FINAL REPORT FOR THE AUSTRALIAN GOVERNMENT DEPARTMENT OF THE ENVIRONMENT AND WATER RESOURCES

Emerging amphibian diseases and disease surveillance in Queensland – Stage 1 (January 2006 – January 2007)

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Cover images: left - photo of a healthy mature adult *Litoria infrafrenata* by S. Young; right – photo of a mature *Litoria infrafrenata* affected by the wasting syndrome, a previously undescribed disease syndrome affecting tree frogs in far northern Queensland, by S. Young.

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Australian Government

Department of the Environment and Water Resources

Executive Summary

The aim of this project was to commence investigations into Emerging Amphibian Diseases and Disease Surveillance in Queensland, a three-year research programme being carried out by the Amphibian Disease Ecology Group at James Cook University. Stage 1 of this project has three components, as outlined below. All research activities were carried out under an approved Queensland Parks and Wildlife Service Scientific Purposes Permit (WISP03866106) and James Cook University Animal Ethics Application (A1085).

Declines and extinctions of amphibian populations have been increasing globally over the past three decades. Over 30 percent of amphibian species are threatened and at least 43 percent are experiencing population declines (IUCN, 2001, 2004; Stuart *et al*., 2004). Since 1980, rapid declines have been reported in over 400 species, 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). Many of these declines have now been linked to the emerging infectious disease, chytridiomycosis, of which the impact on frog populations is thought to represent the most spectacular loss of biodiversity resulting from disease in recorded history (Berger *et al*., 1998; Bosch *et al*., 2001; Carey *et al*., 2003; Daszak *et al*., 2003; Lips *et al*., 2006; Schloegel *et al*., 2006).

Community wildlife care groups exist in many countries throughout the world for the purpose of 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. The Frog Decline Reversal Project runs the Cairns Frog Hospital (CFH), a small, non-profit community wildlife group which has been receiving injured and diseased amphibians from the public since 1998. During this time, information has been collected about cases (including photographic and geographic details), individuals have been treated with a view towards recovery and return to the wild, and limited diagnostic pathology has been carried out. When the CFH first started to consult experts in amphibian disease, the early evidence clearly demonstrated that several of the disease syndromes present had not been seen or documented previously. The most important of these syndromes awaiting investigation is an immunodeficiency-like wasting syndrome in the white-lipped tree frog, *Litoria infrafrenata*, which appears to have had a major impact on the status of this species in northern Queensland. There has been previous collaboration between the CFH and the Amphibian Disease Ecology Group at James Cook University, Townsville, for amphibian disease investigation. The CFH represents a model for passive community surveillance of amphibian diseases in northern Queensland.

Objectives outlined in the Funding Agreement

1. To determine the aetiology of the wasting syndrome in *Litoria caerulea* and *L. infrafrenata*, whether this disease has extended outside Cairns, and to assess its significance to amphibian populations in the wet tropics.

2. To determine what diseases occur in peri-urban amphibians in the wet tropics and whether any are undescribed.

3. To develop suitable techniques for wildlife care groups to collect disease data that is relevant for surveillance for emerging diseases, and to determine how this data can be transmitted in a cost-effective way to the Australian Wildlife Health Network using the Cairns Frog Hospital as a model for the surveillance of amphibian disease.

Investigating new and emerging amphibian diseases in Queensland, with a particular focus in the region of the port city of Cairns, will further the knowledge base regarding amphibian diseases and the risks they pose to amphibian populations globally. Identification of new wildlife diseases in the Cairns region is of particular importance since entry of emerging diseases into countries often occurs through ports. Evaluating disease surveillance techniques and integration of community surveillance into the Australian Wildlife Health Network (AWHN) will be of benefit to 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 such as the CFH to deal with amphibian diseases, and the ability of the AWHN to monitor and diagnose important and emerging diseases affecting these species.

This research is significant to conservation and is an important contribution to the field of wildlife disease investigation and management. It is an opportunity to study emerging diseases that may be relevant to global efforts to preserve amphibians. Recently, another emerging 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 will help maintain this position.

Summary of Progress Towards Meeting the Objectives

Objective 1. To determine the aetiology of the wasting syndrome in Litoria caerulea and L. infrafrenata, whether this disease has extended outside Cairns, and to assess its significance to amphibian populations in the wet tropics.

Preliminary evidence suggests that an undocumented disease involving immune deficiency, with a primary clinical presentation of wasting (emaciation), has caused significant decline in free-ranging *L. infrafrenata* populations in far northern Queensland. In a number of cases, severe infections with secondary pathogens, particularly the tapeworm *Spirometra erinacei*, are present. Populations appear to be affected throughout the entire range of *L. infrafrenata*, indicating that a population reduction sufficient to render the species endangered is possible, particularly should other emerging pathogens occur concurrently. To research this disease syndrome, investigations into the function of the immune system in these species, along with extensive diagnostic, pathological and epidemiological investigations, are needed. Many of these tests have not been used previously in Australian frog species.

Laboratory and field studies have been developed in mammalian and avian species as measures of immune structure and function. Commonly used methods include assessment of immune organs, total and differential peripheral white blood cell counts and serum protein concentrations, and a range of more complex *in vivo* and *in vitro* tests (Horton *et al*., 1976; Rollins-Smith & Cohen, 1982; Gearing *et al*., 1984; Hsu & Du Pasquier, 1984; Rollins-Smith *et al*., 1984; Zettergren *et al*., 1991; Rollins-Smith & Blair, 1993; Whittington & Speare, 1996; Whittington *et al*., 1997; Zupanovic *et al*., 1998; Work *et al.*, 2001; Grasman, 2002; Rosenberg *et al.*, 2002; Gantress *et al*., 2003; Berger *et al*., 2005; Burnham *et al.*, 2005; Kinney & Cohen, 2005). Although there has been much progress made in understanding innate and acquired immunity in many vertebrates (Du Pasquier & Flajnik, 1999), little is known about the mechanisms of defense against viral and fungal pathogens that have been causally implicated in global amphibian population and species declines (Berger *et al*., 1999; Carey *et al*., 1999; Daszac *et al*., 1999; Robert *et al*., 2005). There are no reports describing acquired immunity in *Litoria* species.

To date, over 100 frog specimens have been collected through the passive amphibian disease surveillance system established at JCU as a direct result of this project, and via Cairns Frog Hospital (CFH) submissions. Twenty five percent of specimens (26/102) have been affected with the wasting syndrome. A number of wild *L. infrafrenata* specimens have been submitted directly to JCU with the wasting syndrome and thorough physical examination, necropsy and sample collection protocols have been established and used for each case. A large range of diagnostic samples has been collected from each case and analyses have been carried out including haematology, serum biochemistry, and parasite identification. Formalin-fixed tissues have been processed from each case and histological analysis has been carried out and will continue throughout Stage 2 of the project. Similarly, a range of tissue samples from each specimen has been frozen for future diagnostic testing. Some specimens have been received from the CFH since the project commenced, but the diagnostic value of these frogs has been severely limited due to confounding factors including prior treatment with various medications and the effects of long-term captivity. Nevertheless, these cases have been processed for a thorough diagnostic work-up where possible.

A large component of this project to date has involved sourcing equipment and setting up and

validating various diagnostic techniques for use in amphibians, including routine haematology and serum biochemistry analyses and immunological testing. With the recent exception of investigating the emerging infectious disease, chytridiomycosis, little research has been carried out on diseases of Australian frogs. In order to thoroughly investigate an unknown disease syndrome, baseline values for various diagnostic tests in healthy individuals of the same species must be determined. The majority of published reports on amphibian haematology have little clinical relevance due to the wide range of reported normal values resulting from variations in sampling techniques, sampling conditions, restricted sample size, analytical techniques, physiological state, gender, season and unrecognised pathologies (Wright, 2001). Few clinical reports based on controlled studies of normal values for anurans exist and there are no baseline values published for *Litoria* species. Amphibian biochemistry values have received little attention in the literature, with the exception of plasma glucose levels which vary widely in the northern leopard frog *(Rana pipiens)* due to 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).

Extensive preliminary work has been done in determining baseline values for a range of diagnostic tests. A VetScan VS2^{TM} Chemistry Analyser has been purchased and validated for use in Australian tree frogs (*Litoria* species), allowing a range of serum biochemistry analytes to be measured using only a very small volume (0.1 ml) of blood (Figures 1 and 2). This is the first known use of this machine in amphibians in Australia. Without the VetScan, we would not have been able to measure nearly as many blood parameters (each of which provides valuable information about the health status of the individual) due to limitations associated with the small sample volume that can be collected from frogs. Future research will involve the use of these measurements as an aid in disease diagnosis.

Figure 1. The compact in-house VetScan VS2^{TM} Chemistry Analyser installed and running in the laboratory at James Cook University. The blood sample is loaded into the rotor and then the rotor is placed in the drawer. Sample analysis takes approximately 8 minutes, at the end of which a print out listing the values for 12 individual analytes is produced. Photo by S. Young.

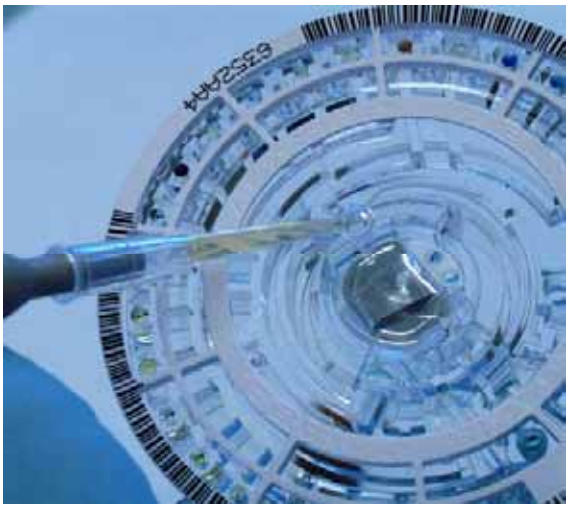

Figure 2. Loading a blood serum sample into the VetScan VS2™ Chemistry Analyser rotor. Note the extremely small sample size required (100 microlitres, or 0.1 ml), allowing the full panel of analytes to be run even with the small sample volumes collected from frogs. Photo by S. Young.

A retrospective spatial and temporal analysis of CFH submission data from January 1999 to December 2004 has been carried out, the results of which will be submitted in a manuscript *Community Surveillance for Amphibian Diseases in Northern Queensland, Australia* to the journal EcoHealth (currently in preparation). This manuscript not only reports results from an extensive analysis of the CFH amphibian disease surveillance data, but also forms a preliminary scientific paper documenting the wasting syndrome as a previously unidentified disease syndrome in tree frogs in far northern Queensland, establishing a detailed case definition and quantifying the range of parasite infections present in affected individuals. The results from this analysis are summarised below.

We obtained submission data from the CFH cases over a six-year period, from January 1999 through to December 2004. *Litoria infrafrenata* cases were classified according to information recorded about presenting signs, origin and season. There were four categories for the primary reason for presentation based on physical examination: injury, sparganosis (infection with the cestode *Spirometra erinacei*), emaciation (irrespective of concurrent sparganosis), and miscellaneous (e.g. dermatitis, neoplasia, healthy frogs with no clinical signs of disease). These categories were necessarily simplistic because diagnosis was made using clinical signs only by the CFH curator, Deborah Pergolotti, who has no formal training in disease diagnosis. There were four origin classifications: Cairns city suburbs, coastal suburbs immediately north of Cairns, surrounding rural or remote towns, and unknown; and four seasons: summer (December to February), autumn (March to May), winter (June to August) and spring (September to November).

During the six years from 1999 – 2004, 1451 post-metamorphic amphibians were submitted to the CFH. These were comprised of 877 (60%) *L. infrafrenata*, 300 (20%) *L. caerulea* (common green tree frog) and 15 other species including *Litoria* sp., *Limnodynastes* sp. and *Bufo marinus* (the introduced cane toad). Over the six years, *L. infrafrenata* cases were submitted most frequently during spring, followed by winter, autumn and then summer. A significantly higher mean number of cases was submitted during winter ($P = 0.008$) and spring ($P = 0.005$), compared with autumn (Figure 3). Other seasonal comparisons were not significantly different ($P > 0.0083$,

which was the Bonferroni-adjusted significance level). At least 69% of all cases originated from urban areas within and immediately north of Cairns. A significantly higher number of cases originated from Cairns city suburbs ($P < 0.001$) and coastal suburbs immediately north of Cairns ($P < 0.001$), compared with rural and remote areas (Table 1). There was no significant difference between the two urban categories ($P = 0.02$, Bonferroni-adjusted significance level 0.0167), but the *P*-value was only slightly greater than the adjusted significance level.

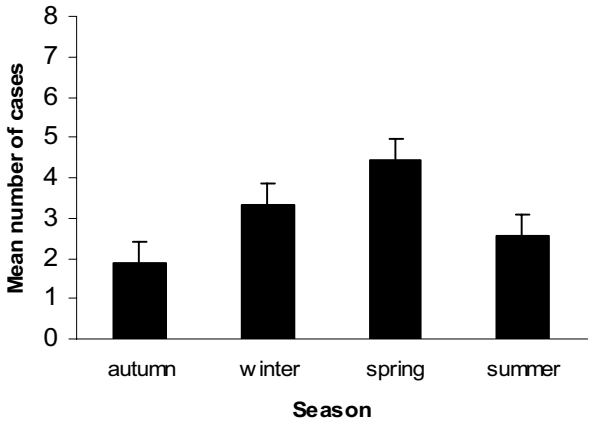

Figure 3. Mean number of white-lipped tree frog (*Litoria infrafrenata*) cases submitted to the Cairns Frog Hospital each season over the six years from January 1999 to December 2004. Error bars represent the standard error.

	Reason for presentation				
Origin	Injury	Emaciation	Sparganosis	Miscellaneous	Total
Cairns city suburbs	117 (40)	22 (28)	115 (53)	159 (56)	413[a]
Coastal suburbs north of Cairns	66 (22)	15 (19)	44 (20)	73 (26)	198[a]
Surrounding rural and remote areas	13 (4)	3 (4)	5 (2)	13 (5)	34[b]
Unknown	102 (34)	39 (49)	54 (25)	37 (13)	232
Total	298 (100)	79 (100)	218 (100)	282 (100)	877

Table 1. Total *Litoria infrafrenata* case numbers submitted to the Cairns Frog Hospital from January 1999 to December 2004, classified according to origin and presenting sign. Values in parentheses are the relative percentages of cases from each area. Values with different superscripts are significantly different ($P < 0.001$).

The most common presenting category was injury, followed by miscellaneous, sparganosis and then emaciation (Table 1). A significantly higher mean number of cases presented with injury ($P < 0.001$), miscellaneous ($P < 0.001$) and sparganosis (P = 0.008), compared with emaciation. Other presenting category comparisons were not statistically different ($P > 0.0083$, which was the Bonferroni-adjusted significance level). A significantly increased mean number of emaciated frogs presented during spring ($P = 0.007$) compared with winter, but there were no significant differences between other seasons ($P > 0.0083$, the Bonferroni-adjusted significance level) (Figure 4). Of the cases presenting with emaciation, 28% had visible concurrent sparganosis. *Spirometra erinacei* occurred predominantly in one or both thigh muscles, and occasionally in subcutaneous locations over the body (Figure 5). There were no significant seasonal differences within the injury, sparganosis or miscellaneous categories ($P > 0.0083$) (Figure 4).

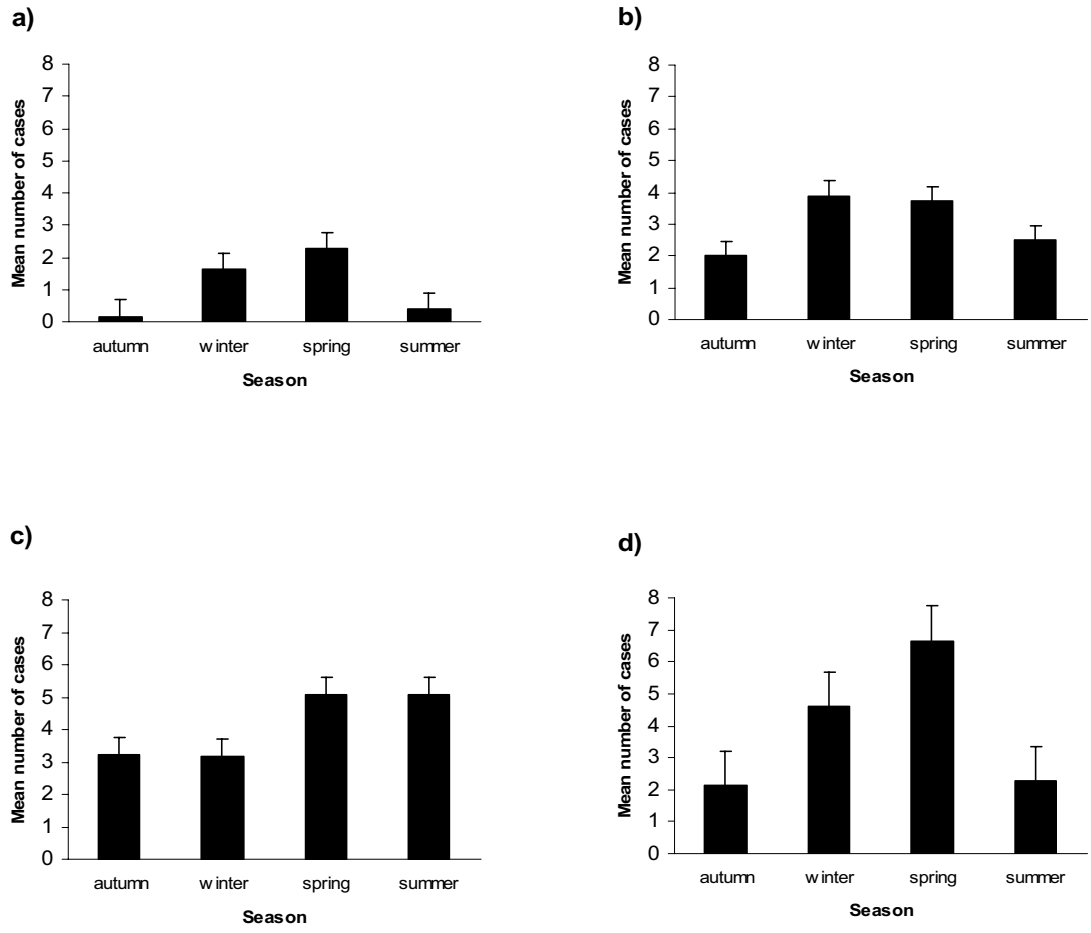

Figure 4. Mean number of white-lipped tree frog (*Litoria infrafrenata*) cases submitted to the Cairns Frog Hospital each season from January 1999 to December 2004 for each of the four presenting categories: a) emaciation, b) sparganosis, c) injury, d) miscellaneous. Error bars represent the standard error.

Figure 5. A mature adult white-lipped tree frog (*Litoria infrafrenata*) with a severe infection with the tapeworm *Spirometra erinacei*. The skin has been reflected during a necropsy examination to reveal the parasites overlying the thigh muscles (left lower quadrant) and significant swelling and haemorrhage of the infected tissues. Photo by S. Young.

The clinical syndrome of emaciation in *L. infrafrenata* was defined as frogs presenting in poor body condition with no obvious clinical cause (Figure 6). While affected frogs originated predominantly from urban areas, cases were documented over a wide geographic area of Queensland from the Cairns area, north to Cape York, south to Townsville and west to the Atherton Tablelands. Cases maintained in captivity post-submission became progressively emaciated, despite supportive nutritional care and repeated shallow immersion in praziquantel (50 mg/l suspension for 30 minutes every two-four weeks; Droncit® 50 mg tablets, Bayer Australia Ltd) for treatment of *S. erinacei* infection. Most of these cases became irreversibly emaciated and died over a period of weeks to months post-submission. Necropsy of 40 specimens presenting with emaciation (26 of which were received during this project) showed few gross abnormalities with the exception of generalized emaciation, depletion of fat bodies, and heavy burdens of *S. erinacei*. Plerocercoids were found predominantly overlying and within the thigh muscles, but also in the coelomic cavity and dorsal musculature. In 50% of cases, there were concurrent infections with other parasites including *Rhabdias* sp. and intestinal nematodes.

Figure 6. A mature adult white-lipped tree frog (*Litoria infrafrenata*) with the wasting syndrome, in the terminal stages of the disease. Affected individuals present clinically in poor body condition with no obvious cause, and become progressively emaciated despite treatment, eventually dying. Photo by S. Young.

In summary, we found a high overall prevalence (27%) of sparganosis in *L. infrafrenata* populations in northern Queensland. and describe a possible new disease in this species that manifests as emaciation and has caused morbidity and mortality over a wide geographic region. While we found a high prevalence (28%) of *Spirometra erinacei* infection in emaciated frogs, this was not significantly different from that in healthy frogs (25%). Although the cause of emaciation described here is not yet clear, the disease will be investigated further in stage 2 of this project.

Objective 2. To determine what diseases occur in peri-urban amphibians in the wet tropics and whether any are undescribed.

This objective involves collecting specimens via passive and active surveillance, along with analysing previously preserved specimens from JCU, QPWS and the CFH. Since more specimens may be received via the disease surveillance systems than are able to be analysed in the scope of the three stages of this project, priority will be given to the following: endangered species; live specimens from which a detailed history and clinical presentation, and clinical pathology and immunology results, can be obtained; and individuals which have received no prior treatment to avoid confusion and bias in results.

A passive amphibian disease surveillance system has already been established at JCU, and surveillance data from the CFH, a second model of passive surveillance for amphibian diseases, has been extensively analysed. Results from the two passive surveillance systems will be compared. Active surveillance will be carried out and repeated at a number of sentinel sites in the region over the next 18 months. Results from the active surveillance system will be evaluated and compared with the results from the passive surveillance models.

As described in Objective 1, many frog specimens have already been collected and for each case, thorough physical examination, necropsy and sample collection protocols have been established and carried out. A large range of diagnostic samples has been collected from each case and analyses have been carried out including haematology, serum biochemistry, and parasite identification (Figures 7 & 8). Formalin-fixed tissues have been processed from each case and histological analysis has been carried out and will continue throughout Stage 2 of the project. Similarly, a range of tissue samples from each specimen has been frozen for future diagnostic testing. Table 2 summarises the diagnostic findings to date in the frog specimens received during this project.

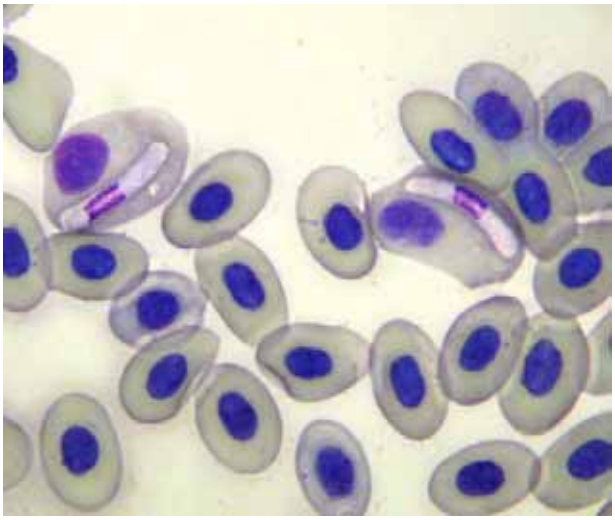

Figure 7. Blood smear from a mature adult white-lipped tree frog (*Litoria infrafrenata*) stained with Wright's stain (x 1000 magnification). Two red blood cells contain a large elongated parasite within their cytoplasm belonging to the Genus *Hepatozoon*. Heavy burdens of this parasite can cause significant debilitation in a number of vertebrate groups but little is known about their effect in amphibians. Photo by S. Young.

Figure 8. Unstained wet preparation of a faecal sample from a mature adult white-lipped tree frog (*Litoria infrafrenata*) (x 100 magnification). Note the heavy burden of parasitic larvae (*Rhabdias* sp.) present even without the use of diagnostic concentrating techniques. Photo by S. Young.

Case ID	Species	Age/Sex	Origin	Diagnostic Findings
FDRQ 001	*Litoria infrafrenata*	Mature adult female	Cooktown	Emaciation *Spirometra erinacei* infection
FDRQ 002	*Litoria infrafrenata*	Mature adult female	Edge Hill Cairns	Injury
FDRQ 003	*Litoria infrafrenata*	Mature adult female	Holloways Beach Cairns	Injury Ascites
FDRQ 004	*Litoria infrafrenata*	Mature adult female	Trinity Beach Cairns	Emaciation *Spirometra erinacei* infection
FDRQ 005	*Litoria infrafrenata*	Mature adult female	Edmonton Cairns	Injury – abdominal hernia Hyperbiliverdinaemia
FDRQ 006	*Litoria infrafrenata*	Mature adult	Trinity Bay Cairns	Emaciation
FDRQ 007	*Litoria infrafrenata*	Mature adult	Brinsmead Cairns	Emaciation
FDRQ 008	*Litoria infrafrenata*	Mature adult	Edmonton Cairns	Emaciation
FDRQ 009	*Litoria infrafrenata*	Mature adult	Trinity Beach Cairns	Emaciation *Spirometra erinacei* infection
FDRQ 010	*Litoria infrafrenata*	Young adult	Brinsmead Cairns	Emaciation
FDRQ 011	*Litoria infrafrenata*	Mature adult	Yarrabah	Emaciation *Spirometra erinacei* infection
FDRQ 012	*Litoria infrafrenata*	Mature adult	Edmonton Cairns	Emaciation *Spirometra erinacei* infection
FDRQ 013	*Litoria infrafrenata*	Mature adult	Manunda Cairns	Emaciation *Spirometra erinacei* infection
FDRQ 014	*Litoria caerulea*	Mature adult male	Mooroobool Cairns	Skin condition
FDRQ 015	*Litoria infrafrenata*	Mature adult male	Manoora Cairns	Anasarca (blue-tinged fluid) Fair body condition
FDRQ 016	*Litoria caerulea*	Young adult male	Edmonton Cairns	Gastrointestinal parasitism
FDRQ 017	*Litoria infrafrenata*	Mature adult female	Mooroobool Cairns	Mild anasarca (blue-tinged fluid)
FDRQ 018	*Litoria infrafrenata*	Mature adult female	Mooroobool Cairns	Mild anasarca (blue-tinged fluid)
FDRQ 019	*Litoria infrafrenata*	Mature adult female	Mooroobool Cairns	Mild anasarca (blue-tinged fluid)
FDRQ 020	*Litoria infrafrenata*	Mature adult male	Mooroobool Cairns	*Spirometra erinacei* infection

Table 2. Summary of case details and diagnostic findings in the frog specimens received during the project.

Case ID	Species	Age/Sex	Origin	Diagnostic Findings
FDRQ 021	*Litoria infrafrenata*	Mature adult	Gordonvale Cairns	Emaciation *Spirometra erinacei* infection
FDRQ 022	*Litoria infrafrenata*	Mature adult	Edge Hill Cairns	Skin condition
FDRQ 023	*Litoria infrafrenata*	Mature adult	Machans Beach Cairns	*Spirometra erinacei* infection Coelomic mass
FDRQ 024	*Litoria infrafrenata*	Mature adult	Cairns	*Spirometra erinacei* infection Hepatic mass
FDRQ 025	*Bufo marinus*	Mature adult	Edmonton Cairns	Open pending further investigation
FDRQ 026	*Litoria infrafrenata*	Mature adult	Edge Hill Cairns	Emaciation
FDRQ 027	*Litoria infrafrenata*	Mature adult	Cairns	Injury
FDRQ 028	*Litoria infrafrenata*	Mature adult female	Cooktown	Open pending further investigation
FDRQ 029	*Litoria infrafrenata*	Mature adult male	Stratford Cairns	Poor body condition after prolonged captivity
FDRQ 030	*Litoria infrafrenata*	Mature adult female	Mooroobool Cairns	Emaciation *Spirometra erinacei* infection
FDRQ 031	*Litoria infrafrenata*	Mature adult male	Mooroobool Cairns	Anasarca (blue-tinged fluid) Hyperbiliverdinaemia
FDRQ 032	*Litoria infrafrenata*	Mature adult male	Portsmith Cairns	Anasarca (blue-tinged fluid) Poor body condition (captive)
FDRQ 033	*Litoria infrafrenata*	Mature adult female	Mooroobool Cairns	Hyperbiliverdinaemia
FDRQ 034	*Litoria infrafrenata*	Mature adult female	Bayview Heights Cairns	Poor body condition (captive) Renal parasitism
FDRQ 035 – 048	*Cyclorana novaehollandiae*	Metamorphs x 11 Tadpoles x 3	Mount Carbine	Hepatic necrosis Skeletal abnormalities Ascites Lipaemia Suspect toxic or nutritional aetiology
FDRQ 049	*Litoria infrafrenata*	Mature adult female	White Rock Cairns	Injury Hyperbiliverdinaemia Renal parasitism
FDRQ 050	*Litoria infrafrenata*	Mature adult female	Edge Hill Cairns	Injury Coelomic nematodes
FDRQ 051	*Litoria infrafrenata*	Mature adult female	Mooroobool Cairns	Injury Hyperbiliverdinaemia

Table 2 (cont.). Summary of case details and diagnostic findings in the frog specimens received during the project.

Case ID	Species	Age/Sex	Origin	Diagnostic Findings
FDRQ 052	*Litoria infrafrenata*	Mature adult female	Manoora Cairns	Emaciation *Spirometra erinacei* infection
FDRQ 053	*Litoria caerulea*	Mature adult female	Peachester SE Qld via QPWS	Chytridiomycosis
FDRQ 054	*Litoria caerulea*	Mature adult female	SE Qld via QPWS	Open pending further investigation
FDRQ 055	*Litoria caerulea*	Mature adult male	Brighton SE Qld via QPWS	Injury
FDRQ 056	*Litoria infrafrenata*	Mature adult female	Mooroobool Cairns	Emaciation *Spirometra erinacei* infection Hyperbiliverdinaemia
FDRQ 057	*Litoria infrafrenata*	Mature adult female	Yorkeys Knob Cairns	Hepatic and renal cysts Encysted nematodes - bladder
FDRQ 058	*Litoria infrafrenata*	Mature adult male	Manunda Cairns	Emaciation *Spirometra erinacei* infection
FDRQ 059	*Limnodynastes terraereginae*	Mature adult female	Calliope SE Qld via QPWS	Open pending further investigation
FDRQ 060	*Litoria caerulea*	Mature adult male	Redland Bay SE Qld via QPWS	Dermatitis Renal disease
FDRQ 061	*Litoria caerulea*	Subadult male	Sunshine Coast via QPWS	Open pending further investigation
FDRQ 062	*Litoria caerulea*	Subadult male	Brighton SE Qld via QPWS	Injury
FDRQ 063	*Bufo marinus*	Mature adult male	Nth Maleny SE Qld via QPWS	Hepatic disease Pulmonary *Rhabdias* infection
FDRQ 064	*Adelotus brevis*	Mature adult female	Greenslopes SE Qld via QPWS	Chytridiomycosis
FDRQ 065	*Litoria peronii*	Mature adult male	Peachester SE Qld via QPWS	Chytridiomycosis
FDRQ 066	*Litoria nasuta*	Mature adult female	SE Qld via QPWS	Chytridiomycosis
FDRQ 067	*Litoria nasuta*	Mature adult male	SE Qld via QPWS	Injury Bilateral blindness
FDRQ 068	*Litoria nasuta*	Mature adult female	Fernvale SE Qld via QPWS	Injury
FDRQ 069	*Litoria rubella*	Mature adult	Sunshine Coast via QPWS	Open pending further investigation
FDRQ 070	*Litoria nasuta*	Mature adult male	Fernvale SE Qld via QPWS	Injury

Table 2 (cont.). Summary of case details and diagnostic findings in the frog specimens received during the project.

Case ID	Species	Age/Sex	Origin	Diagnostic Findings
FDRQ 071	*Litoria infrafrenata*	Mature adult female	Clifton Beach Cairns	Emaciation *Spirometra erinacei* infection Pulmonary *Rhabdias* infection
FDRQ 072	*Litoria caerulea*	Mature adult female	Redlynch Cairns	Coelomic mass
FDRQ 073	*Litoria caerulea*	Mature adult male	Gordonvale Cairns	Poor body condition after prolonged captivity
FDRQ 074	*Litoria infrafrenata*	Mature adult female	Machans Beach Cairns	Injury
FDRQ 075	*Litoria infrafrenata*	Mature adult male	Manoora Cairns	Emaciation *Spirometra erinacei* infection Pulmonary *Rhabdias* infection Gastrointestinal nematodes *Hepatozoon* sp. infection
FDRQ 076	*Litoria nasuta*	Mature adult male	Kuranda	Open pending further investigation
FDRQ 077	*Litoria infrafrenata*	Mature adult female	Manoora Cairns	Emaciation *Spirometra erinacei* infection Hyperbiliverdinaemia *Hepatozoon* sp. infection
FDRQ 078	*Litoria infrafrenata*	Mature adult male	Machans Beach Cairns	Otic mass – suspect chondrosarcoma
FDRQ 079	*Litoria infrafrenata*	Mature adult female	Kuranda	Emaciation *Spirometra erinacei* infection Pulmonary *Rhabdias* infection Encysted coelomic nematodes
FDRQ 080	*Litoria infrafrenata*	Mature adult male	Edge Hill Cairns	Injury *Hepatozoon* sp. infection Microfilaria infection
FDRQ 081	*Litoria caerulea*	Mature adult female	Mooroobool Cairns	*Spirometra erinacei* infection
FDRQ 082	*Litoria infrafrenata*	Mature adult female	Mooroobool Cairns	Emaciation *Spirometra erinacei* infection
FDRQ 083	*Litoria infrafrenata*	Mature adult male	Cooktown	Massive cloacal prolapse *Hepatozoon* sp. infection
FDRQ 084	*Litoria infrafrenata*	Mature adult male	Brinsmead Cairns	Injury *Hepatozoon* sp. infection
FDRQ 085	*Litoria infrafrenata*	Mature adult female	Clifton Beach Cairns	Dermal mass – suspect squamous cell carcinoma *Spirometra erinacei* infection *Hepatozoon* sp. infection
FDRQ 086	*Litoria lesueuri*	Mature adult female	Kuranda	Injury Gastrointestinal nematodes

Table 2 (cont.). Summary of case details and diagnostic findings in the frog specimens received during the project.

Case ID	Species	Age/Sex	Origin	Diagnostic Findings
FDRQ 087	*Litoria infrafrenata*	Mature adult female	Holloways Beach Cairns	Mandibular mass Pulmonary *Rhabdias* infection Encysted coelomic nematodes
FDRQ 088	*Litoria infrafrenata*	Mature adult female	Holloways Beach Cairns	Injury Gastrointestinal nematodes
FDRQ 089	*Litoria infrafrenata*	Mature adult female	Edmonton Cairns	Emaciation *Spirometra erinacei* infection
FDRQ 090	*Litoria infrafrenata*	Subadult	Wonga Beach	Severe skeletal deformities after prolonged captivity
FDRQ 091	*Litoria infrafrenata*	Subadult	Aeroglen Cairns	Severe skeletal deformities after prolonged captivity
FDRQ 092	*Litoria caerulea*	Mature adult male	Edmonton Cairns	Emaciation *Spirometra erinacei* infection Pulmonary *Rhabdias* infection Gastrointestinal nematodes
FDRQ 093	*Litoria infrafrenata*	Mature adult male	Freshwater Cairns	Injury
FDRQ 094	*Litoria infrafrenata*	Mature adult male	Edge Hill Cairns	Pulmonary *Rhabdias* infection Encysted coelomic nematodes
FDRQ 095	*Litoria caerulea*	Subadult male	Mossman	Injury Gastrointestinal nematodes
FDRQ 096	*Litoria infrafrenata*	Mature adult female	Manunda Cairns	Emaciation *Spirometra erinacei* infection *Hepatozoon* sp. infection Gastrointestinal nematodes Hyperbiliverdinaemia
FDRQ 097	*Litoria infrafrenata*	Mature adult male	Smithfield Cairns	Injury
FDRQ 098	*Litoria infrafrenata*	Mature adult female	Machans Beach Cairns	Emaciation *Spirometra erinacei* infection Pulmonary *Rhabdias* infection *Hepatozoon* sp. infection
FDRQ 099	*Litoria infrafrenata*	Mature adult	Forest Gardens Cairns	Injury
FDRQ 100	*Litoria infrafrenata*	Young adult	Brinsmead Cairns	Injury
FDRQ 101	*Litoria infrafrenata*	Mature adult	Manunda Cairns	Open pending further investigation
FDRQ 102	*Litoria infrafrenata*	Mature adult	Trinity Beach Cairns	Emaciation *Spirometra erinacei* infection

Table 2 (cont.). Summary of case details and diagnostic findings in the frog specimens received during the project.

The first suspected case of a chondrosarcoma (a type of malignant tumour) in an amphibian has been found in a *Litoria infrafrenata* specimen as part of the diagnostic analyses carried out in this project (Figure 9). The diagnosis is pending histological confirmation by a specialist veterinary pathologist, and the findings will be reported in a manuscript to be submitted to the Journal of Wildlife Diseases (currently in preparation).

Figure 9. Large mass protruding from the right ear canal of a mature adult white-lipped tree frog (*Litoria infrafrenata*). This mass is suspected to be a type of malignant tumour known as a chondrosarcoma. Photo by S. Young.

An important manuscript reviewing amphibian chytridiomycosis, with a particular emphasis on the role of zoological institutions in the global response to this formidable emerging infectious disease, was produced as part of this project. The final version of this manuscript *Amphibian Chytridiomycosis: Strategies for Captive Management and Conservation* was accepted for publication by the International Zoo Yearbook in January 2007 (Appendix 1).

Objective 3. To develop suitable techniques for wildlife care groups to collect disease data that is relevant for surveillance for emerging diseases, and to determine how this data can be transmitted in a cost-effective way to the Australian Wildlife Health Network using the Cairns Frog Hospital as a model for the surveillance of amphibian disease.

The AWHN is a national initiative, funded and supported by the Australian Wildlife Exotic Disease Preparedness Program and hosted by the Zoological Parks Board of NSW and NSW Agriculture. The aim of the network is to promote and facilitate collaborative links in the investigation and management of wildlife health 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 data base of wildlife health surveillance and diagnostic information, along with a registry of wildlife expertise. 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.

While the response time and capabilities of the wildlife detection system within Australia has improved greatly with the recent implementation of the AWHN, techniques for community wildlife care groups to make the link with the formal AWHN appear not to have been well

established. This project will use amphibian disease and its surveillance in Cairns as a model to evaluate how community-based wildlife care groups can be used in the national surveillance of wildlife disease. It will explore practical strategies to make the data collected by community groups undertaking wildlife disease surveillance available in a meaningful way for processing by the AWHN, and to be made available on a national and global scale. This component of the research will enhance both the capacity of community groups such as the CFH to deal with emerging and epidemic amphibian diseases, and the ability of the AWHN to monitor and diagnose important and emerging diseases affecting these species.

Extensive preliminary research has been carried out in evaluating different surveillance systems. Researcher skills in this specialised field have been enhanced through attendance at the International Symposium on Veterinary Epidemiology and Economics (Cairns, August 2006) and participation in the Australian Biosecurity Cooperative Research Centre workshop *Evaluation of Surveillance Systems* (Brisbane, December 2006). Extensive consultation will be required to establish formal links with the AWHN and the CFH in order to achieve this objective, and this consultation is well underway. The practicalities of integrating community wildlife surveillance data into the AWHN will be effected throughout stage 2 of this project.

Future Directions

There will be a direct continuation of this research to progress further towards completing the objectives under the Department of the Environment and Heritage and James Cook University funding agreement *Emerging Amphibian Diseases and Disease Surveillance in Queensland – Stage 2 (February 2007 – June 2008)*. Specific components and future milestones of this research that will address current knowledge gaps include the following:

- Blood collection from *L. infrafrentata* and *L. caerulea* over two consecutive wet and dry seasons (total 160 samples) to establish a comprehensive panel of baseline haematology and biochemistry values for each of the two species, taking into account the effect of season;
- Experimental laboratory investigations to characterise the acquired immune response in healthy *L. infrafrenata* and *L. caerulea;*
- Experimental laboratory investigations to characterise the acquired immune response to the disease chytridiomycosis in *L. infrafrenata* and *L. caerulea;*
- Experimental laboratory investigations to determine if protective immunity is present following reinfection with the amphibian chytrid fungus in *L. caerulea*;
- Experimental laboratory investigations to characterise the acquired immune response in *L. infrafrenata* affected by the wasting syndrome and comparison with healthy specimens;
- Further pathological investigations into the aetiology of the wasting syndrome in *L. infrafrenata*, including blood collection, immune testing, microbial culture, histology, PCR, ultramicroscopy, and an experimental case-control study to compare syndrome-affected and healthy individuals;
- Determination and implementation of epidemiological techniques for surveying *L. infrafrenata* populations affected with the wasting syndrome, to investigate the epidemiology of this disease;
- Evaluation and comparison of passive and active surveillance techniques for amphibian diseases in the Cairns region;
- Development of suitable techniques for collection of relevant disease surveillance data by community wildlife care groups and determining how this can be transmitted in a practical and cost-effective way to the Australian Wildlife Health Network; and
- Identification of amphibian diseases in Queensland via ongoing passive and active surveillance and subsequent pathological investigations.

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APPENDIX 1: Amphibian Chytridiomycosis: Strategies for Captive Management and Conservation

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ABSTRACT

Dramatic declines and extinctions of amphibian species have occurred worldwide over the last three decades owing to the introduction of chytridiomycosis. This emerging infectious disease is caused by the chytrid fungus *Batrachochytrium dendrobatidis,* a virulent water-borne pathogen of many amphibian species. It 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. The prevalence of chytridiomycosis in the international amphibian trade is high and importation of infected frogs into zoos has caused disease epidemics in established amphibian collections. Management of disease spread requires effective national and international quarantine and control strategies. Although *B. dendrobatidis* is susceptible to a range of commonly used disinfectants, there is no universally effective treatment regime for infected amphibians. Zoological institutions can play a key role in preventing pathogen spread between captive facilities, and in disease surveillance, captive-breeding and reintroduction programmes, to limit the impact of this formidable disease on wild amphibian populations.

KEY WORDS

amphibian, *Batrachochytrium dendrobatidis*, captive management, chytridiomycosis, decline, disinfection, frog, quarantine

INTRODUCTION

Over 30% of amphibian species are threatened and at least 43% are experiencing population declines worldwide (Stuart *et al*., 2004). Since 1980, rapid declines have been reported in over 400 species, with just over half of these attributed to habitat degradation and overexploitation. In at least 200 species declines were considered enigmatic, predominantly affecting stream-associated frogs in protected forests and tropical montane habitats in the Neotropics and Australia, where environmental problems were not detected (Stuart *et al*., 2004). Many of these declines have now been linked to the formidable emerging infectious disease chytridiomycosis, caused by the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Berger *et al*., 1998; Bosch *et al*., 2001; Lips *et al*., 2006; Schloegel *et al*., 2006). Chytridiomycosis has been detected in at least 144 species, over-represented by four anuran families: Bufonidae, Hylidae, Myobatrachidae and Ranidae (Speare & Berger, 2005).

Chytridiomycosis has been recorded in wild amphibian populations in Australia, New Zealand, the Caribbean, Europe, Africa, and South, Central and North America (e.g. Berger *et al*., 1998; Bosch *et al*., 2001; Waldman *et al*., 2001; Muths *et al*., 2003; Hanselmann *et al*., 2004; Weldon *et al*., 2004; Lips *et al*., 2006; Schloegel *et al*., 2006). Epidemiological evidence supports the hypothesis that *B. dendrobatidis* originated in Africa and subsequently spread from that continent via the global trade in African clawed frog *Xenopus laevis* (Weldon *et al*., 2004). In Australia, *B. dendrobatidis* has been associated with dramatic frog population declines and extinctions particularly in the high altitude rainforest areas of Queensland (Berger *et al.,* 1998; Schloegel *et al*., 2006).

EFFECTS AND DETERMINANTS OF CHYTRIDIOMYCOSIS

Infection with *B. dendrobatidis* occurs through waterborne zoospores that invade the superficial keratinised epidermal layers of amphibian skin, causing hyperkeratosis, sloughing and erosions of the epidermis, and occasional ulcerations, in post-metamorphic frogs (Berger *et al*., 1998, 1999).

Affected frogs may display discoloured and reddened skin, abnormal posture, lethargy, anorexia, delayed response to stimuli, loss of righting reflex, seizures and death (Nichols *et al*., 1998, 2001; Berger *et al*., 1999). The mechanisms by which the pathogen causes death are unknown, but may include toxin release or host osmoregulatory disruption secondary to skin damage (Berger *et al*., 1998). *Batrachochytrium dendrobatidis* can be carried in the keratinised mouthparts of tadpoles, many of which exhibit oral abnormalities, particularly jaw sheath depigmentation (Berger *et. al*., 1999; Fellers *et al*., 2001; Rachowicz & Vredenburg, 2004; Obendorf, 2005; Knapp & Morgan, 2006). Populations of wild tadpoles may have a high prevalence of infection (Fellers *et al*., 2001; Knapp & Morgan, 2006). While tadpoles of most species studied do not die from infection, experiments have demonstrated a reduced growth rate and smaller size at metamorphosis (Parris, 2004). Interestingly, infection with *B. dendrobatidis* in Cope's gray tree frog *Hyla chrysoscelis* tadpoles caused slower development only when predators were present, demonstrating the cumulative effect of stressors (Parris & Beaudoin, 2004).

Morbidity and mortality rates in post-metamorphic amphibians vary greatly among species (Ardipradja, 2001; Nichols *et al*., 2001; Woodhams *et al*., 2003; Berger *et al*., 2004; Carey *et al*., 2006). 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, 2005; Ardipradja, 2001; Nichols *et al*., 2001). The Common green tree frog *Litoria caerulea* and Great barred frog *Mixophyes fasciolatus* have been shown experimentally to be highly susceptible host species (Ardipradja, 2001). Incubation times are generally between 18 and 70 days, but may be shorter (Berger, 2001; Nichols *et al*., 2001). Some species can survive infection, and apparently healthy amphibians may frequently carry light infections in the wild (Hanselmann *et al*., 2004; Retallick *et al*., 2004; McDonald *et al*., 2005). Introduction of *B. dendrobatidis* to naïve susceptible populations has caused epidemics of high mortality. If populations survive, the pathogen persists and becomes endemic, with reduced mortality rates

and, in some cases, recovery (McDonald *et al*., 2005). This suggests that there is selection for host resistance against the disease.

Experimentally, the fungus has been shown to persist and survive in environmental samples, independent of its host, for varying periods (Johnson & Speare, 2003, 2005). Suspected mechanisms of spread of *B. dendrobatidis* include movement through water bodies and via surface water during precipitation, movement of individual infected amphibians and translocation on fomites and vectors, such as moist substrate or possibly birds (Speare *et al*., 2001; Johnson & Speare, 2003, 2005). The role of alternative hosts in disease transmission is currently being investigated.

Survival and growth of the chytrid fungus is temperature-dependent, the optimal range being 17–25°C (Piotrowski *et al*., 2004). *Batrachochytrium dendrobatidis* is highly sensitive to elevated temperatures, dying in 4 hours at 37°C (Berger, 2001; Johnson *et al*., 2003), and may be unable to persist outside of the host when soil and water temperatures exceed 25°C for an extended period of time. Host behaviour influences temperature regimes experienced by the pathogen, and basking to elevate host body temperature may play a curative role (Woodhams *et al*., 2003). Prevalence of infection and mortality rates in wild populations increase during cooler months (Berger *et al*., 2004; Retallick *et al*., 2004; McDonald *et al*., 2005; Kriger & Hero, 2006). Experimentally, lower temperatures enhanced pathogen virulence in *M. fasciolatus*, with 100% mortality in frogs maintained at 17–23°C, but only 50% at 27°C (Berger *et al*., 2004). Woodhams *et al*. (2003) reported elimination of *B. dendrobatidis* by exposing infected Red-eyed tree frog *Litoria chloris* to 37°C for two 8 hour periods.

DIAGNOSIS

Clinical signs of chytridiomycosis in post-metamorphic frogs manifest as abnormal behaviour, neurological signs and skin lesions (Berger *et al*., 1998, 1999; Nichols *et al*., 1998, 2001) (Plate 1). However, these signs are non-specific and the disease cannot be diagnosed clinically.

Diagnosis of chytridiomycosis requires laboratory confirmation by routine histological examination of skin sections (e.g. toe clips), direct examination of unstained skin smears, immunohistochemical staining of skin sections, or polymerase chain reaction (PCR) assay of skin swabs (Berger *et al*., 1999, 2000, 2002; Berger, 2001; Boyle *et al*., 2004) (Plates 2, 3, 4 and 5). Diagnostic samples can be collected from live, frozen or preserved frogs. While all tests can accurately detect infection in sick frogs with severe chytridiomycosis, PCR assay of skin swabs is recommended for screening healthy frogs due to its increased sensitivity and non-invasive nature (Table 1). Live tadpoles can be tested by collecting mouthpart swabs for PCR assay (Obendorf, 2005; A. Hyatt, unpubl. data). Visual inspection of tadpole mouthparts may also be a reliable indicator of *B. dendrobatidis* infection (Fellers *et al*., 2001; Rachowicz & Vredenburg, 2004).

MANAGEMENT OF CHYTRIDIOMYCOSIS

Disinfection Effective disinfection protocols are essential for the management of chytridiomycosis in captive facilities and in the field. *Batrachochytrium dendrobatidis* is susceptible to a wide range of physical and chemical treatments (Johnson *et al*., 2003; Webb *et al*., 2007) (Table 2). It is highly sensitive to temperatures above 32°C, with 100% mortality within 4 hours at 37°C and within 30 minutes at 47°C. Equipment can be disinfected by immersion in hot water (60°C for ≥5 minutes). Desiccation is also effective, but requires extended periods to ensure that any water has evaporated completely. A combination of heating and drying is recommended for some objects, such as clothing and equipment.

The pathogen is susceptible to several chemical disinfectants, but concentration and time of exposure are important (Johnson *et al*., 2003; Webb *et al*., 2007). Recommended chemicals include the quaternary ammonium compound didecyl dimethyl ammonium chloride (DDAC), benzalkonium chloride, Virkon®, F10SC Veterinary Disinfectant®, TriGene®, ethanol and sodium hypochlorite (household bleach). These are particularly useful for disinfection of equipment in

the field, but care must be taken to prevent environmental contamination. Although sodium hypochlorite is an effective disinfectant, it may damage some equipment.

Treatment Effective treatment protocols for chytridiomycosis are necessary to ensure success of captive-breeding programmes for threatened species and to reduce risks associated with amphibian movements. Infected tadpoles survive and remain at sites after adults have declined or disappeared following an outbreak of chytridiomycosis. Treatment of tadpoles collected from these sites would enable an emergency response to mortality and declines in threatened species through captive-rearing of tadpoles and reintroduction. Effective treatment would also prevent pathogen spread during translocation of amphibians between wild and captive populations (Australian Government Department of the Environment and Heritage, 2006). To date, no treatments have been consistently effective across species.

Itraconazole has been used successfully both orally (2-13 mg/kg daily for 9–28 days) and via shallow immersion (0.01% suspension for 5 minutes daily for 11 days) to treat some adult amphibians (Taylor *et al*., 1999; Nichols & Lamirande, 2000; Taylor, 2001). A commercial solution of malachite green (0.1 mg/litre) and formaldehyde (25 ppm) (Formalite III®) effectively treated African clawed frog *Xenopus tropicalis* (Parker *et al*., 2002), but malachite green can cause developmental deformities and therefore is not recommended for use in threatened species. Although temperature elevation to 37°C was effective in treating *L. chloris* (Woodhams *et al*., 2003), this treatment has not worked in other species (G. Marantelli, unpubl. data). Furthermore, some species may not tolerate these high temperatures. Itraconazole, fluconazole and F10SC Veterinary Disinfectant® baths for tadpoles were either ineffective or toxic (Marantelli *et al*., 2000; B. McMeekin, unpubl. data). Treatment of water with terbinafine hydrochloride (2–4 mg/litre for 7 days) appears to be non-toxic to tadpoles and its efficacy in eliminating infection is currently being investigated (B. McMeekin, unpubl. data).

Quarantine Routine quarantine procedures are critical for controlling chytridiomycosis in captivity, in the field and during translocations. Infected frogs may appear clinically normal and their transport internationally has been implicated in disease spread. There is a high prevalence of chytridiomycosis in the international amphibian trade including the pet trade (Berger *et al*., 1999; Cunningham *et al*., 2005), the scientific trade (e.g. Weldon *et al*., 2004), the food trade (e.g. Mazzoni *et al*., 2003; Hanselmann *et al*., 2004), the ornamental trade (Daszak *et al*., 1999), and the introduction of frogs into zoological collections (Nichols *et al*., 1998, 2001; Pessier *et al*., 1999; Banks & McCracken, 2002; Schloegel *et al*., 2006). Infected frogs imported into zoos have caused epidemics of chytridiomycosis in established amphibian collections, but few of these cases have been published (Nichols *et al*., 1998; Pessier *et al*., 1999). This may reflect potential problems with quarantine and hygiene procedures within zoological collections.

Amphibians should be maintained in quarantine as individuals in separate containers for at least 2 months, whether moving between field sites, captive collections or countries. During quarantine individuals should be regularly examined for signs of disease and a thorough necropsy must be performed on any animals that die during the quarantine period (Daszak *et al*., 2001; Lynch, 2001). Published protocols suggest temperature during quarantine should be maintained between 17–23°C to increase the chance of infection becoming clinically apparent (Lynch, 2001). However, owing to the sensitivity of *B. dendrobatidis* to elevated temperatures and the high sensitivity of the PCR assay, the authors recommend holding heat-tolerant species at the maximum temperature they can tolerate, and collecting skin swabs for PCR assay on arrival and 7 weeks post-arrival.

Important hygiene practises include wearing disposable gloves and changing gloves between enclosures, disinfection of equipment between use, attending to animals in a consistent order starting with threatened species and those least likely to be infected, use of automated husbandry systems, and disinfection of water used in enclosures before disposal (Marantelli & Berger, 2000; Lynch, 2001). Adhering to quarantine guidelines can prevent transmission of

chytridiomycosis between frogs housed in close proximity, but even small numbers of zoospores are highly infectious and great care must be taken to prevent contamination of groups of animals with drops of water.

Hygiene protocols for fieldwork should be stringently followed, including disinfection of footwear and equipment between sites, the use of disposable gloves and plastic bags for handling frogs and disinfection of equipment between frogs. Adults should never be held together in the same container, and tadpoles for release should never be held with batches of tadpoles from other sites, even if they originate from a common water body (New South Wales National Parks and Wildlife Service, 2000; Speare *et al*., 2004).

Control '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 minimize 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.

Captive breeding can play a critical role in limiting the impact of chytridiomycosis on wild amphibian populations by providing supplemental numbers of threatened species for restocking and research (Australian Government Department of the Environment and Heritage, 2006). It may be possible to select for innate disease resistance by collecting tadpoles from declining populations, breeding from individuals that survive metamorphosis and restocking field sites with their progeny. Individuals must not be returned to their point of origin unless they can

be shown to be disease-free, and diagnostic screening procedures must be implemented to ensure this. Augmenting remnant populations through restocking has been successfully used in Australia with Corroboree frog *Pseudophryne corroboree* and this strategy appears to have played an important role in slowing the decline of this Critically Endangered species (IUCN, 2006; G. Marantelli, unpubl. data). An intensive captive-breeding programme for the last remaining Wyoming toad *Bufo baxteri* population in Wyoming prevented extinction and efforts to reintroduce this species to the wild are under way (AmphibiaWeb, 2006). The success of this programme was largely a result of early recognition of the precarious status of wild populations, extensive collaborative captive-breeding efforts involving government and zoological institutions, and habitat protection through land purchase. The urgent need for captive-breeding programmes for a number of other threatened species has been recognized but many of these programmes are in their infancy owing to limited captive-breeding success.

Restocking of threatened species may increase their chance of survival when high mortality rates threaten to cause extinction. This strategy can increase the time available for a species to develop disease resistance and maintain population numbers during adverse conditions that favour the pathogen. Captive-reared amphibians used for restocking must be kept disease-free, and restocking must be implemented before the final population of a species is under threat, or extinction may not be preventable (Australian Government Department of the Environment and Heritage, 2006). This is demonstrated by the case of Sharp-snouted day frog *Taudactylus acutirostris* in Australia, where frogs and tadpoles were transferred from the last remaining population into captivity. As the wild population crashed from chytridiomycosis, the captive specimens also died from the disease, resulting in extinction of the species (Banks & McCracken, 2002; Schloegel *et al*., 2006; Australian Government Department of the Environment and Heritage, 2007).

Batrachochytrium dendrobatidis continues to spread through naïve populations in Panama, causing severe declines and threatening large numbers of frog species (Lips *et al*., 2006).

Emergency response to this situation has involved moving as many individuals as possible into captivity (Goodman, 2006), but without established captive-breeding programmes and treatment and quarantine protocols, this strategy may not be successful in preventing extinctions. Until successful treatments are identified, prevention of pathogen spread, captive breeding for restocking well in advance of disease outbreaks in the wild, and captive rearing and breeding from tadpoles to select for innate resistance, are the most important strategies to limit the impact of chytridiomycosis on wild populations.

FUTURE DIRECTIONS

Dedicated resources on a global scale must be made available in zoological and other institutions for captive breeding of threatened species and for emergency response to population declines. Researching the natural history and biology of these species in the wild and in captivity must be prioritized to enable captive-breeding success. To date, few captive-breeding programmes for threatened species have been successful owing to a lack of critical knowledge about the species. 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. Although cryopreservation of amphibian semen has been achieved, the large size and thick capsules of eggs and embryos makes successful freezing unlikely (Sargent & Mohun, 2005).

Further research is needed to determine effective treatments for tadpoles and adults, and whether selection for innate disease resistance is a feasible management strategy for both captive and wild populations. 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.

Finally, while zoos can play a key role in contributing to amphibian conservation, maintaining threatened species in captivity must be recognized 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 maximize 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.

For detailed information about chytridiomycosis, including diagnosis and management, see the Amphibian Disease Home Page:
http://www.jcu.edu.au/school/phtm/PHTM/frogs/ampdis.htm
Some related amphibian conservation websites include:

- AmphibiaWeb: http://www.amphibiaweb.org
- Declining Amphibian Populations Task Force: http://www.open.ac.uk/daptf/index.htm
- IUCN Global Amphibian Assessment: http://www.globalamphibians.org
- IUCN Species Survival Commission Conservation Breeding Specialist Group: http://www.cbsg.org/amphibian/php
- World Association of Zoos and Aquariums: http://www.waza.org/conservation/campaigns21.php?view=campaigns&id=1

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PRODUCTS MENTIONED IN THE TEXT

F10SC Veterinary Disinfectant®: broad-spectrum multi-purpose disinfectant and sanitizer, manufactured by Health and Hygiene Pty Ltd, PO Box 34, Sunninghill, 2157, South Africa.

Formalite III®: aquarium antimicrobial in-water treatment, manufactured by Aquatronics, PO Box 2457, Oxnard, CA 93034, USA.

TriGene: broad-spectrum disinfectant concentrate, manufactured by MediChem International (Manufacturing) Ltd, Unit 3, Stalham Business Centre, Rushenden Rd, Queenborough, Kent ME11 5HE, UK.

Virkon®: broad-spectrum disinfectant effective against viruses, bacteria and fungi, manufactured by Antec International Ltd, Sudbury, Suffolk CO10 2XD, UK.

DIAGNOSTIC SERVICES OFFERING CHYTRID PCR TESTING

Australian Animal Health Laboratory: CSIRO Division of Livestock Industries, 5 Portarlington Road, Geelong VIC 3220, Australia. Contact Alex Hyatt (alex.hyatt@csiro.au) prior to sample submission.

Institute of Zoology: Zoological Society of London, Regent's Park, London NW1 4RY, UK. Contact Matt Perkins (matthew.perkins@ioz.ac.uk) and Clyde Hutchinson (clyde.hutchinson@ioz.ac.uk) prior to sample submission.

Pisces Molecular: 5311 Western Avenue, Suite E, Boulder CO 80301, USA. Contact John Wood (jwood@pisces-molecular.com) prior to sample submission.

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Plate 1. Captive-bred Great barred frog *Mixophyes fasciolatus* metamorph with naturally acquired chytridiomycosis in the terminal stages of disease. Note depressed attitude, half-closed eyes and accumulations of sloughed skin over the body. Bar = 0.5 cm. Reprinted from Berger (2001).

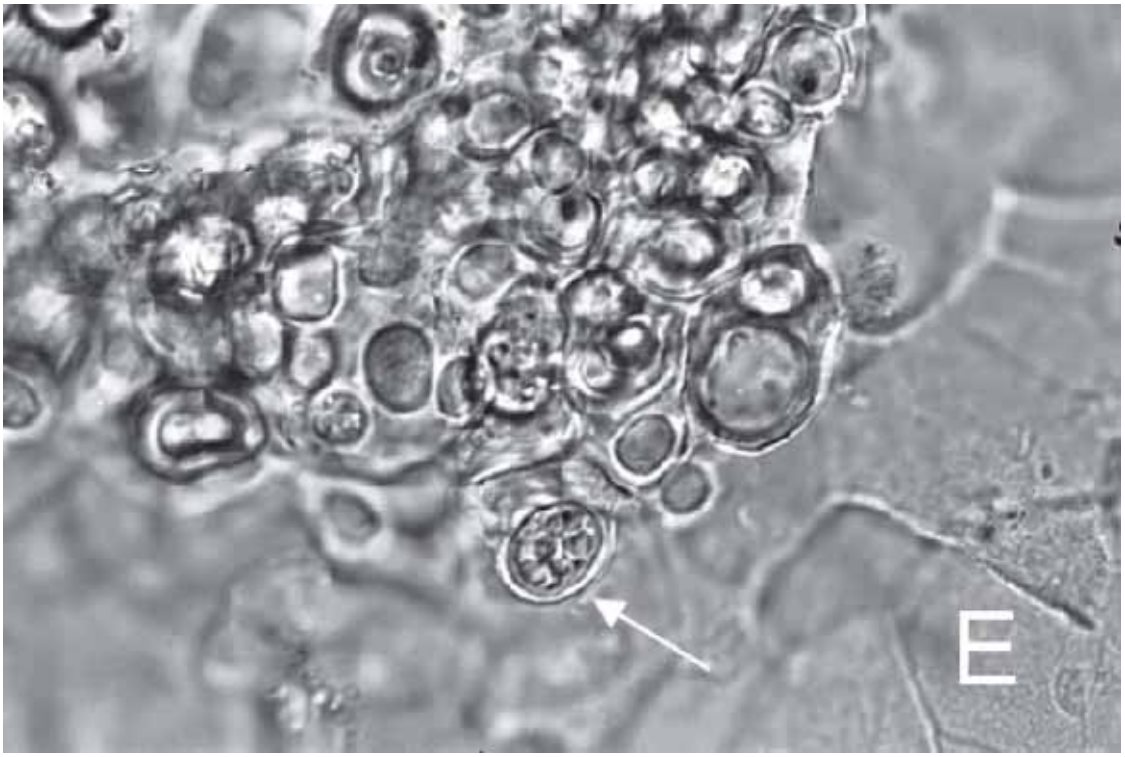

Plate 2. Unstained wet mount of shedding skin from a Common green tree frog *Litoria caerulea* infected with *Batrachochytrium dendrobatidis*. Note refractile round and oval sporangia. Most sporangia are empty but one contains developing zoospores (arrow). E = epidermal cell. 1000 x magnification. Reprinted from Berger (2001).

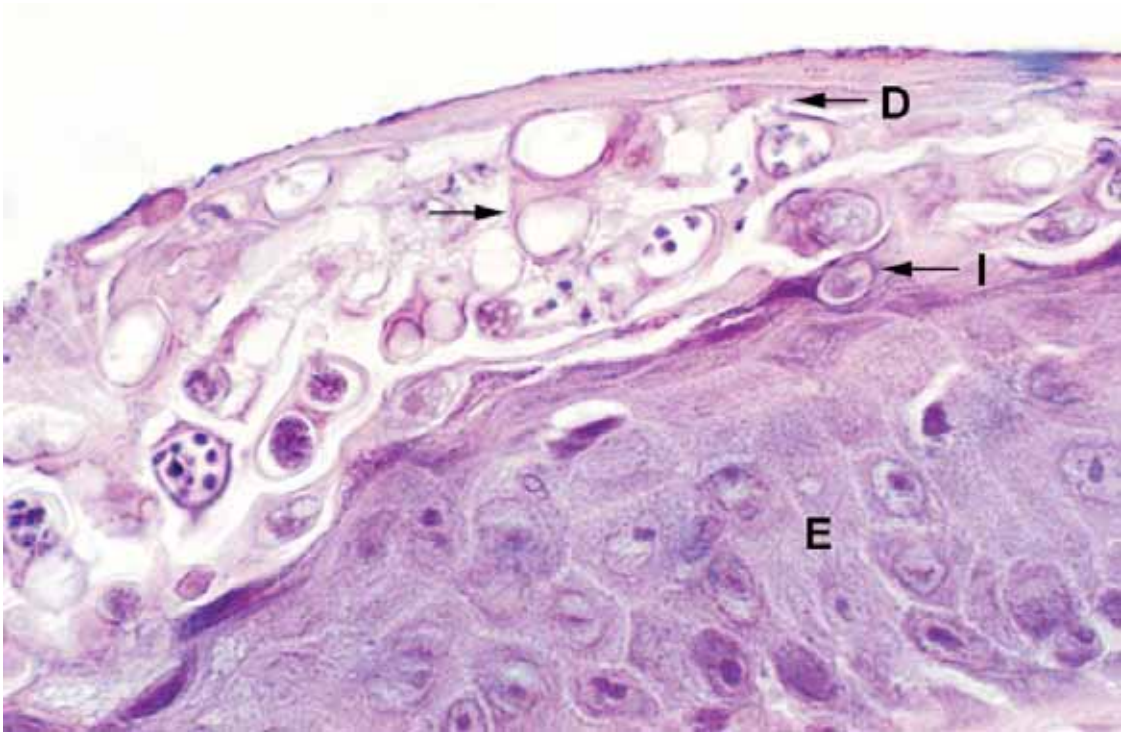

Plate 3. Histological section of skin from a Common green tree frog *Litoria caerulea* heavily infected with *Batrachochytrium dendrobatidis*. Note homogenous immature stage (I), larger multinucleate stages, zoosporangium with discharge tube (D) containing zoospores, and empty zoosporangium after zoospores have discharged (arrow). E = epidermis. Stained with haematoxylin and eosin, 1000 x magnification. Reprinted from Berger (2001).

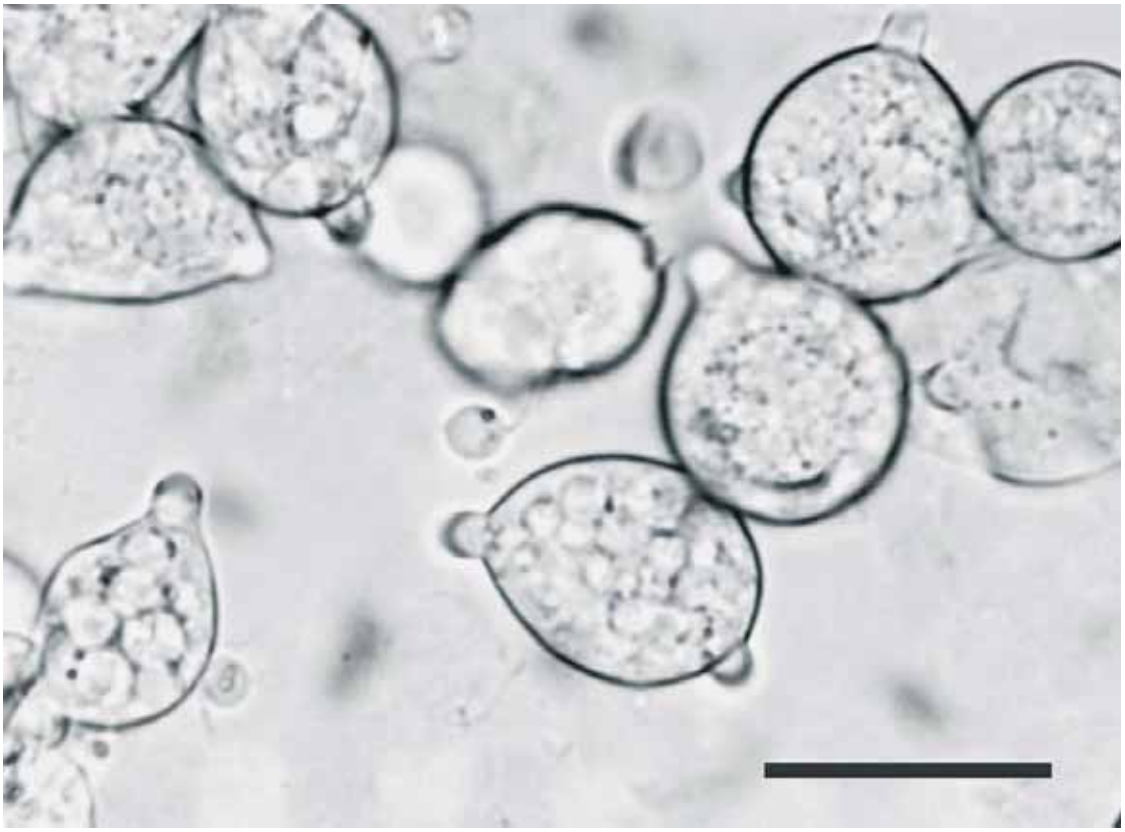

Plate 4. Live cultured *Batrachochytrium dendrobatidis* sporangia. The infective zoospores form internally and then escape by swimming out through the discharge tubes when the plugs dissolve. Bar = 20 μm. Reprinted from Berger (2001).

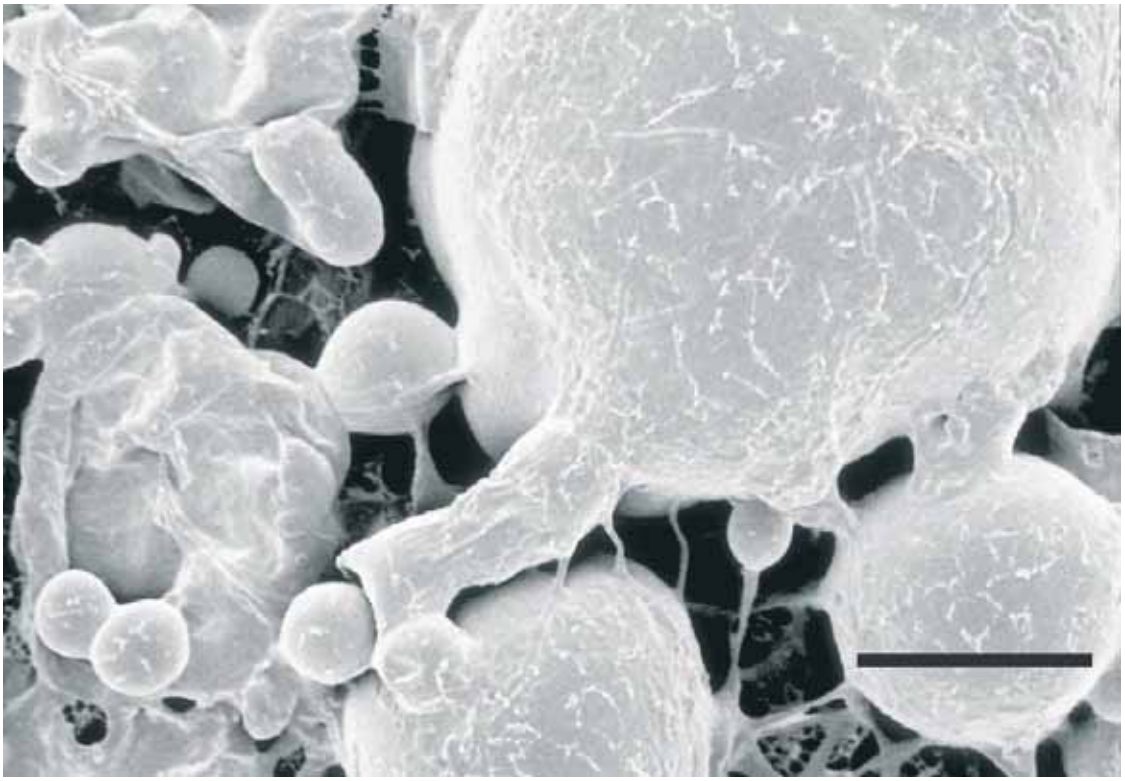

Plate 5. Scanning electron micrograph of a *Batrachochytrium dendrobatidis* zoosporangium on agar releasing a zoospore through a long discharge tube. Bar = 10 μm. Reprinted from Berger (2001).

Diagnostic test	Unstained skin scrapings/smears	Histology of skin sections	Immunostaining of skin sections	PCR assay of skin swabs
Complexity and cost of preparation	1+	2+	3+	4+
Resources required	1+	2+	3+	5+
Ease of interpretation	1+	2+	3+	5+
Sensitivity	2+	2+	3+	5+
Use for healthy frogs	Limited use	Limited use	Limited use	Very useful

Table 1. Comparison of the characteristics of diagnostic tests for amphibian chytridiomycosis. All four methods of diagnosis are useful, but each has various advantages and disadvantages. Note that frogs with clinically severe disease typically have very heavy *Batrachochytrium dendrobatidis* infections and highly sensitive tests are not required for diagnosis. 1+ = lowest, 5+ = highest. Adapted from Australian Government Department of the Environment and Heritage (2006) and Berger *et al*. (2007).

Disinfectant	Concentration	Exposure Time
Ethanol	70 %	1 min
Virkon®	1 mg/ml	1 min
Benzalkonium chloride	1 mg/ml	1 min
Sodium hypochlorite	1 %	1 min
Didecyl dimethyl ammonium chloride	1:1000 dilution	30 sec
F10SC Veterinary Disinfectant®	1:1000 dilution	1 min
TriGene®	1:5000 dilution	1 min
Complete drying	-	3 h or more
Heat	60°C	5 min

Table 2. Disinfection methods suitable for killing *Batrachochytrium dendrobatidis*, showing minimum effective concentrations and exposure times. From Johnson *et al.* (2003) and Webb *et al*. (2007).