8%) and ease of interpretation.

A wide range of antifungal drugs and compounds were effective against *B. dendrobatidis in vitro*, but when fluconazole and benzalkonium chloride were selected for testing in an animal trial they were found to be ineffective for treating infected frogs. Sporangia and zoospores were sensitive to three common disinfectants when used at routine levels - 70% ethanol, 0.1% benzalkonium chloride and 0.1% Virkon. Cultures were also killed by drying for an hour, and by incubating at temperatures at 32°C or above.

Chytridiomycosis is a highly pathogenic emerging infectious disease that has caused mass mortality of wild frogs leading to population declines and extinctions in protected montane rainforest areas. The most plausible explanation for the extreme susceptibility of some amphibian species giving rise to unsustainably high mortality rates is that they have not co-evolved with this pathogen, and that *B. dendrobatidis* was introduced to Australia and has spread through a naïve population.

# ACKNOWLEDGEMENTS

I am forever grateful to Rick Speare for giving me the opportunity to work on frog disease and for his continuous inspiration, generosity and support that has made so many things possible. I thank Alex Hyatt for his enthusiastic encouragement and the opportunity to work in his fantastic laboratory, and for his guidance while introducing me to the world of electron microscopy. It was great collaborating with herpetologists whose concern for the frogs was always the highest priority. Gerry Marantelli and the Amphibian Research Centre's unique and generous contributions and ideas were indispensable to the project, especially the animal experiments. The tremendous support from the start by Harry Hines was responsible for the outcomes of the disease survey, by finding many of the sick frogs and taking the time to investigate outbreaks reported by the public and collate information. I thank Keith McDonald for his role in initiating the project, which arose out of his dedicated research and monitoring of declining populations. I'm also indebted to many other froggers who contributed their time and skills, especially Craig Williams, John Clarke, Mike Tyler, Raelene Hobbs, Nick Sheppard, David Page, Graeme Gillespie, Michael Mahony, Michelle Christy, Lance Tarvey, Monica Wangmann, Paul O'Calloghan, Marc Hero, Kathy Taylor, Jenny Holdway, D. Stewart, Richard Retallick, Lothar Voigt, and everyone else listed in the thesis who sent in specimens. Joyce Longcore helped enormously by sharing her mycological findings on *B. dendrobatidis*. We were lucky that someone as generous as her described the frog chytrid and had such a crucial role. Thanks to everyone in the electron microscopy lab: Veronica Olsen for her hard work and care of the frogs in the last experiment, Helen Parkes for helpful discussions, Sandy Crameri and Terry Wise for advice and help with EM and photography, and Donna Boyle for attempting virus isolations. The histology lab made me welcome. Thanks to Megan Braun for preparing a huge number of excellent histology slides and for her immense wit, Gail Russell for her good advice and tolerance, and Peter Hooper, Deborah Middleton and Mark Williamson for sharing their pathology expertise. Thanks also to all those who gave advice, identified parasites and collaborated on various projects, including Peter Daszak, Louise Goggin and Mark Ragan for their groundbreaking work on identification of the frog chytrid, as well as Annette Thomas, Frank Mutschmann, Di Barton, Andrew Cunningham, Jess Morgan, Peter O'Donoghue, David E. Green, Norman Cheville, Robin Gasser, Xingquan Zhu and Peter Timms. Frank Filippi did

fantastic frog photography and was a huge help in preparing the figures. Thanks to Julia Hammond for bacteriology and for listening. The library staff at AAHL were very helpful. Thanks to Peter Kavenagh for his patience and assistance with setting up the frog disease database. Thanks to John Humphrey for helping me get started. Gary Rowe and Trevor Kelly in the stores always located the frogs that lost their way. John Muschialli and Don Carlson helped set up the animal rooms. Leigh Keegan assisted with tadpole pathology. Bruce Parry generously did some haematological analysis. Kate Steinmann translated some French publications and was a great help with the database. Marilyn Skerratt kindly proof-read the whole thesis in 24 hours. I thank Kerry Kelly and the whole Speare family for their hospitality in Townsville over the years. Thanks to Emma Homes and Stanley Isaiah for the food and exercise. Environment Australia via Queensland Parks and Wildlife Service with guardianship by Keith McDonald provided funding throughout the project.

I'm forever grateful to Linda Berger, Clive Berger, Karen Berger and Dawn Powell for their love and for taking care of me. I thank Lee Skerratt for a great many things, but in general because I would not have even started this without him.

# PREFACE

All procedures reported in the thesis received the approval of the AAHL Ethics Committee or the Amphibian Research Centre Ethics Committee.

Some of the research required multidisciplinary skills, and was conducted in collaboration or consultation with other scientists. In general, my supervisors Rick Speare and Alex Hyatt gave advice on the design of the research, in interpretation of results and in preparing the thesis. People who gave technical assistance are thanked in the acknowledgements. The specific scientific contributions of others to each chapter are summarised below.

CHAPTER 4

Electron microscopy was performed in collaboration with Alex Hyatt and Sandra Crameri. Although I took most of the photos, operation of the microscopes and preparation and processing of samples was a joint effort. Alex Hyatt processed the samples that were freeze-substituted, and did the work at Adelaide University on freeze-fractured samples. Sandra Crameri evaluated different buffers for fixing cultured fungi. Joyce Longcore commented on a draft of this chapter.

CHAPTER 5

Norman Cheville was consulted for interpretation of the ultrastructural pathology, and Lee Skerratt advised on the statistical analysis in the study on distribution of infection. Helen Parkes first noticed possible fungal sporangia in the mouthparts of tadpoles, while examining *Bufo marinus*.

CHAPTER 6

This chapter consists of two multi-authored papers. The paper on histological diagnosis (Berger et al., 2000) was written with Rick Speare and Andrew Kent based on shared observations. The paper on production of polyclonal antibodies (Berger et al., 2001) included advice from Alex Hyatt, immunoperoxidase staining by Veronica Olsen, gold labelling by Sandra Crameri, fluorescence staining by Donna Boyle, a frog transmission

experiment conducted with Gerry Marantelli, and utilised various fungi isolated by Joyce Longcore and Kaye Humphreys.

CHAPTER 7

Four of the seven transmission experiments were conducted at the Amphibian Research Centre (ARC). For these experiments, I prepared the zoospores, infected the frogs and did the diagnostic work, while husbandry, observations, euthanasia and preservation of samples were conducted by Gerry Marantelli, Raelene Hobbs and other staff at the ARC. Lee Skerratt advised on the statistics.

CHAPTER 8

This chapter describes frogs with chytridiomycosis collected during the disease survey (see Chapter 10). The most significant frogs were collected from Big Tableland by Keith McDonald, Rick Speare, Kelly Field and Andrew Dennis in 1993, and were necropsied by Rick Speare. Harry Hines commented on a draft of this chapter and Vivienne Lewis advised on the statistics.

CHAPTER 9

The treatment trial was conducted at the ARC in collaboration with Gerry Marantelli, Raelene Hobbs and other staff.

CHAPTER 10

The survey of frogs for disease involved over 50 herpetologists around Australia. The most active collectors are included in Chapter 3, and everyone involved is listed in Appendix 1 with details of the frogs submitted for testing. Harry Hines collected the most frogs and investigated many reports of mortality from the public. Various experts were consulted for identification of parasites by PCR or morphological examination and are referred to in the results section, as well as in Chapter 3. Peter Daszak originally identified *B. dendrobatidis* as a chytrid. At AAHL, assistance with the search for viruses was provided by Donna Boyle who attempted virus isolations on specimens submitted after 1997, and Jacqui Kattenbelt performed the ranavirus PCR.

CHAPTER 11

For the paper on chlamydiosis (Berger et al., 1999b), identification of *Chlamydia pneumoniae* by PCR was performed by Peter Timms, Kym Volp and Sarah Mathews. Rick Speare made the initial presumptive diagnosis of chlamydiosis based on histology.

CHAPTER 12

This chapter is comprised of four multi-authored papers. The description of fungal disease in cane toad tadpoles (Berger et al., 2001) involves tadpoles that were collected and described grossly by Rick Speare, and mycological culture by Annette Thomas. Alex Hyatt assisted and advised on the electron microscopy. John Humphrey helped with the post mortem in the case report on mucormycosis in a green treefrog (Berger et al., 1997). For the other two papers I was not the primary investigator. For Speare et al. (1997), most of the work was done by Rick Speare. I contributed results from three post mortems, took the photos and assisted with preparing the manuscript for publication. For Creeper et al., (1998), my role was minor and I helped with the diagnosis and in writing the paper.

CHAPTER 13

For the manuscript on spargana, Lee Skerratt identified many of the parasites. The section on metacercariae includes tadpoles collected by Harry Hines, and the metacercariae were identified by Di Barton.

CHAPTER 14

Rick Speare and Deborah Middleton assisted with diagnosis and description of the neoplasms.

CHAPTER 15

Rick Speare identified the iridophores and Frank Mutschmann contributed the results on Tanzanian frogs.

# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES