CHAPTER 8

Hosts, distribution, incidence and impact of chytridiomycosis: relevance to population declines

8.1 Introduction

The only time that dead frogs were collected and found infected with *B. dendrobatidis* during a significant population crash in Queensland was at Big Tableland in 1993 (Berger et al., 1998). In previous declines in Queensland dead frogs were not found, although the rapidity of the declines indicated that mass mortality of adults had occurred. A wave of declines appeared to progress from southern to northern Queensland between 1979 and 1993. As stream-dwelling adult frogs disappeared while tadpoles remained, a theory was proposed that a waterborne infectious agent, fatal to adults was spreading at an overall rate of 100 km/year (Laurance et al., 1996). The hypothesis proposed that the agent could exist at high and low altitudes, but had greater impact on populations at higher altitude (Laurance et al., 1996). Populations of some species disappeared at high altitude sites but survived in the lowlands (Richards et al., 1993). The explanation suggested for the altitudinal difference was that lower temperature enhanced pathogenicity of the disease by having a negative effect on the frogs' immune system and favouring growth of the pathogen (Laurance et al., 1996). As a variety of unrelated frog species were affected, the agent needed to have a broad host range.

B. dendrobatidis has many characteristics that are consistent with causing the epidemiological pattern of declines seen in coastal Queensland. Infection is often fatal to adults but tadpoles remain healthy carriers (Chap 5); survival of tadpoles with death of post-metamorphic stages in upland streams was the usual pattern of disease in the wild. The temperature effects on *in vitro* growth of *B. dendrobatidis* have now been tested. In culture *B. dendrobatidis* grew slowly at 6°C, developed most rapidly at 23°C but died if kept at temperatures above 29°C (Longcore, 2000; Chap 9). An infection experiment using *M. fasciolatus* showed that while all frogs kept at 23°C and 17°C died, four out of eight frogs kept at 27°C survived (Chap 7). For the first time recovery from infection was seen in infected frogs with three of these surviving frogs eliminating a

confirmed infection. This pattern was consistent with the hypothesis that mortality in the wild was higher in upland areas owing to lower temperature. Also, the infective zoospores of chytridiomycetes require water for dispersal, matching the association with declines in populations of stream-dwelling frogs.

To gain an understanding of frog diseases in the wild, sick frogs were surveyed from declining and non-declining populations in Australia. Chytridiomycosis was found to be the most common disease of Australian frogs and infection was prevalent in some wild tadpoles. *B. dendrobatidis* has a broad amphibian host range and is widespread around Australia, occurring in a variety of habitats at high and low altitudes. An increased incidence of chytridiomycosis occurred in winter, supporting the experimental findings that lower temperatures enhanced the pathogenicity of this disease.

8.2 Methods

The survey of sick and dead frogs was conducted opportunistically and relied on collaboration with a network of herpetologists and other scientists at government environmental departments, universities and frog interest groups who submitted sick and dead frogs to the laboratory for diagnosis. Diagnostic methods and the network for collection of frogs are included in the general methods section in Chapter 3. Information sheets were disseminated that described how to identify sick frogs, how to preserve frogs, and what information should be recorded (Berger and Speare, 1998). Briefly, dead frogs found by herpetologists were preserved in 10% formalin or 70% ethanol, or were frozen. Sick frogs were identified by their unusual behaviour (e.g. lethargy, sitting unprotected during the day) or by the presence of lesions (e.g. reddening, ulcers). Sick frogs were either euthanased and preserved, or were couriered alive to the laboratory. For most specimens, the collector identified the species of amphibian. At the laboratory, routine post mortems were performed to diagnose the cause of illness. Chytridiomycosis was diagnosed by histopathology and/or examination of skin scrapings, and internal organs were examined for concurrent disease. Tadpoles were examined for *Batrachochytrium* by cutting them in half sagitally and embedding with cut surface downward to obtain histological sections through the mouthparts. Cases were entered on a frog disease database in Microsoft Access.

Statistical analysis of the incidence of chytridiomycosis was performed using a goodness-of-fit test to compare the mean number of frogs diagnosed with chytridiomycosis in July and August with the mean for the rest of the year. Using five years of data for Queensland and NSW, the difference between months was compared using a one-way analysis of variance (ANOVA).

8.3 Results

8.3.1 Overview of hosts with chytridiomycosis

Chytridiomycosis occurred in 201 (56.5%) of 356 post-metamorphic amphibians that could be examined fully. This included 135 (55.6%) of 243 free-living animals and 66 (58.4 %) of 113 captive animals. An overview of other diseases found is included in Chapter 10, and Appendix 1 contains a complete list of frogs from the database with the name of the submitter for each case.

Frogs were from 20 Hylid species, 15 Myobatrachid species and 1 Bufonid species. Numbers of wild (and captive) post-metamorphic anurans with chytridiomycosis from each species were: 11 *Adelotus brevis*, 1 *B. marinus*, 1 *Crinia georgiana*, (1) *Cyclorana platycephala*, 1 *Heleioporus australiacus*, 1 *Lechriodus fletcheri*, 5 (1) *L. dumerilii*, 1 *Lim. peronii*, 6 *L. tasmaniensis*, 1 *L. terraereginae*, 5 *L. aurea*, 40 (6) *L. caerulea*, 2 *L. chloris*, (4) *L. citropa*, 2 *L. ewingii*, 3 *L. genimaculata*, (1) *L. gracilenta*, (3) *L. infrafrenata*, 14 *L. lesueuri*, 1 *L. moorei*, 3 *L. nannotis*, 8 *L. pearsoniana*, 8 *Lit. peronii*, (1) *L. raniformis*, 2 *L. rheocola*, 7 *L. spenceri*, (1) *L. tyleri*, 1 *L. verreauxii*, 1 (31) *M. fasciolatus*, 4 *M. fleayi*, (9) *Neobatrachus kunapalari*,1 *Nyctimystes dayi*, (1) *P. pengilleyi*, 2 (6) *T. acutirostris*, 3 *T. eungellensis*, and (1) *Uperoleia laevigata.*

Of 130 frogs where sex was determined, 66 (50.8%) were females and 64 (49.2%) were males. Of the 135 free-living frogs, 125 (92.6 %) were adults and 10 (7.4 %) were juveniles or metamorphs. Of the 66 captive animals, 19 (28.8 %) were adults and 47 (71.2 %) were juveniles or metamorphs.

8.3.2 Concurrent diseases

Most diseased frogs were in reasonable body condition and 77 (72.6 %) of 106 were considered to have moderate or large fat bodies. Twenty-five (12.4 %) out of the 201 frogs with severe chytridiomycosis had concurrent diseases. The contribution of these diseases to the pathogenesis in these frogs is unknown. In most frogs chytridiomycosis appeared to be the major disease process, although pre-existing chronic diseases could have predisposed these frogs to overwhelming chytrid infection, and acute terminal infections may have contributed to the cause of death. Chronic diseases were biliary hyperplasia (5 frogs), localised mucormycosis (2 frogs) and foreign body myositis (1 frog). More acute diseases were bacterial septicaemia (2 frogs), bacterial pneumonia (2 frogs), bacterial cystitis (1 frog), hyphal mycotic dermatitis (3 frogs) and acute nephrosis (2 frogs). Microsporidiosis (3 frogs), immunosuppression (2 frogs), dermocystidiosis (1 frog) and a heavy spargana infection (1 frog) may have been significant in the demise of these frogs.

Of the 155 post-metamorphic sick frogs that were diagnosed as having other primary diseases or were undiagnosed, 9 (5.8%) were found to have light chytrid infections that appeared to be incidental to their primary disease.

8.3.3 *B. dendrobatidis* in tadpoles

The occurrence of *B. dendrobatidis* in the mouthparts of healthy tadpoles was initially discovered in *M. fasciolatus* (3/4) and *B. marinus* (3/8) tadpoles that were euthanased from groups of captive anurans that died with chytridiomycosis after metamorphosis (Berger et al., 1998; Chap 5). The mouthparts of 17 wild tadpoles and 52 captive tadpoles, that were collected during the amphibian disease survey or were healthy animals sacrificed for disease screening, were subsequently checked for chytrids. Infections were found in a high proportion of wild tadpoles in some areas and these infections were not considered to have caused disease. Infections were found in the following wild tadpoles: 9/10 *Mixophyes* sp. from Goomburra and Cunningham's Gap collected as healthy or with other diseases such as skin lesions or biliary hyperplasia, 3/4 *Mixophyes* sp. from Scrubby Creek in the Conondales that were collected due to metacercaria infection, and 1/1 *M. fasciolatus* from Eungella with metacercaria.

Infections were not found in 2/2 healthy *L. tasmaniensis* from Paralana Springs, Flinders Ranges. Infections were not found in the following captive tadpoles: 5 *L. lesueuri* from the ARC, 9 *M. fleayi* and 1 *M. fasciolatus* from Lone Pine Koala Sanctuary, 5 *M. fasciolatus* from the ARC, 3 *L. aurea* from Taronga Zoo, and 26 *P. corroboree* from 3 groups of wild-collected spawn raised at the ARC.

8.3.4 Distribution

In Australia, chytridiomycosis has been found causing disease in at least three geographic zones: a large east coast zone from Big Tableland near Cooktown in north Queensland to the Southern Highlands in Victoria, an Adelaide zone (Berger et al., 1998) and a zone in south west Western Australia (Aplin and Kirkpatrick, 2000) (Fig. 8.1). Infection was also detected in the Kimberley in northern Western Australia (Aplin and Kirkpatrick, 2000). Disease occurred in populations that have declined, as well as areas where no declines have been documented. Chytridiomycosis occurred in a wide range of habitats at high and low altitude, including protected rainforests and other forests, as well as disturbed areas such as rural and urban areas. Chytridiomycosis has not been detected in the Northern Territory, and there have only been some unconfirmed reports from Tasmania.

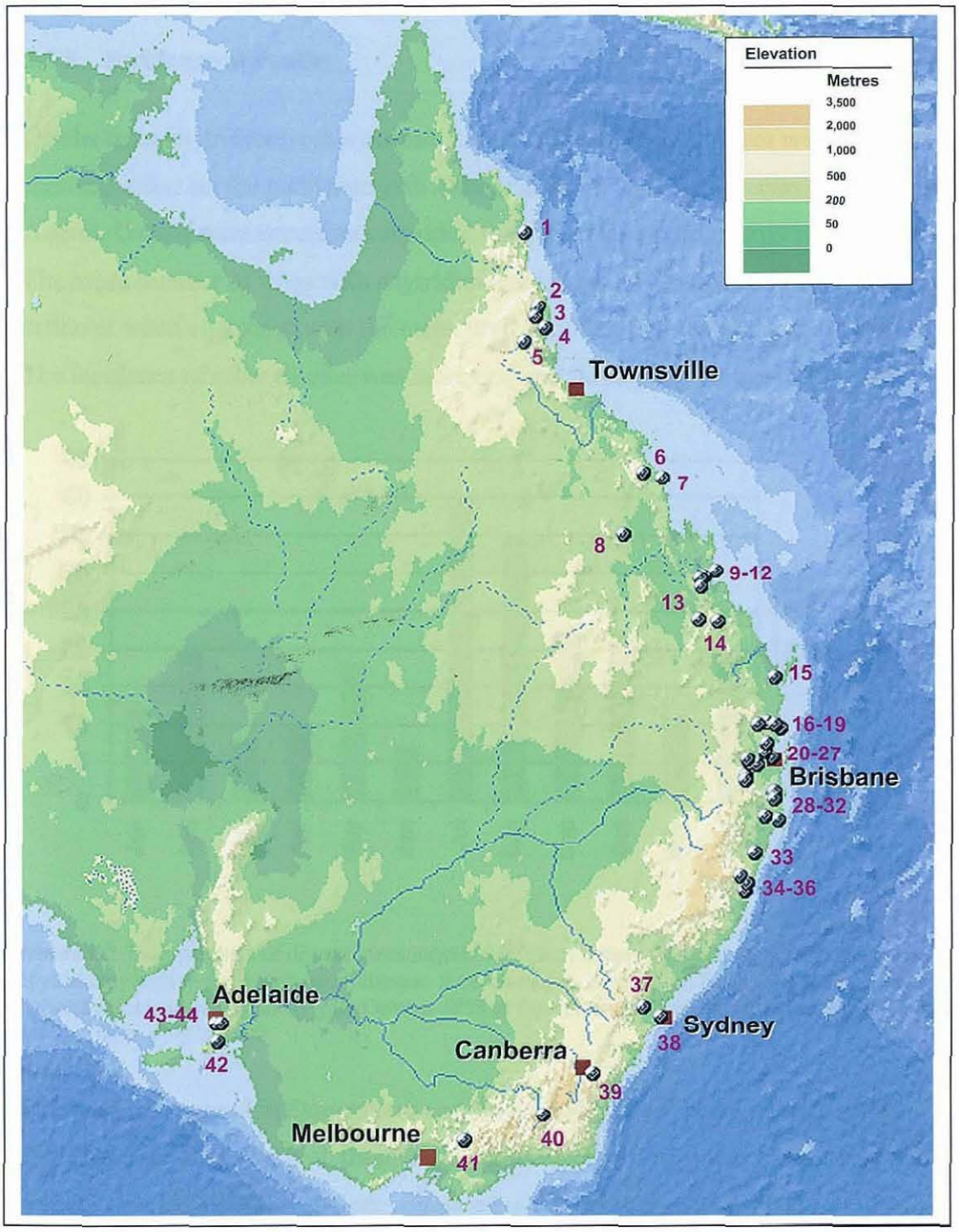


Figure 8.1 Map of eastern Australia showing where ill, wild amphibians were found infected with *Batrachochytrium dendrobatidis*. This distribution correlates with searching intensity.

1. Big Tableland
2. Topaz
3. Sth Johnstone R
4. Tully
5. Kirrama
6. Eungella NP
7. Mackay
8. Dysart
9. Bouldercoumbe
10. Gracemere
11. Rockhampton
12. Kinka Beach
13. Biloela
14. Kroombit Tops
15. Maryborough
16. Six Mile Ck
17. Mary R
18. Triunia SF
19. Buderim
20. Mt Mee
21. Mt Glorious
22. Ashgrove
23. Lockrose
24. Laidley
25. Thagoona
26. Goomburra
27. Cunningham's Gap
28. Canungra Ck
29. Lamington NP
30. Mebbin SF
31. Casino
32. Alstonville
33. Grafton
34. Dorrigo
35. Bellingen
36. Bowraville
37. Springwood
38. Homebush
39. Hoskinstown
40. Kosciuszko NP
41. Southern Highlands
42. Mt Compass
43. Woodville
44. Campbelltown

8.3.5 Incidence of deaths

Deaths from chytridiomycosis occurred at any time of year but there was a distinct seasonal effect on the incidence with 48% of cases in wild frogs occurring in July and August. Deaths were sometimes reported to occur after a sudden drop in temperature. The mean number of frogs with chytridiomycosis for July and August was significantly different when compared with the mean level for other months of the year ($P < 0.01$). The incidence of other disease was more uniform throughout the year (Fig. 8.2).

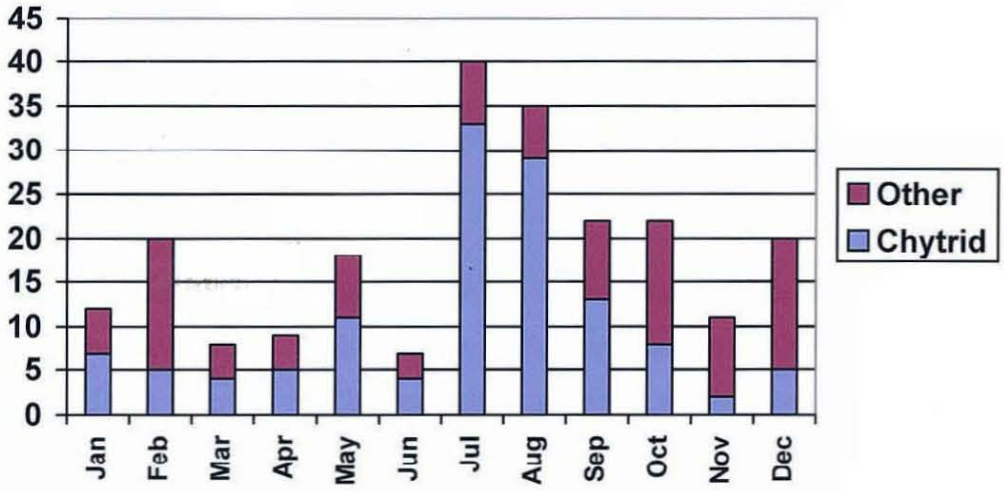


Figure 8.2 Total numbers of ill post-metamorphic wild frogs examined, comparing frogs diagnosed with chytridiomycosis to frogs with other diseases for each month. Includes all frogs used in the survey (where collection dates were provided) from October 1993 to December 2000, although searching intensity was greatest from 1996 to 2000.

This pattern occurred in Queensland and New South Wales where most frogs were collected (Fig. 8.3). For both these states the numbers of frogs that died in July and August were significantly greater compared with the other months ($P < 0.01$). Frogs collected from Victoria and South Australia are included in this graph but as these frogs were taken from only a few outbreaks, this data may not be representative of seasonal effects. However, the only frogs collected from February and March were from the foothills of the Southern Highlands in Victoria in 1998. Frogs from Western Australia were submitted to the Western Australian Museum and are not included here.

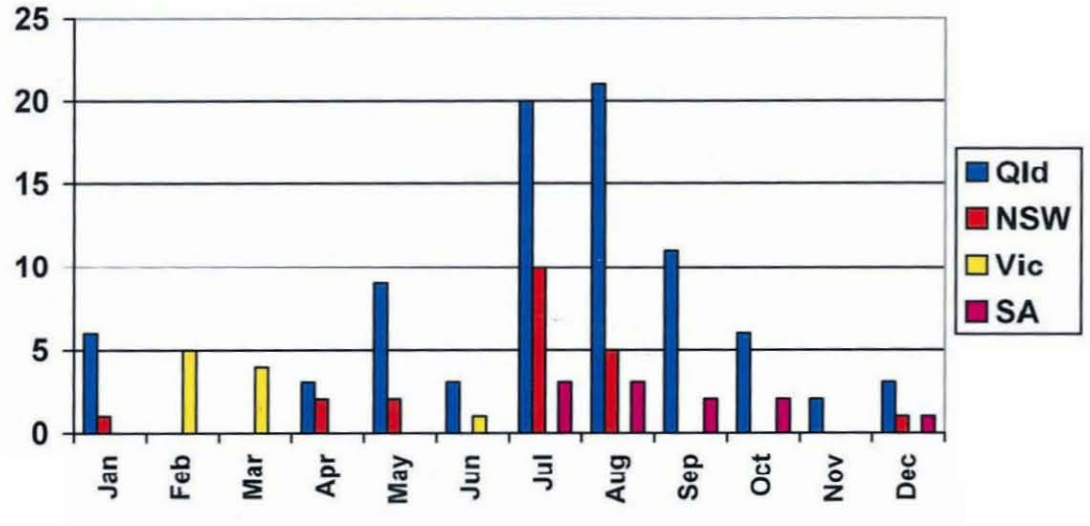


Figure 8.3 Total numbers of wild sick frogs diagnosed with chytridiomycosis from each state per month between October 1993 and December 2000.

When the number of frogs received with chytridiomycosis is graphed over five years, it can be seen that there is a peak in winter each year (Fig. 8.4). When each month was considered as a group, the difference between months was highly significant ($P < 0.001$)

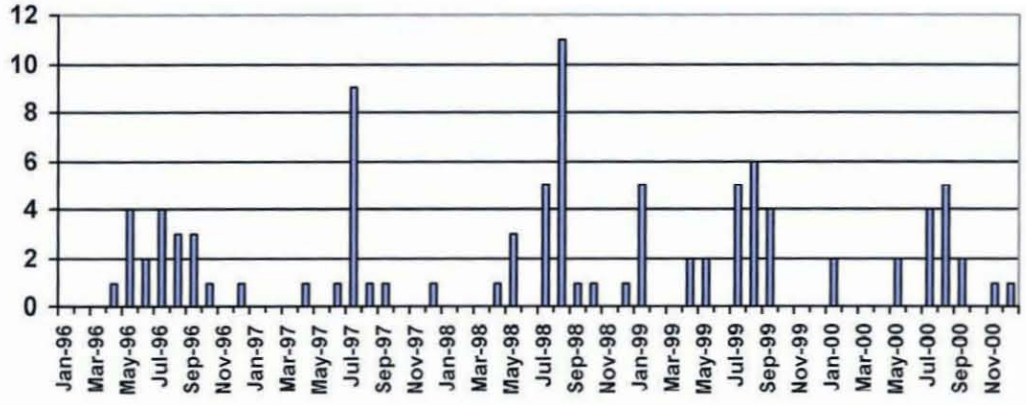


Figure 8.4 Numbers of wild frogs from Queensland and NSW diagnosed with chytridiomycosis from 1996 to 2000. The trend of increased disease in winter was repeated each year.

8.3.6 Impacts

In this survey, chytridiomycosis was identified in mass mortality events at the time of declines, and in individual and mass deaths of frogs in populations that had previously

declined or were apparently stable. Mortality occurred in both high and low altitude sites, in undisturbed areas and in populated areas. Included here are selected examples of cases where I examined frogs with chytridiomycosis, together with published or unpublished observations from herpetologists on the numbers of ill frogs found and affects on population levels. Accession numbers are provided as a link to the full list of cases in Appendix 1, which includes more detail on each frog.

8.3.6.1. Mass mortality and declines in endangered frogs at protected sites

In two instances, chytridiomycosis was diagnosed in a mass mortality of endangered frogs in protected high altitude sites leading to local extinction of some species and total extinction of one species.

Epidemic at Big Tableland: An outbreak of chytridiomycosis associated with extinction of *T. acutirostris* at Big Tableland (altitude 620 m) near Cooktown in north Queensland (15°42'S, 145°16'E). This species occurred only in a small range that extended 310 km in upland (altitude 300-1300 m) wet tropics from Mt Graham north to Big Tableland (McDonald, 1992). Populations had been abundant until declines began in 1990 in the southern end of the range and progressed to the north. A precipitous decline occurred in the last population at Big Tableland in late 1993 with most frogs disappearing over three months (Fig. 2.1). *L. nannotis* and *L. rheocola* at Big Tableland declined in a similar pattern at this time and became locally extinct, however lowland populations on the same watercourses persisted (McDonald and Alford, 1999). Frogs from all three species were found ill or dead and diagnosed with chytridiomycosis from October 1993 to January 1994. These were 2/2 *T. acutirostris*, 2/3 *L. rheocola* and 2/4 *L. nannotis* that were examined (93 290) (Berger et al., 1998). Numbers of *L. genimaculata* at this site also declined (McDonald and Alford, 1999) but subsequently recovered to close to former levels (Speare et al., 2001). Surviving tadpoles of *T. acutirostris* that had been collected in September and December 1993 for raising in captivity died after metamorphosis due to chytridiomycosis (Slocombe, pers comm 1997; Mahony et al., 1999;). Wild adults collected in September and October 1993 that were taken to James Cook University also died within weeks of collection, and 5/7 were diagnosed with chytridiomycosis (93 290) (Speare, 1995; Berger et al., 1998; Mahony et al., 1999).

Epidemic at Kosciuszko National Park: A sudden decline of the endangered frog *L. spenceri* occurred in 1996 in a previously stable and abundant population at Bogong Creek (altitude 1100 m) in Kosciuszko National Park and only one frog remained in 1999 (Gillespie and Hines, 1999; Gillespie and Marantelli, 2000). In the summer of 1995/1996 several frogs were found dead and chytridiomycosis was diagnosed in one that was found moribund (96 370/1). In a retrospective survey of 95 toe clips from 1994 (16 toes), 1995 (59 toes) and 1996 (20 toes), the first appearance of the fungus was in March 1996, the last time that frogs were seen in high numbers. In February and March 1998, chytridiomycosis was also found in 4/4 dead *L. spenceri* (98 320/9,10,11,12) and 3/3 dead *L. lesueuri* (98 320/6,7,8) from lowland sites in Victoria at the other end of the distribution of *L. spenceri*, but these populations did not decline (Gillespie and Hines, 1999).

8.3.6.2 Deaths in a stable upland population

Sporadic deaths of individuals or groups of rainforest frogs occurred in upland areas where declines had previously occurred and also in lowland areas where populations appeared unaffected.

Deaths at Main Range: Goomburra (27°59'S, 152°22'E) and Cunningham's Gap (28°3'S, 152°22'E) are 700 m above sea level on Main Range in south-east Queensland. These areas have been regularly monitored since 1996 as they contain significant populations of the endangered frog *M. fleayi*. *M. fleayi* is a poorly known species that disappeared from some sites at which it was known and may have declined at others (Hines et al., 1999). The timing of these declines is not known but is likely to be in the period from the late 1970s to 1980s (Harry Hines, pers comm 2001). Sick and dead frogs from endangered and common species were occasionally found at these sites, but the population of *M. fleayi* has remained stable since monitoring began (Harry Hines, pers comm 2001). The following frogs were found sick and diagnosed with chytridiomycosis - four *M. fleayi* in August and September 1996 (96 962/1,4,5,14), one *L. dumerilii* in December 1996 (96 1429/1), one *L. lesueuri* in April 1997 (97 574/1), one *L. fletcheri* in January 1999 (99 951/3), and one *L. chloris* in August 1999 (99 1385/13). Nine out of ten tadpoles of *Mixophyes* sp. were found to be carrying *B. dendrobatidis* in their mouthparts (96 1428, 99 951/7).

8.3.6.3 Mass mortality in common lowland frogs

Outbreaks that occurred in winter on private properties in south-east Queensland were often associated with local declines in common frogs. Deaths in frogs around houses were often closely observed by members of the public, and reports from residents of greater than 20 dead frogs in a few weeks were not uncommon. Four examples are given below that were investigated by Harry Hines of Queensland Parks and Wildlife Service.

Deaths of *L. caerulea* at Laidley: At a property in a rural residential area in Laidley (27°37'S, 152°23'E), south-east Queensland, *L. caerulea* were found dying in June and July of 1996. The residents had not observed any unusual mortality in the previous ten years. Nine ill or dead frogs were collected in June and two in early July. A search on the 21st June did not reveal any live *L. caerulea*, although usually there were large numbers (about 100) around the house and garden. A few healthy *L. rubella* were found. Three dead *L. caerulea* that were examined had chytridiomycosis (96 961/12,18,22). Recently rat bait containing brodifacoun had been used around the house and diazinon fly spray was used in nearby cattle yards. Deaths in *L. caerulea* were also reported at a nearby property at this time.

Deaths of *L. caerulea* at Thagoona: At a property in a rural residential area in Thagoona (27°37'S, 152°37'E), in south-east Queensland, mass die-offs in *L. caerulea* occurred in the winters of 1996 and 1997. In 1996 over 100 dead frogs were counted. About 20 subadults and adults were seen alive at the end of winter, but metamorphs, which were abundant the previous year, were not seen. A significant reduction in the numbers of *B. marinus* and *L. rubella* was also noted although these species were not found dead. In 1997 at least ten *L. caerulea* were found ill or dead in June and July and chytridiomycosis was confirmed in four out of five examined (97 845/4,8,11,12). This area is registered as contaminated with heptachlor, an organochlorine insecticide used against termites. Residents suspected this was the cause of the deaths, and it is possible the frogs were weakened by this toxin which is known to cause mortality and reduced reproduction in birds (Wobeser, 1994).

Deaths of *A. brevis* in Brisbane: In Ashgrove in urban Brisbane (27°26'S, 152°59'E), many dead *A. brevis* were found in a garden pond in 1998 and chytridiomycosis was

confirmed in 7/8 submitted for testing (98 1159). Five were seen dead in early July and six more were found between 7th and 26th August. *Lim. peronii* and *L. gracilenta* also used the pond but none were found dead. In August about eight *Lim. peronii* were heard calling as well as two *A. brevis*.

Deaths of *Lit. peronii* at Lockrose: On a property at Lockrose (27°29'S,152°29'E) in a rural residential area in south-east Queensland, at least eight *Lit. peronii*, four *L. caerulea* and two *L. terraereginae* were found dead or dying in 2000. Deaths began in April with most occurring in July and August. The *L. caerulea* were usually found alive showing typical signs of chytridiomycosis while the *Lit. peronii* were usually found floating in a pond dead. Chytridiomycosis was confirmed in all seven *Lit. peronii* that were submitted (00 1879). Several other species of frogs were present that were not found ill including *L. nasuta*, *L rubella*, *C. parinsignifera*, *L. alboguttata* as well as *B. marinus*. The residents reported the abundance of *Lit. peronii* was reduced.

8.4 Discussion

8.4.1 Host range

The broad host range suggests *B. dendrobatidis* can infect all amphibian species, although there may be differences in susceptibility to disease. In addition to the 36 species that were submitted ill in this survey, *B. dendrobatidis* has been found on 8 other native species (Speare et al., 2001) as well as pet axolotyls. Worldwide it has recently been found in at least 50 other amphibian species (Speare et al., 2001).

Of the 44 Australian species found with chytrid infections, one (*T. acutirostris*) is listed as extinct (Environment Protection and Biodiversity Conservation Act, 1999). Eight species are listed as endangered and five as vulnerable (Speare et al., 2001).

The broad host range is important in understanding the declines. It may explain how the organism persists even when numbers of declining species are very low. The rapid spread of *B. dendrobatidis* worldwide would have been assisted by its ability to infect all amphibians. Despite its low host specificity, only some frog species have declined. The reasons may be innate characteristics of the species and/or environmental factors

operating at their geographic location. The declining species from high altitude rainforests in eastern Australia have significantly smaller clutch sizes, occupy restricted geographic ranges, have aquatic larvae associated with streams, and many spend a large proportion of their time in or adjacent to streams (Williams and Hero, 1998; McDonald and Alford, 1999). Populations of these species are therefore less able to recover from declines due to any cause, and also inhabit environments that would support *Batrachochytrium* i.e. cooler, riparian habitats. Other aspects of host biology, genetic factors (see Leberg and Vrijenhoek, 1994) and complex ecological factors affecting the lifecycle of the chytrid, could be important in determining the selectivity of the declines.

The large number of *L. caerulea* found with chytridiomycosis is likely to be due to this species being highly conspicuous and commensal with humans. However, as recent experiments have shown that *L. caerulea* is a highly susceptible species (Katie Ardipraja and Gerry Marantelli, unpub 2001), the large numbers may be a true indication that this species suffers higher mortality in the wild.

Few frogs that were diagnosed with other diseases were infected with *B. dendrobatidis*, demonstrating that *Batrachochytrium* is not a ubiquitous secondary infection.

The high prevalence of non-pathogenic infection in tadpoles in Queensland shows the pervasiveness of this disease. The infection did not appear to affect the tadpoles, which were collected for other reasons. It is not known if these infected animals would have survived metamorphosis, as 100% mortality occurred in captive *M. fasciolatus* metamorphs from infected tadpole groups at the ARC (Chap 5). However, the three ill wild metamorphs of *Mixophyes* sp. that were examined from these sites were not infected, and recruitment was occurring (Harry Hines, pers comm. 2001). During the decline at Big Tableland, mortality rate of adults and metamorphs was high, but tadpoles remained for months after adult frogs disappeared (McDonald and Alford, 1999). However, these tadpoles were likely to be carrying infection as when tadpoles were collected for rearing in captivity few survived past metamorphosis due to chytridiomycosis (Slocombe pers comm, 1997; Mahony et al., 1999).

As chytridiomycosis affects adults, it would be predicted to have a more dramatic effect on population levels than if it killed tadpoles. Adult survival is a major limiting factor

on populations, whereas tadpole mortality is normally high, for example only 5% of *Rana aurora* reached metamorphosis, due to density dependent predation (Calef, 1973).

8.4.2 Distribution

The distribution of chytridiomycosis as determined from examination of sick frogs shows the disease to be widespread in Australia, stretching from near Cooktown in the north down the coast to Victoria, with zones around Adelaide and in WA. These regions include some of the wetter areas of Australia and generally have lower maximum temperatures than inland areas (Bureau of Meteorology, 2001). Frogs and humans are more abundant in these areas, which include sites where declines have been documented and where there is greatest intensity of monitoring.

Many additional records of infected frogs have been found in surveys of toe-clips collected from apparently healthy frogs in the wild or from archived museum specimens. The earliest case found in Australia is from December 1978 in south-east Queensland (Speare et al., 2001). The earliest case in Western Australian is in 1985 in the south, and 612 samples tested before this date were negative for chytridiomycosis (Aplin and Kirkpatrick, 2000). Chytridiomycosis is now distributed over large areas of Western Australia, with many positive samples collected in the southwest and one record from the Kimberley in the north. Data collected on the temporal distribution supports the theory that *B. dendrobatidis* has been introduced to Australia.

In Queensland the appearance of *B. dendrobatidis* in museum specimens coincides with the spread of the declines (Speare et al., 2001). Declines occurred in mountainous areas, but *B. dendrobatidis* was able to spread across lowland areas where declines were not observed. The mechanism of spread of *B. dendrobatidis* is unknown but may involve normal movement of individual infected amphibians, streams, and surface water possibly during rain. Since no resting stage of *B. dendrobatidis* has been found (Longcore et al., 1999), and sporangia and zoospores are killed by drying (Chap 9) it seems likely that spread of disease into new distant zones occurs via the translocation of infected amphibians. Accidental movement of amphibians occurs frequently in transportation of fruit (Marantelli and Hobbs, 2000; O'Dwyer et al., 2000) and amphibians are transported in the pet trade and as laboratory animals (Speare et al.,

2001). Disposal of contaminated water into the environment from aquariums could also be a mechanism of spread.

Recent studies have investigated the prevalence of infection in various habitats. In a survey of stream, pond and terrestrial frogs in eastern NSW, *B. dendrobatidis* was found to be widespread in a range of habitats at low and high altitudes, but was most prevalent in stream frog communities (Mahony, 2000).

Chytridiomycosis has been found in at least three *L. aurea* populations, and although this disease has not been confirmed as a significant factor in declines of this species, consideration of the changes in distribution of *L. aurea* has led to the formulation of some ideas on the impact of the environment on *B. dendrobatidis* (Ross Wellington, pers comm 2001). Most populations at high altitudes and in large permanent water bodies have disappeared. However, *L. aurea* has a relatively high tolerance to salinity and most populations now occur close to the coast or an estuary (Mahony, 1999), perhaps as *B. dendrobatidis* is less salt tolerant than this frog species (Chap 9). The population remaining at the highest altitude is at Hoskintown, NSW at a site contaminated with heavy metals from a gold mine, perhaps as these pollutants have antifungal effects (Ross Wellington, pers comm 2001). Several of the lowland coastal populations are also exposed to contamination, such as from a copper smelter near Port Kembla, NSW and from chromium deposits from an old tannery at a site near Clyde, NSW (Ross Wellington, pers comm 2001). Investigations into the distributional trends revealed by remnant *L. aurea* populations and how pollutants and salinity affect the distribution and impacts of chytridiomycosis may increase our understanding of amphibian declines.

Globally, chytridiomycosis has been recorded from New Zealand, Spain, Germany, Africa, and South, Central and North America, from a broad range of habitats (Speare et al., 2001).

8.4.3 Incidence of deaths

There was a distinct seasonal effect on the incidence of chytridiomycosis with most frogs from Queensland and NSW dying during winter. This pattern was repeated during

the five years of active surveying from 1996 to 2000. Frogs with other diseases came in more regularly throughout the year, demonstrating the seasonal effect is particular to chytridiomycosis and not an effect on incidence of disease in general. The small numbers of frogs collected from South Australia and Victoria preclude conclusions on the incidence in these states. However, wild frogs from Victoria were mostly from a specific die-off in foothill forests in summer.

Experimental studies in upland wet tropics rainforest also demonstrated high rates of mortality in winter from chytridiomycosis in *L. rheocola* translocated from lowland sites (Retallick and Dwyer, 2000).

The increase in chytridiomycosis in winter seems most likely related to colder temperatures. Experimental transmission at different temperatures showed that mortality rate was decreased at 27°C compared with 23°C and 17°C (Chap 7). Deaths in the wild were reported to occur after a cold snap (Keith McDonald, unpub). In Brisbane, July is the coldest month and the mean daily minimum temperatures in July and August are 9.5 and 10.3°C respectively, and the mean daily maximum temperatures are 20.4 and 21.8°C (Bureau of Meteorology, 2001). Although 23°C was the optimum temperature for growth of an isolate of *B. dendrobatidis* (Longcore et al., 1999), perhaps colder temperatures encourage epidemic fungal growth and adversely affect the immune system of frogs. The metabolic rate of a frog is slower at lower temperatures and both acquired and innate (e.g. dermal antimicrobial peptides) defences may be reduced, as discussed in Chapter 7 of this thesis. Outbreaks of saprolegniasis in farmed channel catfish (*Ictalurus punctatus*) are known as "winter kill". This fungal disease occurs due to immunosuppression and increased zoospore concentrations associated with a 10°C drop in water temperature after a severe cold weather front, but fish do not become infected if they are acclimatised to the colder temperatures (~11°C) (Bly et al., 1993, 1996). Immunosuppression was characterised by low mitogen responses. Winter saprolegniasis is considered to be unnatural as the catfish ponds are shallow allowing large temperature fluctuations and the fish are unable to thermoregulate by moving toward preferred temperatures, unlike free-living fish (Bly et al., 1993). High mortality rates in frogs with chytridiomycosis in winter may also be considered unnatural if the disease has been introduced. *B. dendrobatidis* may be the only important fungal

pathogen of Australian frogs with a low temperature preference, so their immune systems may have not been selected by evolution to cope with a high load of pathogens in winter.

There are other seasonal effects besides temperature to be considered. In Queensland, winter is the dry time of year. Shrinkage of water bodies could affect the disease by increasing the concentration of both the fungus and the amphibian hosts. However, less rain may reduce the spread of the waterborne fungus. Seasonal behavioural changes in the interaction between frogs could also affect transmission. Although many frogs are not breeding in winter, some species may congregate in cracks to gain protection from extreme temperature and humidity fluctuations (McDonald and Davies, 1990).

Many acute infectious diseases of humans also occur on a regular seasonal cycle, such as influenza, rotaviral disease and meningococcal disease (Dowell, 2001). Pathogens that cause disease seasonally can be detected in the off-season, but the incidence of disease is low and epidemics do not occur (Dowell, 2001). Dowell (2001) suggests that photoperiod has a large effect on host immunity and may be more important than seasonal weather changes or changes in host behaviour in explaining the seasonal occurrence of some diseases in mammals. Large seasonal variations in immunity occur in ectotherms, generally with maximal immune reactivity occurring in summer (Zapata et al., 1992). These variations occur even when animals are kept at constant temperature and photoperiod, suggesting an endogenous rhythm regulates these processes (Zapata et al., 1992).

However, as *B. dendrobatidis* probably grows in the environment, seasonal effects on the occurrence of the fungus in water bodies are likely to be important. Many other chytridiomycetes are stimulated to bloom with temperature changes, with different species showing different temperature preferences (Sparrow, 1968). If tests are developed to detect *B. dendrobatidis* in the environment, the distribution and incidence of the putative free-living organism can be studied. A combination of reduced host immunity and increased pathogen abundance, as occurs with winter saprolegniasis, appears the most likely hypothesis for explaining the seasonal epidemics. These effects are likely to be mediated by temperature, but there may also be other factors involved.

As with winter saprolegniasis, the rapidity of the decrease in temperature may be important.

Although the increased disease occurrence in colder months generally supports the theory that high altitude populations have disappeared due to lower temperatures and hence greater susceptibility to chytridiomycosis, it is difficult to explain how this effect is consistent over various latitudes. Within Queensland there is overlap between temperatures at low altitudes in the south and at high altitudes in the north. There is also overlap between temperatures at high and low altitudes during the year (Alford and Richards, 1997; Bureau of Meteorology, 2001). As the "high altitude" sites of frog disappearance are as low as 400 m, it is interesting that slight differences in temperature or other environmental factors could be so significant. In addition, large epidemics in *L. caerulea* occurred at low altitudes in south-east Queensland, so the effects of altitude are not straightforward. In these populations of *L. caerulea* there may be other predisposing factors such as unnaturally high densities of frogs and permanent water supplies associated with humans (Harry Hines, pers comm 2001).

Altitude also affects the biology of the host, causing differential growth and longevity (Esteban and Sanchiz, 2000). The distributions of other diseases in wildlife have been affected by altitude, but often due to the distribution of insect vectors (Wobeser, 1994). Although *Batrachochytrium* has not been found in non-amphibian hosts, it is possible that other cold-blooded animals with keratinised surfaces could harbour infections and play a role in the persistence and spread of the disease.

8.4.4 Impact

Catastrophic declines that occurred with outbreaks of chytridiomycosis are likely to have occurred with the initial arrival of this pathogen, for example at Big Tableland and Bogong Creek. Frogs at these sites had been abundant until these outbreaks were witnessed, and populations of some species did not survive. In all other areas where sick frogs were collected, the disease appeared to have arrived at an earlier time and become endemic in the population, with deaths in individuals or outbreaks being detected sporadically. In these areas season had a large effect on the outbreaks of disease. Documented declines previously occurred in many areas within the distribution of

Batrachochytrium, and many more declines probably went unnoticed. In populations that survived the initial declines the ongoing effects of chytridiomycosis are mostly unknown, although increased mortality rates are likely to reduce overall population levels. Some species have managed to recover to near former numbers after an initial sudden decline, such as *L. genimaculata* that declined on Big Tableland. Possibly a balance between *L. genimaculata* and *B. dendrobatidis* has been reached (Speare et al., 2001).

Observations of particular frog species surviving at times of mass mortality suggests there are distinct differences in vulnerability to chytridiomycosis.

Mortality rates in the wild are difficult to assess by discovery of sick and dead animals, as only a small proportion are ever found. Small animals rapidly disappear after death; for example 75% of passerine bird carcasses were found to disappear within one day (Wobeser, 1994). Significant numbers of dead animals are only found when they are dying at rapid rates so that normal scavenging mechanisms for removal are overloaded (Wobeser, 1994).

Observations of mass mortality and population declines demonstrate that chytridiomycosis can cause high mortality rates in the wild (as in captivity), probably most severe when first arriving in an area, and in winter in endemic areas. Even if chytridiomycosis did not cause 100% mortality, some populations may have been so reduced after the arrival of *B. dendrobatidis* that their vulnerability to extinction due to stochastic events was greatly increased, or numbers were too low for successful breeding to occur.

8.4.5 Future directions

This study has tackled broad aspects of host range, distribution, incidence and impacts of chytridiomycosis around Australia. As it was conducted opportunistically, the analysis presented above is simplistic. More focussed investigations of epidemiology within a population remain to be done. Current studies on healthy animals will determine prevalence in populations. Although the host range is broad, differences in susceptibility between species need to be studied experimentally and in nature. More

thorough analysis of distribution including habitat and climatic differences could be examined. The complete distribution of *B. dendrobatidis* needs to be determined to assess the risks of the disease to frogs in areas that may be free of infection. Incidence could be compared with weather patterns. The mechanism of how colder temperature effects host and pathogen to result in increased disease needs to be elucidated. Population impacts could be determined by looking at prevalence in wild tadpoles and adults and correlating this with abundance, recruitment, life expectancy and survival rates, as well as more subtle changes in breeding success and behaviour. However, this data will be more useful if data is available from the same populations before the arrival of *B. dendrobatidis* for comparison.

As chytridiomycosis is the most common disease of Australian frogs and is widespread, it would be expected to have a large impact on the abundance of frogs. A few frog species have not survived this threat, and although the majority of species remain, the current impact of chytridiomycosis is yet to be determined. This situation is still dynamic and although the final balance between *B. dendrobatidis* and various host species cannot be predicted, the abundance of frogs may be permanently reduced and additional species could disappear.

CHAPTER 9
Treatment and disinfection

9.1 Introduction

A treatment for chytridiomycosis would be useful in a number of situations. An obvious use would be for treating epidemics in captive frogs such as collections of endangered species as well as pet or research amphibians. In establishing a captive colony of a declining species, treatment of remaining tadpoles may be more important than treatment of adults which may already be ill or few in number (Marantelli et al., 2000). A method for eliminating infection would have important applications in preventing spread of infection, as apparently healthy animals may be carrying or incubating the disease. The risks of spread are greatest when frogs are being moved - such as during release or translocation of animals in the wild, or movement of animals into or between captive situations.

Wildlife managers are concerned about the potential for movement of *B. dendrobatidis* both within and between countries. Hence, if a regime was available that was safe and could effectively eliminate *Batrachochytrium* from the skin of amphibians, it could be used on all amphibians prior to movement. This could be made a regulatory requirement associated with permits. International movements of amphibians will come under increasing attention since amphibian chytridiomycosis was approved for inclusion in 2001 on the OIE Wildlife List of diseases (Stephanie Haigh, pers comm 2001). Therefore, developing a safe, effective regime for elimination of *Batrachochytrium* in amphibians in captivity is an important tool in strategies to reduce the risks of chytridiomycosis in wild amphibians.

The essential characteristics of an antifungal regime are that it is highly effective, is able to be used in bath treatment of numerous animals, and has a high safety margin for use in valuable breeding stock of endangered frogs and tadpoles. Ideally it would be cheap, readily available and work rapidly so that it was effective in a single application. Frogs in late stages of disease are unlikely to survive even with treatment, but it is hoped

methods to eliminate infection in healthy carriers and in frogs showing early signs of disease can be developed.

Treatments currently recommended for fungal infections in amphibians involve bathing in cheap, non-prescription compounds such as methylene blue, sodium chloride, malachite green and benzalkonium chloride, injection of drugs such as miconazole and amphotericin B, or oral treatment with ketoconazole (Raphael, 1993; Hulst, 1999). Oral itraconazole was used successfully for treatment of a mycotic dermatitis that was diagnosed as due to *Basidiobolus ranarum* (Taylor et al., 1999c), but was likely to be chytridiomycosis. Recently, bathing in 0.01% itraconazole suspension for 5 minutes a day for 11 days was reported to successfully treat chytridiomycosis in *Dendrobates tinctorius* (Nichols and Lamirande, 2000).

The work described here on sensitivity of *B. dendrobatidis* to antifungal compounds *in vitro* was used to choose drugs for a treatment trial using tadpoles of *M. fasciolatus* (McInnes, 1999). Itraconazole was found to be highly toxic to tadpoles even at low concentrations and was discarded, while safe levels of three drugs were tested on infected tadpoles. Fluconazole (6.6 mg/L, 6 hours per day for 7 days) and benzalkonium chloride (1 mg/L, 3 hours per day for 6 days) moderately reduced prevalence of infection, but methylene blue (3 mg/L for 72 hours, then 6 mg/L for 72 hours) had no significant effect (McInnes, 1999). Methylene blue and benzalkonium chloride were toxic to tadpoles at higher levels.

Disinfectants are used for preventing spread of amphibian disease in the wild by cleaning equipment that comes in contact with frogs such as nets and toe-clipping instruments, as well as cleaning boots and tyres. In captivity disinfection is a crucial part of quarantine and is performed when cleaning enclosures before reuse, and for equipment that is moved between enclosures. An ideal disinfectant would work rapidly, would be safe for the human operator, would have minimal effect on equipment, would have a low risk of contaminating the environment, and any residual concentration would have no effect on amphibians. Most commonly used disinfectants are effective against fungi. As a resistant resting spore has not been detected for *B. dendrobatidis* (Longcore et al., 1999), an effective disinfection regime needs to kill zoospores and zoosporangia only.

Methods to determine the sensitivity of *B. dendrobatidis* to antifungal drugs and disinfectants were developed. The test for antifungals was aimed at finding the lowest concentration that inhibited zoospore encystment and growth. Routinely used concentrations of three disinfectants were tested to find the minimum time needed to kill cultures containing all stages.

The effects of heat and drying on survival of *B. dendrobatidis* were also investigated. Longcore et al. (1999) found that cultures grew slowly at 28°C but did not grow substantially at 29°C. In this study I investigated the time required to kill cultures at temperatures from 32°C to 100°C.

In an experiment conducted with Gerry Marantelli at the Amphibian Research Centre, fluconazole and benzalkonium chloride were used to treat experimentally infected frogs.

Results from this work showed that standard disinfectants, heat and drying were effective in killing sporangia and can be used for disinfection. Many antifungal compounds were effective in preventing zoospore encystment and growth *in vitro*. The treatment regimes using benzalkonium chloride and fluconazole *in vivo* were not effective.

9.2 Methods

9.2.1 Evaluating antifungals *in vitro*

An isolate of *B. dendrobatidis* obtained from a wild *N. dayi* from Tully (98 1810/3) was used for all *in vitro* experiments described below. The culture was isolated and maintained on TGhL agar and broth by routine methods (Longcore et al., 1999; Chap 3).

A number of methods are used for testing antifungal chemotherapeutics on pathogenic fungi, but these did not work with *Batrachochytrium*. For rapidly growing fungi, a method similar to that used for testing bacterial antibiotic sensitivity is used in diagnostic mycology laboratories. Tablets containing antifungals are placed on agar

plates covered by a diffuse inoculum of fungi. The antifungal compound diffuses into the agar forming a gradient, and the zone of inhibition is measured after one or two days. Tablets were obtained containing amphotericin B, fluconazole and itraconazole (Rosco Neo-Sensitabs) to trial this method. Due to the slow growth of *Batrachochytrium*, a one or two day incubation did not reveal a distinct zone of inhibition, and when left for longer times, too much diffusion of the compound had occurred. For yeasts and yeast-like fungi such as *Candida* and *Cryptococcus*, a kit is available commercially (Sensititre YeastOne Antifungal Panel) consisting of multi-well plates of drugs at various concentrations to which the test organism is added. A colour change in the medium indicates growth. These tests use RPMI as the growth medium, and unfortunately this medium did not support *B. dendrobatidis*.

A method was developed to evaluate the sensitivity of *B. dendrobatidis* to antifungals by observing inhibition of zoospore encystment and growth. Flat bottom 96 well plates (Nunclon, Denmark) were prepared with doubling dilutions of antifungal compounds, similar to a dilution method used previously for testing effects of skin peptides (Louise Rollins-Smith, unpub). Compounds were diluted in distilled water to 1 mg/ml. This was diluted to 200 μg/ml in TGhL broth, and then serially diluted in doubling dilutions of broth 6 times down to 3.125 μg/ml. 50 μl of each dilution was placed in 5 wells, along with 5 control wells of 50 μl TGhL broth only.

Sixteen compounds were tested, including human antifungal drugs and aquarium medications as well as three peptides isolated from frog skin (Table 9.1).

Zoospores were collected in distilled water from 4-5 day old TGhL agar plates and counted using a haemocytometer. 50 μl containing about 50,000 zoospores in water was added to each well. This resulted in final concentrations of the compounds ranging from 100 μg/ml down to 1.56 μg/ml, with 5 wells at each dilution. Although this diluted the TGhL broth to half strength, cultures still grew well. Initially zoospores were collected in broth instead of distilled water, but they did not survive after being added to wells.

Plates were sealed with Parafilm® to prevent evaporation and were incubated at 18-20°C. They were examined with an inverted microscope to determine the minimum

concentrations that 1) caused loss of zoospore motility after 30 minutes and 2) inhibited encystment and growth (as examined after 4 days).

Each assay was repeated on a separate day. If growth occurred at all concentrations (as with povidone iodine, ethanol and sodium chloride), higher concentrations were tested. Benzalkonium chloride was tested down to 0.78 μg/ml.

9.2.2 Disinfection

9.2.2.1 Chemicals

A method was used to determine the minimum time required to kill fungi *in vitro* using set concentrations of disinfectants. Cultures were grown as monolayers in 96 well plates, by adding 100 μl of 4 day old broth cultures with about 10,000 sporangia to each well and incubating for four days. The monolayers were exposed to disinfectants by replacing the broth with 100 μl of 70% ethanol, 1 mg/ml Virkon or 1 mg/ml benzalkonium chloride. For each disinfectant, 8 wells were left for 20 seconds and 8 for 5 minutes. Each well was then washed three times with 100 μl broth, then incubated for one week at 18-20°C. Plates were examined for growth and the presence of motile zoospores on an inverted microscope. Eight wells were washed with fresh broth and kept as controls.

9.2.2.2 Heat

Flasks of broth cultures were incubated at various temperatures to determine the time required to kill all sporangia. 5 ml of 4 day old broth culture with about 50,000 sporangia was added to 25 cm^2 plastic tissue culture flasks (Corning). These were placed in incubators at 23°C, 26°C, 32°C, 37°C, 47°C, 60°C or immersed in water at 100°C. Prior calibration of incubators showed they varied up to 1°C from the set temperature. After some pilot trials, zoospores and sporangia in flasks were exposed to the 6 temperatures for a range of the following times: 2 minutes, 5 minutes, 30 minutes, 1 hour, 2 hours, 15 hours, 24 hours, 48 hours or 96 hours. Two flasks were used at each temperature-time point. Flasks were then incubated at 23°C for 2 weeks and checked for growth. Growth was obvious by the opacity of the broth and a layer of sporangia over the surface of the plastic. Flasks containing proliferating cultures were discarded

whereas flasks with no observable growth were incubated for another two weeks. This experiment was repeated with flasks left in incubators over the crucial times.

In addition, to test the effect of rinsing with water at 100°C, a monolayer of culture was grown in a 24 well plate (Becton Dickinson) with about 20,000 sporangia per well. After three days, broth was removed from 18 wells, replaced with 3 ml boiled water for 1 minute, then the broth replaced. Six wells were kept as controls.

9.2.2.3 Drying

To test the effect of drying, monolayers of culture were grown in a 24 well plate with 20,000 sporangia per well. After 3 days, broth was removed from 18 wells and the plate was left open in a Class 2 biosafety cabinet at 22°C. Broth was removed and replaced with fresh broth in 6 control wells. After one hour broth was replaced on 12 dried wells, and after 5 hours broth was replaced for the remaining 6 wells. Initially drying was attempted using monolayers in 96 well plates, however water remained along edges of wells for over 40 minutes. Plates were monitored for growth and the presence of motile zoospores for two weeks using an inverted microscope.

9.2.3 Testing antifungals in frogs

An *in vivo* treatment experiment was designed and conducted in collaboration with Gerry Marantelli. The animals were housed at the Amphibian Research Centre (ARC). I infected the frogs and analysed the skin samples, while treatments, husbandry, observations, euthanasia and preservation of specimens were performed by Gerry Marantelli, Raelene Hobbs and other staff at the ARC. After consideration of the *in vitro* sensitivity work and of McInnes (1999), fluconazole and benzalkonium chloride were selected for bath treatment of experimentally infected frogs. Field workers had requested a short-term treatment that could be used when translocating animals, so treatment with each drug was tried for three and seven days.

For animal infections, an isolate of *B. dendrobatidis* was used that had been cultured from a wild *L. caerulea* from Rockhampton, Queensland (accession no. 99 1385/12), nine months previously. The culture had been maintained in TGhL broth at 4°C.

Zoospores were collected off a 5 day old culture on TGhL agar by rinsing the plate with sterile distilled water. Zoospores were counted with a haemocytometer and were stored in a 25 cm^2 plastic tissue culture flask and kept in an esky with an ice block at about 15°C for 6 hours until frogs were exposed.

For this experiment we used three to six week old captive bred metamorphs of *L. caerulea* that had been bred from banana box frogs transported from Queensland. They were housed individually in isolated plastic tubs with an automated flow through of tap water. Room temperature varied between 16 - 20°C. The frogs were fed crickets dusted with a vitamin and mineral supplement.

Frogs were exposed to zoospores by placing them in yellow-top 45 mm x 50 mm specimen containers (Labserv) with 5 ml distilled water. Air holes had been punched in the lids. Fourteen frogs were kept as controls and were given water washed off an agar plate, and 50 frogs were exposed to 50,000 zoospores each. Frogs were moved to individual tubs after 24 hours, however *L. caerulea* spent most of the time sitting on the container wall, so their total contact time with the inoculated water was unknown.

At 19 days after exposure, toe-clips were removed for histology. Infection was confirmed in 21/40 toes examined, and treatment with antifungals commenced. Benzalkonium chloride was used at 1 mg/L and fluconazole at 25 mg/L. Frogs were sprayed thoroughly in these solutions twice a day, and water in the enclosure was kept at this concentration. For each drug, nine frogs were treated for three days and nine for seven days. Fourteen infected frogs were left untreated. Frogs were observed daily and were euthanased by bathing in 0.2 % MS222 if they showed obvious clinical signs of disease. They were preserved in 10% buffered neutral formalin. The experiment was terminated at day 85 with euthanasia of uninfected controls. Chytridiomycosis was diagnosed by examination of skin scrapings from all frogs and histology on four frogs from each group. Groups were compared for significant differences using the Student's non-paired two sample *t*-test one-tailed in Microsoft Excel.

9.3 Results

Cultured *B. dendrobatidis* was found to be highly sensitive to most chemicals and to heat and drying. However, the two treatments we tested were not successful in eliminating infection on the frogs.

9.3.1 Antifungals *in vitro*

Table 9.1 Concentrations of antifungal compounds that inhibited encystment and growth of zoospores of *Batrachochytrium dendrobatidis,* compared with the recommended doses for treatment (where available).

Compound	Minimum concentration to cause loss of motility at 30 minutes	Minimum inhibitory concentration (MIC)	(Tradename) Manufacturer	Recommended concentrations for treatment of amphibians (Hulst, 1999)
Benzalkonium chloride	6.25 µg/ml	0.78 µglml	Apex	0.25 mg/L 72 hrs 2 mg/L 1hr daily
Povidone iodine	312.5 µg/ml	312.5 µg/ml (= 31.25 µg/ml available iodine)	(Betadine) Faulding pharmaceuticals	-
Amphotericin B	50 µg/ml	3.125 µg/ml	(Fungizone) Apothecon	1mg/kg daily by intraperitoneal inoculation
Fluconazole	>100 µg/ml	stunted encystment in all rows	(Diflucan) Pfizer	-
Cetrimide/ chlorhexidine	12.5/1.25 µg/ml	1.56/0.156 µg/ml	Deltawest	-
Ethanol	>10%	>10%	Univar	-
Itraconazole	>100 µg/ml	stunted encystment in all rows	(Sporanox) Janssen	-
Methylene blue	>100 µg/ml	1.56 µg/ml	Aristopet	2-4 mg/L
Mercurochrome	12.5 µglml	6.25 µg/ml	Sigma	3 mg/L bath 72 hrs
Sodium chloride	6.25 mg/ml	12.5 mg/ml (stunted growth 6.25 mg/ml)	AnalaR	4-6 g/L bath 3-5 d 25 g/L dip 10 min
Virkon#	12.5 µg./ml	3.125 (stunted growth 1.56)	Antec International	-
Acriflavin/ malachite green	0.625/0.125 µg/ml	0.156/0.0312 µg/ml	(Antifungus) Alive-O	0.25/0.05 mg/L (recommended dose on bottle)
Enilconazole	1.56 µg/ml	1.56 µg/ml	(Imaverol) Janssen pharmaceutica	-
Citropin 1.1	25 µg/ml	12.5 µg/ml	John Bowie*	-
Caerin 1.9	50 µg/ml	12.5 µg/ml	John Bowie	-
Caerin 1.1	>100 µg/ml	12.5 µg/ml	John Bowie	-

Virkon is the tradename for a mixture of potassium peroxy sulphate compound, sulphamic acid, alkyl benzene sulphate, alkyl hexameta phosphate, malic acid, and sodium chloride (Ausmed material safety data sheet)

* Obtained from John Bowie, Dept of Chemistry, University of Adelaide

Most assays showed a distinct cut-off between the concentration that caused death of all zoospores and the concentration that allowed encystment and growth (Table 9.1). However, with itraconazole and fluconazole, encystment of zoospores occurred in all

wells and spores grew to two to three times the size of zoospores then ceased to grow. Observation of these plates for an additional week showed no further growth or maturation, and *B. dendrobatidis* was considered to be sensitive to these dilutions. Similarly, small fungal colonies that grow within a zone of inhibition around Neo-Sensitabs® containing azole antifungals have not been considered resistant (Mycology Online, 2000).

9.3.2 Disinfection

9.3.2.1 Chemicals

Exposure to 70% ethanol, 1 mg/ml Virkon or 1 mg/ml benzalkonium chloride for 20 seconds resulted in death of all sporangia. Sporangia killed with ethanol appeared shrivelled.

9.3.2.2 Heat

Sporangia were killed in flasks kept at temperatures down to 32°C (Table 9.2). At 37°C or above, death occurred after short incubation times.

When flasks were washed with boiling water for 1 minute, this resulted in detachment and loss of many sporangia from the plastic surface, but none that remained survived.

Table 9.2 Times at which all sporangia were killed at different temperatures.

Temperature	Time at which all sporangia were killed
100°C	1 min
60°C	5 min
47°C	30 min
37°C	4 hr
32°C	96 hr
26°C	no death
23°C	no death

9.3.2.3 Drying

All sporangia were killed by drying for one or more hours. Sporangia appeared shrivelled, and the walls were less defined and less refractile. Cultures in control wells remained healthy for the two weeks of observations.

9.3.3 Antifungals in frogs

The treatments were not effective and all infected frogs became sick, and either died or were euthanased. However, treated frogs as a group lived significantly longer than the untreated group ($P < 0.05$) (Table 9.3). There were no significant differences between treatment groups ($P > 0.05$). Examination of skin scrapings and histology showed that all frogs in the infected groups, that were treated or not, died with chytridiomycosis. Four of the nine frogs treated with benzalkonium chloride had light infections with sporangia. Infections were not seen in skin scrapings of any of the controls demonstrating that each tub was kept effectively isolated, as the controls were randomly placed among infected frogs. Three of 15 uninfected controls died at days 79, 76 and 83 and the rest remained healthy and were euthanased at day 85. Histology from the three control frogs that died did not reveal the cause of death.

Table 9.3 Mean time till death and range of times till death for each treatment.

Treatment	Mean time till death (days)	Time till death for each frog (days)
Untreated	37.9	30, 30, 33, 33, 34, 34, 34, 36, 37, 38, 38, 43, 43, 67
Benzalkonium chloride (3 days)	44.3	37, 37, 37, 38, 44, 44, 48, 54, 60
Benzalkonium chloride (7 days)	42.7	21, 34, 39, 41, 42, 43, 44, 53, 67
Fluconazole (3 days)	44.1	29, 32, 32, 37, 38, 41, 47, 65, 76
Fluconazole (7 days)	43.7	32, 35, 39, 40, 45, 45, 46, 51, 60

9.4 Discussion

A large range of compounds was effective against *B. dendrobatidis in vitro*. Zoospores of *B. dendrobatidis* encysted and grew in 0.625% saline but not in 1.25%. This precludes its use as an antifungal treatment for most amphibian species, but is interesting information when considering the coastal distribution of some remaining populations of *L. aurea* (Mahony, 1999). Three peptides from amphibian skin - caerin 1.9, citropin and caerin 1.1 - had similar activity against *B. dendrobatidis* and all

inhibited growth at 12.5 μg/ml. The concentrations of skin peptides inhibiting growth of a variety of bacteria ranged between 3 and 100 μg/ml (Bowie et al., 1999). The effects of these peptides on other fungi are not known. Although they are effective against *B. dendrobatidis in vitro*, they obviously are not universally protective for frogs, for example *L. caerulea* secretes at least 40 peptides and this species is highly susceptible to chytridiomycosis. Perhaps the concentration of peptides on skin is not high enough. Further investigations could potentially lead to some therapeutic uses for skin peptides, including genetic manipulation (R. Speare, pers comm).

Fluconazole, itraconazole, methylene blue, and benzalkonium chloride were selected for animal trials. The first two were suspected to be safe and effective, the latter two are cheap and easily obtained without the need for prescription. Toxicity of some antifungals was a concern, so malachite green, amphotericin B and ethanol were not considered further for use in frogs. Selected compounds were initially tested on tadpoles and due to the toxicity of itraconazole and the ineffectiveness of methylene blue (McInnes, 1999), these were discarded. For treatment trials using juvenile *L. caerulea*, benzalkonium chloride at 1 mg/L - a dose similar to those previously used in amphibians (Groff et al., 1991; Hulst, 1999) - and fluconazole at 25 mg/L were used. Although these treatments resulted in slightly longer survival times, the mortality rate was still 100%.

Although drugs were highly effective in inhibiting zoospore encystment *in vitro*, they were not effective when used on frogs at suggested doses that were higher than levels tested *in vitro*. The reason for this may be that sporangia in skin are protected and there is a lack of drug penetration into the keratinised cells. Benzalkonium chloride is a quaternary ammonium compound and a surfactant, but activity is reduced in the presence of tissue debris (Fraser, 1991).

The test used here to determine sensitivity to antifungals measured inhibition of zoospores, so it does not determine fungicidal or even fungistatic levels effective on sporangia. It was chosen as it provided a simple method of comparing different drugs at different concentrations, but results should be interpreted with caution as they are likely to overestimate the efficacy of the drugs tested. More than one type of assay is often used for evaluating antifungals (Alderman, 1982; Bly et al., 1996). Methods to test for

lack of sporangial growth and for minimal lethal doses at various times should be developed for *B. dendrobatidis*. These tests could be based on the method used here to test disinfectants. Concentrations required to inhibit sporangia are likely to be greater than those required to inhibit zoospores, which have no cell wall.

Antimicrobial treatment is generally ineffective without assistance from the host immune system. There may be no effective immune reaction in sick frogs with chytridiomycosis, and so a treatment would need to eradicate every sporangium to prevent infections returning to previous levels. Unless a cure is 100% effective, it will be necessary to house frogs individually or in small groups to prevent cross-infection. A one-off treatment appears difficult to achieve. However, a recent experiment has shown that the species we attempted to treat, *L. caerulea*, is highly susceptible to chytridiomycosis compared with other species (Katie Ardipraja and Gerry Marantelli, unpub 2001), and perhaps other species may be more easily treated.

Tadpoles are more sensitive to toxic effects of drugs. Benzalkonium chloride was toxic above 1 mg/l, possibly as it is a surfactant which could interfere with gill function. Itraconazole is not water soluble and forms a precipitate in water and this could also affect the gills.

A subsequent experiment using terbinafine hydrochloride (Lamisil) and raising the temperature to 30°C had promising results (Claire Steel and Gerry Marantelli, unpub, 2001). Other attempts to find a cure could involve using higher doses of fluconazole and itraconazole (post-metamorphic frogs only), new human drugs, or perhaps cheaper compounds used in aquaculture such as trifluralin (Treflan) (Ramasamy et al., 1996), Pyceze (Anon, 1997) and others (Lio-Po et al., 1985). Povidone iodine is used for treating ringworm and retains its activity in the presence of organic matter and may be worth testing. Copper sulphate is a commonly used antifungal for aquatic animals, but can cause increased mortality and reduced growth rates in tadpoles (Lande and Guttman, 1973). Increased temperature used alone and in combination with drugs should be tested.

The three chemical disinfectants tested were effective in killing sporangia when applied for 20 seconds. Ethanol (70%) and benzalkonium chloride (1 mg/ml) were tested at the

recommended concentrations. Virkon, which was tested at 1 mg/ml, is usually used at 5 to 10 times this concentration, i.e. at 0.5 - 1%. Virkon was reported to be safe and effective and has been made compulsory for disinfecting foreign fishing tackle that is brought into Iceland (Randolph, 2001), although there are doubts about its safety (Peter Le Blanc Smith, pers comm 2001). Sodium hypochlorite (0.2%) was not tested *in vitro* but its use has been proven in containing epidemics in captivity (G. Marantelli, unpub 1997). A larger range of disinfectants needs to be tested, although it seems likely *B. dendrobatidis* is sensitive to any broad action compounds that are effective against other pathogens. Disinfectants and protocols for use need to be tested in field situations, such as when mixed with dirt in boots.

Drying and heat were very effective in killing sporangia, and can be incorporated into disinfection protocols, and a combination of both is suggested. Sporangia were killed in less than an hour at temperatures above 47°C. However, these test conditions - using actively growing sporangia from an isolate that had been maintained in culture for many months - may have produced results that are different what may occur in real-life situations. At least twice the incubation times that were effective experimentally should be used when evaluating disinfection. It must also be considered that other pathogens such as ranaviruses are more resistant and can survive in the environment for extended periods even with drying (Langdon, 1989), and a disinfection technique that is effective for *Batrachochytrium* may not be effective for ranaviruses.

The susceptibility of *B. dendrobatidis* to heat and drying is also important when considering the geographic distribution and spread of chytridiomycosis. The lack of a resistant resting spore means that the fungus is less likely to be spread on fomites.

Quarantine protocols for captive collections and disinfection procedures for field work are included in Speare et al. (2001), as well as a list of recommendations to reduce spread of disease. Based on incubation times in our experimental work (Chap 7), quarantine periods should be at least two months when introducing frogs into collections, and frogs in quarantine should be kept at 24°C or less to increase the chance of infected frogs showing signs of disease. Using routine quarantine methods, chytridiomycosis has not spread between frogs housed in close proximity, and no airborne transmission has been observed (G. Marantelli, pers comm, 1997). However,

even small numbers of fungi are highly infectious (Chap 7) and great care is needed to prevent drops of water from contaminating groups of animals. Important quarantine practices include: changing gloves between every enclosure, disinfection of equipment and tubs between use, cleaning and feeding of animals in the same order each time, use of automated husbandry systems, and immediate and safe disposal of water used in enclosures (Lynch, 2001).

A comprehensive hygiene protocol for amphibian field work was produced by NSW National Parks and Wildlife Service (2000) that utilised knowledge gained from the work in this thesis. The protocol includes instructions on how to disinfect boots, tyres, equipment etc. when moving between sites. Strategies to avoid increasing disease exposure when handling frogs involve the use of disposable gloves and plastic bags. Although chytridiomycosis is already widespread, it is important not to increase transmission rates and exposure to new strains above natural levels.

CHAPTER 10

Overview of the disease survey

10.1 Introduction

There are numerous papers on helminth and protozoan parasites of Australian amphibians (Delvinquier and Freeland, 1988; Delvinquier and Freeland, 1989; Barton, 1994). However, until 1995 there had been reports of just four non-pathogenic infections – due to *Myxobolus hylae*, *Batrachomyia*, Bohle Iridovirus and *Mucor amphibiorum* (Johnston and Bancroft, 1918; Elkan, 1965; Speare and Smith, 1992; Speare et al., 1994; Vogelnest, 1994). Since this study commenced in 1995 eight more reports have been published. Hill et al. (1997) described thin *L. caerulea* with biliary hyperplasia associated with the gall bladder protozoan *Myxidium* sp. The other seven papers are included in this thesis and describe diseases caused by *M. amphibiorum* (Berger et al., 1997; Speare et al., 1997; Creeper et al., 1998), *B. dendrobatidis* (Berger et al., 1998, 1999a), *Chlamydia pneumoniae* (Berger et al., 1999b) and *Aphanomyces* sp. (Berger et al., 2001). When declining frogs were found dying in 1993 (Laurance et al., 1996) it was difficult to interpret their pathology without any baseline information. To acquire knowledge of amphibian diseases, we began pathological investigations of wild and captive frogs collected from around Australia. A network was established involving herpetologists and other scientists at universities, government environmental departments and community interest groups, so that any diseased frogs encountered were submitted for diagnostic testing. With the involvement of many people, we obtained ill frogs from widespread areas. Over half the frogs had chytridiomycosis, and this subset is reported on in detail in Chapter 8. This current chapter consists of an overview of all amphibians received and brief descriptions of the variety of diseases encountered. Some of the more significant diseases are described in detail in the following chapters.

10.2 Methods

Diagnostic methods and an outline the network for collection of frogs are included in the general methods section in Chapter 3. Information sheets were disseminated that

described how to identify sick frogs, how to preserve frogs, and what information should be recorded (Berger and Speare, 1998). Briefly, dead frogs found by herpetologists were preserved in 10% formalin, 70% ethanol or frozen. Sick frogs were identified by their unusual behaviour (e.g. lethargy, sitting unprotected during the day) or by the presence of lesions (e.g. reddening, ulcers, lumps). Sick frogs were either euthanased and preserved, or were couriered alive to the laboratory. For most specimens the collector identified the species of amphibian. At the laboratory, routine post mortems were performed with tissue samples collected for histopathology, culture, electron microscopy, PCR and haematology. Small frogs or tadpoles (i.e. <15 mm) were not dissected but were cut in half and sectioned for histology. Many frogs were received whole in formalin or ethanol which precluded culture and identification of infectious agents. Towards the end of the project, chytridiomycosis could be diagnosed confidently by skin scrapings and not all frogs with chytridiomycosis were examined histologically, although their internal organs were examined for gross lesions. Parasites were collected for identification. Specialised tests, such as immunostaining, viral culture, and transmission and scanning electron microscopy, were done when required to reach a diagnosis. Collaborations were established with various experts for assistance with identification of some infectious organisms by PCR, culture or morphological examination. Cases were entered on a frog disease database in Microsoft Access.

10.3 Results

10.3.1 Overview of specimens submitted

Collaboration with Australian herpetologists was very productive and 487 sick amphibians were received (Table 10.1). One hundred and four healthy frogs were also received; these were mainly animals sampled for disease screening before release or experimentation, and others were collected as controls at the time sick frogs were collected. This group is not included in the general analysis of sick frogs received, but is referred to with the disease results. Complete information is not available for all frogs due to autolysis, freezing artefacts, or incomplete reporting by submitters.

Table 10.1 Stages of captive and free-living sick amphibians received.

	Adults	**Juveniles & Metamorphs**	**Tadpoles**	**Total**
Free-living	253	32	38	323 (66.3%)
Captive	72	72	20	164 (33.7%)
Total	325 (66.7%)	104 (21.4%)	58 (11.9%)	487 (100%)

Frogs and tadpoles were from 23 Hylid species, 20 Myobatrachid species and 1 Bufonid species. Numbers of adults and tadpoles from each species were: 19 *A. brevis*, 24 *B. marinus*, 6 *C. georgiana*, 3 *C. riparia*, *3 C. signifera*, 1 *C. platycephala*, 1 *H. australiacus*, 1 *L. fletcheri*, 10 *L. dumerilii*, 1 *L. ornatus*, 11 *Lim. peronii*, 18 *L. tasmaniensis*, 3 *L. terraereginae*, 17 *L. aurea*, 91 *L. caerulea*, 19 *L. chloris*, 5 *L. citropa*, 5 *L. ewingii*, 4 *L. fallax*, 5 *L. genimaculata*, 7 *L. gracilenta*, 10 *L. infrafrenata*, 2 *L. latopalmata*, 17 *L. lesueuri*, 2 *L. moorei*, 10 *L. nannotis*, 20 *L. pearsoniana*, 12 *Lit. peronii*, 3 *L. raniformis*, 7 *L. rheocola*, 1 *L. serrata*, 10 *L. spenceri*, 1 *L. tyleri*, 3 *L. verreauxii*, 52 *M. fasciolatus*, 26 *M. fleayi*, 4 *M. iteratus*, 18 *Mixophyes* sp., 9 *N. kunapalari*,1 *N. dayi*, 2 *P. corroboree*, 1 *P. pengilleyi*, 16 *T. acutirostris*, 5 *T. eungellensis*, and 1 *U. laevigata.*

Sex was not determined for tadpoles, metamorphs or some small frogs sectioned whole. There were 289 amphibians that could be sexed with 142 (49.1%) females and 147 (50.9%) males. Frogs and toads were collected mainly from the eastern states, with 243 from Queensland, 101 from New South Wales, 84 from Victoria, 51 from South Australia and 8 from Western Australia. Of the 323 free-living amphibians, 159 were from wilderness areas, 77 were from urban areas, 54 were from rural areas, and habitat was not described for 33 other frogs submitted.

Most sick frogs were collected in winter, but cases of diseases other than chytridiomycosis were submitted evenly throughout the year (see Chap 8).

10.3.2 Overview of diseases

Selected diseases are listed in Table 10.2, and a complete list of cases including diagnosis, concurrent infections, species, lifecycle stage, sex, location, date collected

and name of submitter is presented in Appendix 1. A list of helminths identified is presented in Appendix 2. Information could not be gained from 32 frogs due to autolysis, and 50 were not fully examined histologically because not all tissues were submitted (e.g. no skin provided), or because they were part of a consistent group and were kept frozen and used for other tests such as viral or bacterial culture. Morphologic diagnoses were determined on 360 (88.9%) of the 405 amphibians examined by histology or skin scrapings, although in many of these the aetiologic agent was not determined. Most diagnoses involved infectious disease, and included several undescribed species of parasites. A wide variety of diseases were found, but apart from chytridiomycosis, none occurred at high prevalence.

10.3.2.1 Bacterial diseases

Unidentified bacteria, mostly gram-negative rods, caused septicaemia or localised infections in 15 specimens from various species. Septicaemia, pneumonia or cystitis occasionally occurred secondary to chytridiomycosis. Massive multi-systemic bacterial infections, where most organs of the frog were invaded by bacteria, occurred in two *L. infrafrenata* (99 1743/2 and 99 1385/3) and a *L. gracilenta* (99 2144/7.1) that were transported to Sydney with bananas, and may have been stressed by refrigeration. One of the *L. infrafrenata* was also parasitised by two larvae of *Batrachomyia* sp. in the subcutaneous space behind the right tympanum. The maggots were surrounded by inflammation and bacteria, and their damage to the skin may have provided the route of infection for bacteria. A captive, gravid *P. corroboree* (01 95/10) died with severe bacterial pneumonia and peritonitis associated with abnormal vitellinogenesis.

Chlamydia pneumoniae was identified in the lung of a free-living *M. iteratus* that died with monocytic pneumonia (Berger et al., 1999b; Chap 11). A captive *L. caerulea* (96 284/4) had a systemic infection with bacteria that appeared similar to *Chlamydia* by electron microscopy but could not be identified by PCR or immunofluorescence (Peter Timms, unpub).

Heavy infections with mycobacteria caused focal to diffuse, multi-systemic, interstitial granulomatous lesions in two 9 month old captive *L. aurea* that were found dead. Affected organs included liver, spleen, lung, kidney, bladder, heart, stomach and small intestine. Granulomatous inflammation was associated with minimal fibrosis. Caseous

foci occurred within areas of inflammation and contained abundant acid-fast bacilli (Fig. 10.3). The mycobacteria occurred singly, in pairs, or in clusters within vacuoles in the cytoplasm of inflammatory cells and were also seen extracellularly. Their mean length was 5.0 μm (range 3.2- 6.3 μm) (n = 10) and they were about 0.8 μm wide. The kidneys were markedly enlarged by masses of granulomatous inflammation between tubules (Fig. 10.1), and in one frog the lumen of the oviduct contained necrotic cell debris and abundant mycobacteria. In the other frog the lungs were also severely affected with massive thickening along serosal edges (Fig. 10.2). Both frogs had flexible bones, suggestive of calcium deficiency. Attempts to identify the mycobacterial species using PCR are continuing.

10.3.2.2 Mycotic diseases

Cutaneous chytridiomycosis occurred in 201 (56.5%) of 356 post-metamorphic amphibians that could be examined fully. This included 135 (55.6%) of 243 free-living animals and 66 (58.4%) of 113 captive animals. Details on the pathology, experimental transmission, incidence and distribution of frogs with chytridiomycosis are described in Part 1 of this thesis. Three frogs with chytridiomycosis also had significant dermal infections with unidentified hyphal fungi. Other mycotic infections were found in 12 juveniles and adults, and 17 tadpoles.

Mycotic infections in juveniles and adults

Severe mucormycosis due to *M. amphibiorum* occurred in two wild frogs, a *L. caerulea* (Berger et al., 1997; Chap 12) and a *Lim. peronii* (Fig. 10.4), and a captive *L. caerulea*. These frogs had disseminated granulomas in most organs containing obvious fungal sphaerules. *M. amphibiorum* was seen as an incidental finding in two wild *L. caerulea* that died with chytridiomycosis – within a large inactive granuloma in the intestine in one, and in a few foci of granulomatous inflammation in the liver and large intestine of the other. Free-living cane toads had a high prevalence of infection with disseminated mucormycosis in 3/9 sick adult toads.

Several cases of severe dermatitis involved unidentified fungal hyphae. A free-living *L. caerulea* had deep skin ulcers with rims of hyphae on dorsal skin. A captive metamorphosing *M. fasciolatus* had a diffuse infection with aseptate, branching hyphae infiltrating through skin and underlying tissue, especially in the resorbing tail (Fig.

10.5). Overwhelming fungal infections occurred in a group of recently collected *C. riparia.* The skin became thickened and wrinkled and a large pale protuberance grew from the side of one frog which died about one week after collection. About twelve days later similar growths appeared on the other five frogs in the tub which soon died. Histology on two of these frogs showed that much of the dermis and epidermis had been replaced by masses of hyphae. The lumps that grew on the side of the frogs consisted almost entirely of fungus.

Mycotic infections in tadpoles

Outbreaks of aphanomycetosis occurred in *B. marinus* tadpoles (Berger et al., 2001; Chap 12). Thirty-seven percent of tadpoles in a dam were infected with tufts of fungi on the skin. All tadpoles had fungus on the head, and some also had tufts growing from skin of the feet and/or tail. Focal ulceration occurred where the fungi attached, but hyphae did not invade through the dermis. Obstruction of the mouth, nostrils and eyes caused debilitation and likely death. Two captive *M. fleayi* had a different mycotic disease with hyphae invading through the deeper subcutaneous tissue of the ulcerated mouthparts. The fungal species was not identified.

10.3.2.3 Protozoal and protistan diseases

An unusual protistan parasite in the CNS was associated with neurological signs. A free-living *L. caerulea* was found hopping in circles. Petechiae in the brain were seen grossly, and histologically there was a severe encephalitis and myelitis, with large foci of haemorrhage, coagulative necrosis, monocytic perivascular cuffing and scattered protists (Figs. 10.6 and 10.7). Another *L. caerulea* with hind limb paralysis had a few protists in the spinal chord but associated lesions were not found. Occasional protists were seen in the brain or spinal cord of four *L. caerulea* that died due to other diseases, and did not have encephalitis or neurological signs (96 692/1, 97 845/12, 97 845/6, 98 320/16). Organisms occurred in spherical cysts (10 - 18 μm) within dilated axons. Cysts contained many 2 μm eosinophilic and basophilic round cells. Between about 15-50 cells were seen in each cyst in sections. Bill Hartley at the Veterinary and Quarantine Centre, Taronga Zoo, Sydney diagnosed a similar infection in dying captive tadpoles of *L. aurea,* and sent a heavily infected specimen to AAHL. Electron microscopy of the spinal chord showed the cysts were surrounded by myelin, and sometimes two or more cysts were contained within the one myelin capsule. The internal cells had tubular

mitochondrial cristae and unusual large, electron dense, membrane-bound organelles in the cytoplasm (Fig. 10.8), but the organism could not be identified.

Myxobolus hylae occurred in ovaries of two *L. caerulea* and a *Lit. peronii*, and in the testes of a *L. lesueuri*. This protozoan did not appear to be the cause of illness but infection resulted in partial replacement of the gonads by large encapsulated cysts (Fig. 10.9). Degenerate cysts were infiltrated with granulomatous inflammation. Identification of this parasite was confirmed by Frank Mutschmann, Tierarztpraxis, Berlin.

Three out of nine captive metamorphs of *N. kunapalari* that died during an outbreak of chytridiomycosis had concurrent infections with a microsporidian identified as a *Pleistophora* sp. (Frank Mutschmann, unpub). These frogs had been collected from the wild as tadpoles. All three had focal infections in skeletal muscles and two frogs also had heavy infections of the liver with spores filling the cytoplasm of enlarged macrophages and hepatocytes, grouped in foci (Fig. 10.10). Spores were oval, refractile and pale. The remainder of the liver appeared unaffected. The frog with the heaviest liver infection also had a few microsporidia within macrophages in the kidney. Electron microscopy revealed the microsporidia had a single row of about 10 polar tube coils (Fig. 10.11). Spores were 2258 nm long and 1564 nm wide on average (n = 12), with a 185 nm thick capsule. A few microsporidia were also found as a secondary infection in the skeletal muscles of a *L. aurea* with a heavy infection with spargana.

Biliary hyperplasia, fibrosis and/or inflammation occurred in ten adults of *L. caerulea* and two adults of *M. fleayi*, although these lesions did not seem to be the primary cause of morbidity in these frogs. Infection with the gall bladder myxozoan *Myxidium* sp. was noticed in four of the *L. caerulea*. Eighteen other frogs with *Myxidium* did not have biliary hyperplasia.

A *C. georgiana* received from Peter Kirkpatrick, of the Western Australian Museum, for confirmation of fatal chytridiomycosis had a concurrent infection with masses of small protozoa in thickened areas of dermis and stomach. This parasite was suspected to be a species of *Dermocystidium* (Frank Mutschmann, unpub).

A large proportion of tadpoles of two species were infected with an eimeriid coccidian. These were in 8 - 12 μm parasitophorous vacuoles in the basal epithelial cells of the gut (Fig. 10.12). These were identified as a probable *Goussia* sp. after examination by electron microscopy (Peter Daszak, unpub). Infections were seen in 8/20 wild and captive *Mixophyes* tadpoles from Queensland and 3/4 wild tadpoles of *L. tasmaniensis* from South Australia. Infected epithelial cells were often swollen and degenerate, but there was no associated inflammation. The impact on tadpole health was difficult to ascertain, but the parasite may be potentially pathogenic. Deaths in a group of captive *L. tasmaniensis* tadpoles (not included in this study) were suspected to be due to overcrowding and heavy coccidian infections (C. Williams and L. Berger, unpub).

Four tadpoles of *Mixophyes* sp. that had *Goussia* infections also had biliary hyperplasia and fibrosis (Fig. 10.13). In two of these tadpoles, severe hyperplasia caused the liver to bulge markedly to the right and distort the abdomen. Gall bladders in tadpoles were not examined for the presence of *Myxidium*.

10.3.2.4 Helminth diseases

Spargana, the intermediate stage of the cestode *Spirometra erinacei*, occurred in 12 adults of *L. caerulea, L. aurea, L gracilenta*, and *Lit. peronii* (Chap 13). Identification of the tapeworm species was confirmed by PCR (Zhu et al., 2001). In at least seven of these cases, the infection was considered to be pathogenic. Heavy infections in muscles, subcutaneous tissue and the peritoneal cavity appeared to cause severe debilitation, and led to secondary bacterial and protozoal infection in one frog. In milder cases of the disease the main clinical signs were behavioural changes and frogs remained in the same unprotected position for about a week. Subcutaneous lumps over thighs and caudal belly were usually obvious. In more advanced cases, frogs were moribund.

Heavy infections with *Rhabdias* sp. occurred in two captive frogs, a *L. dumerilii* and a *L. infrafrenata* and one wild frog, a *L. aurea*. Many blood-filled adult worms occurred in the lungs, and larvated eggs and larvae occurred in the intestines. The captive *L. infrafrenata* had thickened, fibrotic lungs but lesions associated with heavy infections were not seen in the other two frogs, and the significance of the parasites to their deaths is uncertain. Lung worms were found in 28 other frogs with no apparent ill effects.

Subcutaneous and intramuscular encysted metacercariae of *Fibricola* sp. occurred in ten free-living tadpoles of *Mixophyes* sp. (Chap 13) and unidentified metacercaria were present in a metamorph of *M. iteratus*. The cysts were striking orange or black. The tadpoles appeared to be generally healthy, but the high burden of cysts may have made them more obvious to predators. Cysts were contained by fibrous capsules, but there was little associated inflammation except with occasional degenerate cysts that were replaced with granulomatous cells.

10.3.2.5 Neoplastic diseases

Fatal neoplasms were confirmed in three frogs - a basal cell carcinoma in a free-living *L. caerulea*, a squamous cell carcinoma in a captive *L. infrafrenata* and an intravascular lymphoma in a captive *L. caerulea* (Chap 14). Another unconfirmed neoplasm was a possible lymphoma in glomeruli of a *L. chloris* (97 1754/3).

10.3.2.6 Syndromes of unknown aetiology

A number of syndromes were recognised in this collection, but their aetiology was unable to be determined. The most frequent was vacuolating and ulcerative dermatitis found in free-living frogs from Queensland and NSW.

Vacuolating and ulcerative dermatitis

Twenty-one free-living amphibians were found active or ill with dermatitis ranging in severity from mild to severe. Various lesions were observed and there appeared to be more than one type of disease causing skin lesions in different frogs, but overlap between types presented difficulty in classifying different lesions. Undiagnosed dermatitis occurred in *L. chloris*, *L. pearsoniana*, *L. aurea, L. caerulea* and *B. marinus*. Grossly, focal to extensive discolourations, erosions, and ulcers occurred mainly on the dorsal skin of the body and limbs (Fig. 10.14). Histological lesions included vacuolation and degeneration of epidermal cells progressing to vesicles and ulcerations, and breakdown of the basement membrane with pigment cells in the epidermis (Fig. 10.15). Chronic lesions with fibrosis of ulcerated tissue were also seen. Some frogs had necrosis and inflammation in dermis, and degeneration or loss of dermal glands. Bacterial infections of skin and internal organs in a few frogs were suspected to be opportunistic infections. An infectious cause was often suspected, but no organisms were found despite special stains, cell culture, ranavirus PCR and electron microscopy.

In two frogs, a few chytrids were found on examination of skin scrapings but were not seen on histology. It was not known if these were incidental infections or if these cases represented an atypical form of chytridiomycosis with few sporangia. Further work is required to determine the aetiology of this potentially important syndrome.

Chronic enteritis

Two of the cane toads were emaciated and had chronic enteritis with fibrosis and granulomatous inflammation in the thickened submucosa of the large intestine. Cell culture and ranavirus PCR assays did not detect the presence of ranaviruses or other viruses.

Chronic interstitial nephritis and hepatic fibrosis

Two captive *L. infrafrenata* died with incoordination and ascites on separate occasions. Both had chronic nephritis with markedly thickened kidneys due to severe interstitial fibrosis, and moderate hepatic fibrosis. Warthin-Starry stain for spirochaetes was negative.

10.3.2.7 Miscellaneous diseases

Miscellaneous diseases in post metamorphic frogs included opthalmitis, disseminated granulomas due to unseen parasites, osseous metaplasia, calcium deficiency, hydrops, road trauma, and inappropriate ingestion including stones, a feather, a large beetle, and spiders. Pentastomes occurred in tissue around the oesophagus in a *L. infrafrenata* and were found in a subcutaneous lump and in the mesentery in a *L. gracilenta*, but their impact was unknown. Two male *Lim. peronii* had deep, well-demarcated ulcers on dorsal skin thought to have been incurred during fighting, behaviour typical in this species (Gerry Marantelli, pers comm 2000). A high prevalence of deformities occurred in a captive-bred spawning group of *L. chloris*. Two surviving frogs were submitted - one with unilateral micropthalmia and the other with bilateral micropthalmia. A two-headed tadpole of *P. corroboree* raised in captivity from wild spawn was also received. Other diseases of captive tadpoles were multiple discrete granulomas in the anterior peritoneal cavity, and one case of an unresorbed yolk sac infected with bacteria.

Table 10.2 Selected diseases of amphibians submitted to AAHL. "Diagnosis" includes concurrent infections, therefore some animals may be presented twice. See Appendix 1 for a complete list of cases and their source.

Diagnosis	Species (Accession no.)	Number of frogs
Bacteria		
Septicaemia or local infections	Various	15
Mycobacteria	*Litoria aurea* (00 671/10 & 11)	2
Chlamydia pneumoniae	*Mixophyes iteratus* (98 320/1)	1
Fungi		
Batrachochytrium dendrobatidis	Various (36 species)	201
Mucor amphibiorum	*Litoria caerulea* (95 656/2, 96 495/16, 96 961/19, 00 1645/3)	4
	Limnodynastes peronii (99 1562/24)	1
	Bufo marinus (96 1429/3, 99 951/2, 00 1266/14)	3
Aphanomyces sp.	*Bufo marinus* (95 517/1-15) (tadpoles)	15
Hyphal dermatitis	*Mixophyes fasciolatus* (99 2144/2)	1
	Litoria caerulea (98 1231/3)	1
	Crinia signifera (00 782/12 & 13)	2
Hyphal stomatitis	*Mixophyes fleayi* (00 1266/9 & 10) (tadpoles)	2
Protozoa & protists		
Microsporidia	*Neobatrachus kunapalari* (96 1431/40 & 41, 99 2144/17)	3
	Litoria aurea (98 320/14)	1
Protistan encephalitis +/or myelitis	*Litoria caerulea* (96 961/11 & 21)	2
	Litoria aurea (97 1754/7) (tadpole)	1
?*Dermocystidium* sp.	*Crinia georgiana* (99 2144/10)	1
Myxobolus hylae	*Litoria lesueuri* (00 1266/5)	1
	Litoria peronii (00 782/30)	1
	Litoria caerulea (98 1469/13, 00 1645/3)	2
Goussia – like coccidian	*Limnodynastes tasmaniensis* (95 341/11, 13 & 22) (tadpoles)	3
	Mixophyes sp. (96 570/7, 96 1428/18, 99 951/7, 99 2144/4, 01 95/4) (tadpoles)	8
Helminths		
Spirometra erinacei	*Litoria caerulea* (96 961/11, 98 1469/5 & 13, 99 1562/22 & 23, 98 320/16, 98 871/28 & 29)	8
	Litoria gracilenta (98 1159/2)	1
	Litoria peronii (98 1469/11)	1
	Litoria aurea (98 320/14, 00 782/11)	2
Rhabdias sp.	*Litoria aurea* (00 1016/3)	1
	Litoria infrafrenata (96 284/5)	1
	Limnodynastes dumerilii (96 284/2)	1
Fibricola sp.	*Mixophyes* sp. (96 570/7.1-7.9, 01 95/4) (tadpoles)	10
Metacercaria	*Mixophyes iteratus* (98 320/5)	1
Neoplasms		
Basal cell tumour	*Litoria caerulea* (98 320/26)	1
Squamous cell carcinoma	*Litoria infrafrenata* (95 203/1)	1
Intravascular lymphoma	*Litoria caerulea* (99 2144/16)	1
Other		
Vacuolating and ulcerative dermatitis	*Litoria chloris* (97 1754/10, 11, 12 & 18, 98 222/14 &15)	6
	Litoria pearsoniana (98 222/4, 10 & 12)	3
	Litoria caerulea (98 1469/6, 99 146/3)	2
	Bufo marinus (99 1743/3)	1
	Various	9

Biliary hyperplasia +/- fibrosis	*Litoria caerulea* (96 495/8, 96/692/1,2 & 4, 96 961/15, 17 & 11, 97 845/6 & 8, 99 951/14)	10
	Mixophyes fleayi (96 962/8 & 14)	2
	Mixophyes sp. (96/1427/10, 99 951/7.1&7.2, 99 2144/4, 01 95/4) (tadpoles)	5
Chronic interstitial nephritis	*Litoria infrafrenata* (96 144/4, 96 1136/9)	2
Chronic enteritis	*Bufo marinus* (99 2144/11, 00 1879/1)	2
Opthalmitis +/or conjunctivitis	Various	7
Trauma	Various	9
Wounds from fighting	*Limnodynastes peronii* (99 232/29.1 & 29.2	2
Deformities	*Litoria chloris* (96 1431/10 & 11)	2
	Pseudophryne corroboree (00 1645/59)	1
No abnormality found	Various	45
Autolysed	Various	32
Incomplete specimen*	Various	16
Not fully examined*	Various	34

* These classifications only refer to partly examined specimens where abnormalities were not detected.

10.4 Discussion

There have been few widespread systematic surveys of causes of disease among sick amphibians. Taylor et al. (1999c) reviewed the causes of mortality in 278 wild and captive Wyoming toads (*Bufo baxteri*). Seventy-four percent (54/73) of free-living toads and 37% (46/126) of captive toads were reported to have died with mycotic dermatitis due to *Basidiobolus ranarum*, but this infection appears identical to chytridiomycosis and may have been misdiagnosed. The large proportion of Wyoming toads with possible chytridiomycosis is similar to our findings in a variety of frog species. The most comprehensive review on amphibian pathology is by Reichenbach-Klinke and Elkan (1965). Most of this work describes infectious diseases but prevalence figures are not given.

In this study a wide variety of diseases were found, but none occurred at high prevalence apart from chytridiomycosis. Most diseases diagnosed were due to infection. When the diseases are classified into the broad International Classification of Diseases categories, Infectious and Parasitic Diseases (Category 1) make up 79% of all diagnoses (Fig. 10.16), illustrating the overwhelming predominance of infectious diseases. Of the cases of infectious disease, chytridiomycosis accounted for 71.5%.

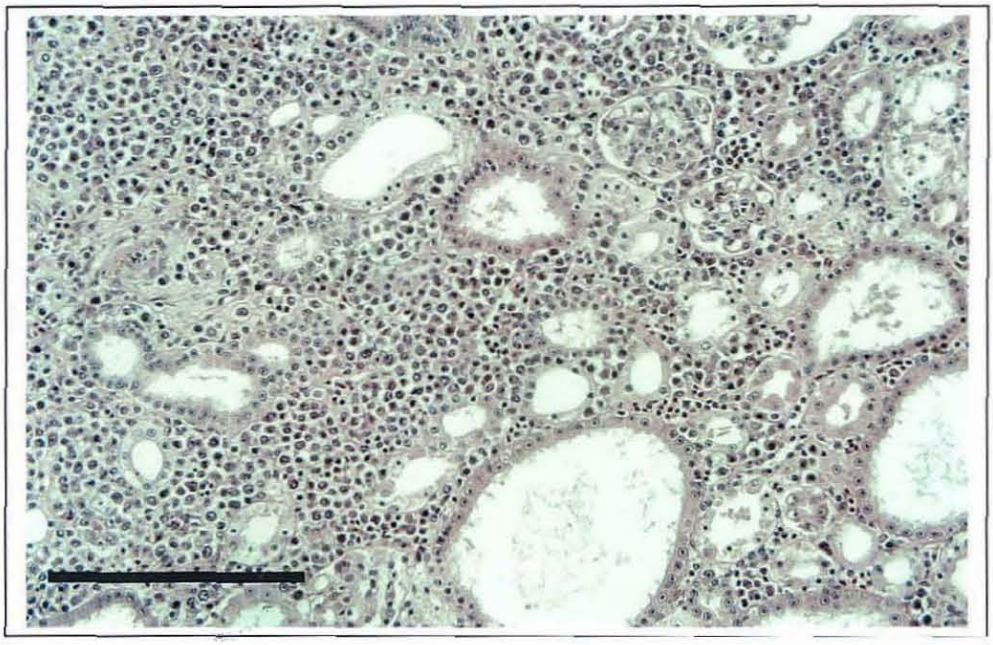
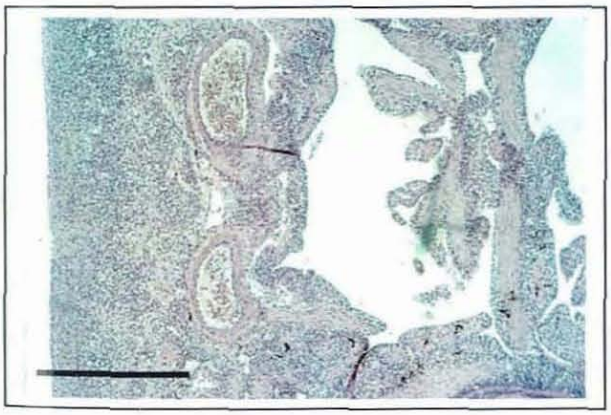


Figure 10.1 Section of thickened kidney from a *Litoria aurea* (00 671/11) with masses of diffuse, interstitial inflammation and dilated tubules due to mycobacteriosis. Bar = 250 µm.



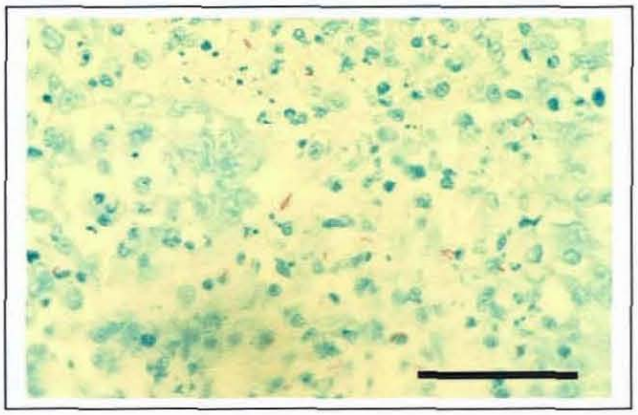


Figure 10.2 Lung of a *Litoria aurea* (00 671/10) with greatly thickened septa due to a mononuclear inflammatory response to *Mycobacteria* sp. Bar = 480 µm.

Figure 10.3 Kidney of a *Litoria aurea* stained with Ziehl Neelsen stain, demonstrating the presence of pairs of intracellular acid-fast bacterial rods. Many pyknotic nuclei are present in this field. Bar = 35 µm.

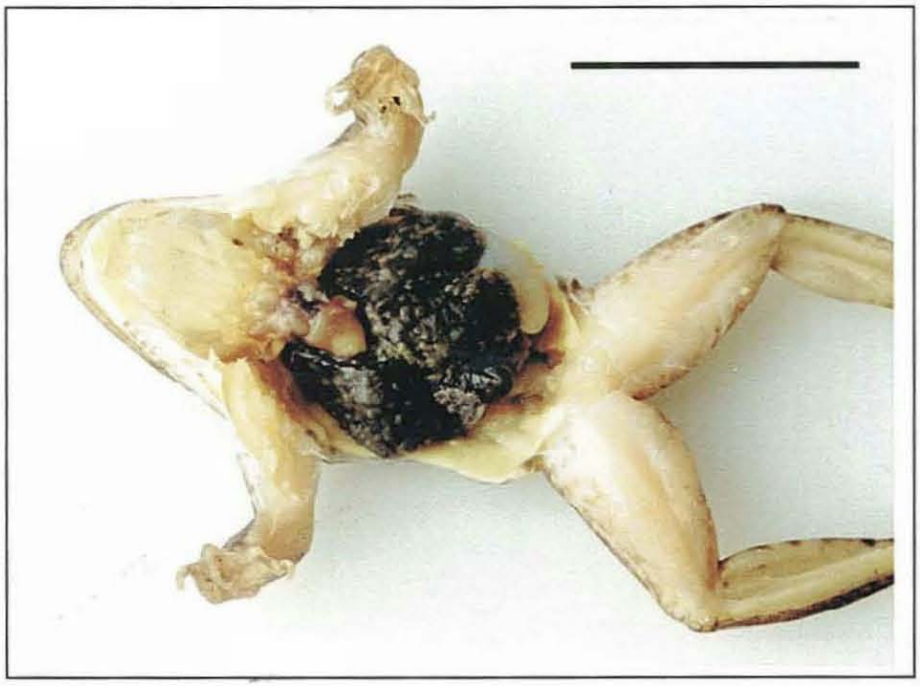


Figure 10.4 A *Limnodynastes peronii* (99 1562/24) with small nodules throughout the enlarged liver caused by *Mucor amphibiorum*. Bar = 25 mm.

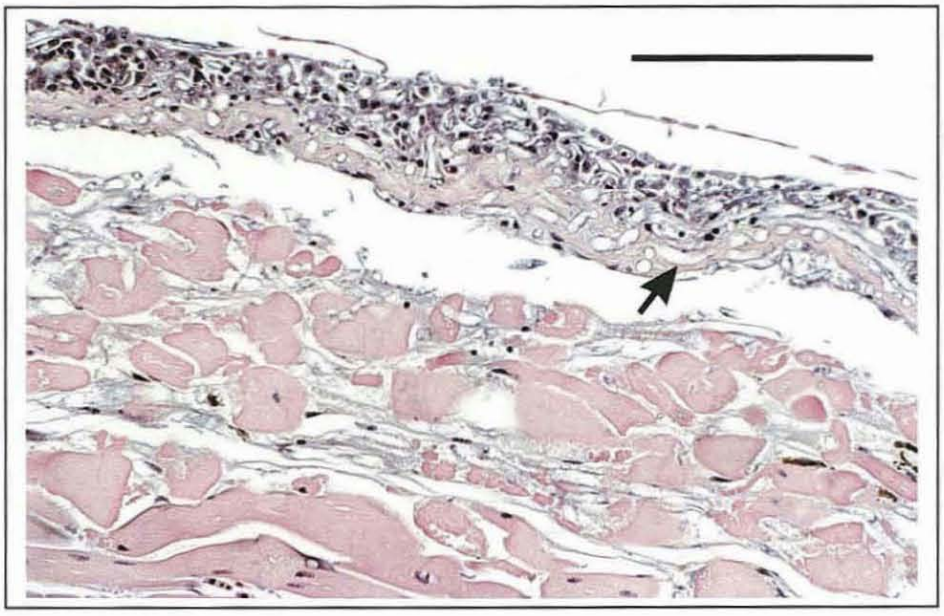


Figure 10.5 Section of skin from a *Mixophyes fasciolatus* (99 2144/2) with mycotic dermatitis. There is a heavy infection with unidentified hyphae throughout the epidermis, dermis (arrow) and underlying muscle, with no associated inflammation. Bar = 80 μm.

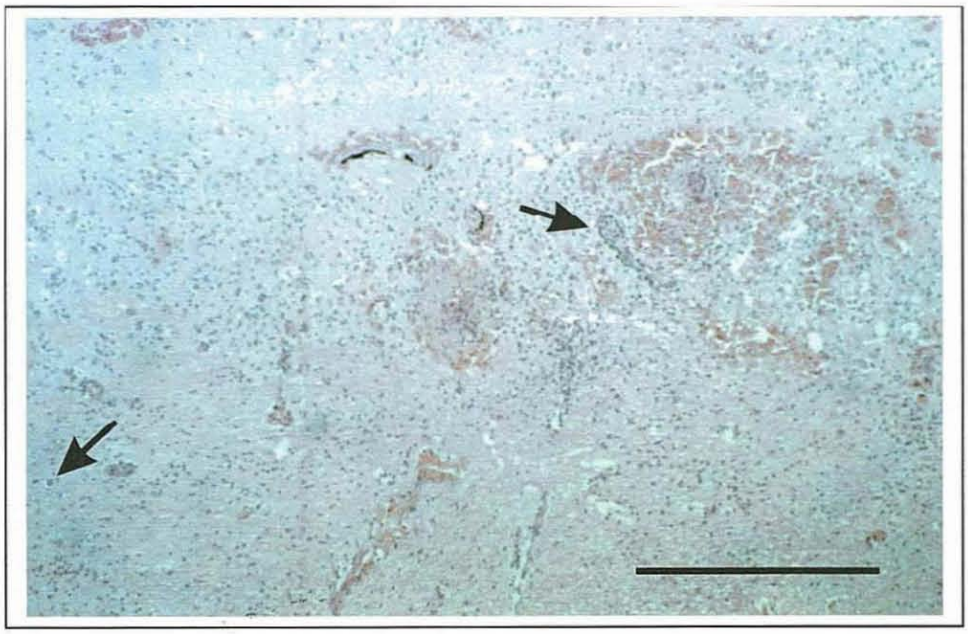


Figure 10.6 Section of brain from a *Litoria caerulea* (96 961/11) with protistan encephalitis, with severe haemorrhage, caseous necrosis and perivascular cuffing (right arrow). A protistan cyst is present distant from these lesions (left arrow). Bar = 450 μm.

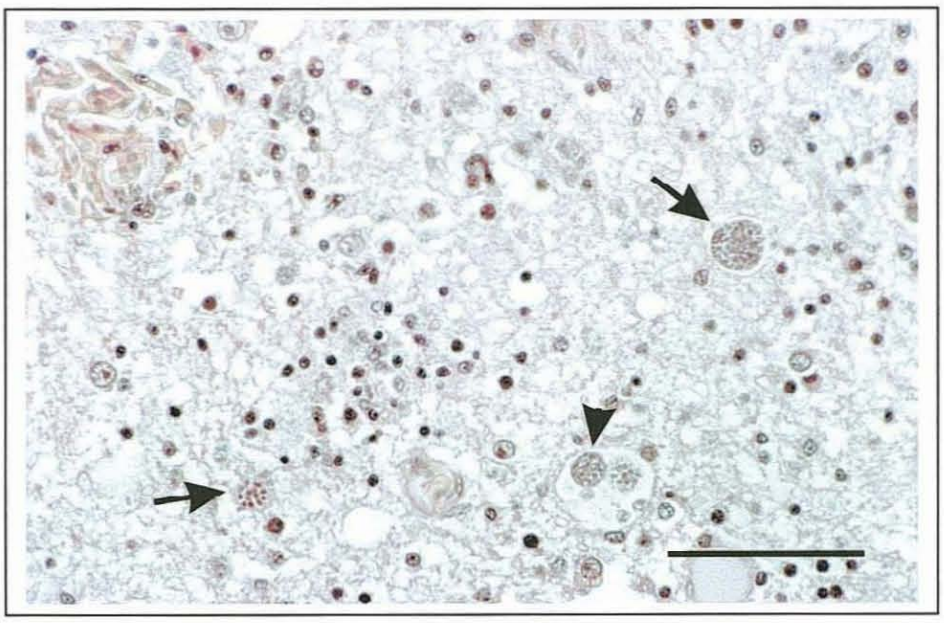


Figure 10.7 Cysts of the unidentified protist (arrows) in the brain of a *Litoria caerulea* (96 961/11). Two cysts are present within a single vacuole surrounded by myelin (arrowhead). Bar = 60 μm.

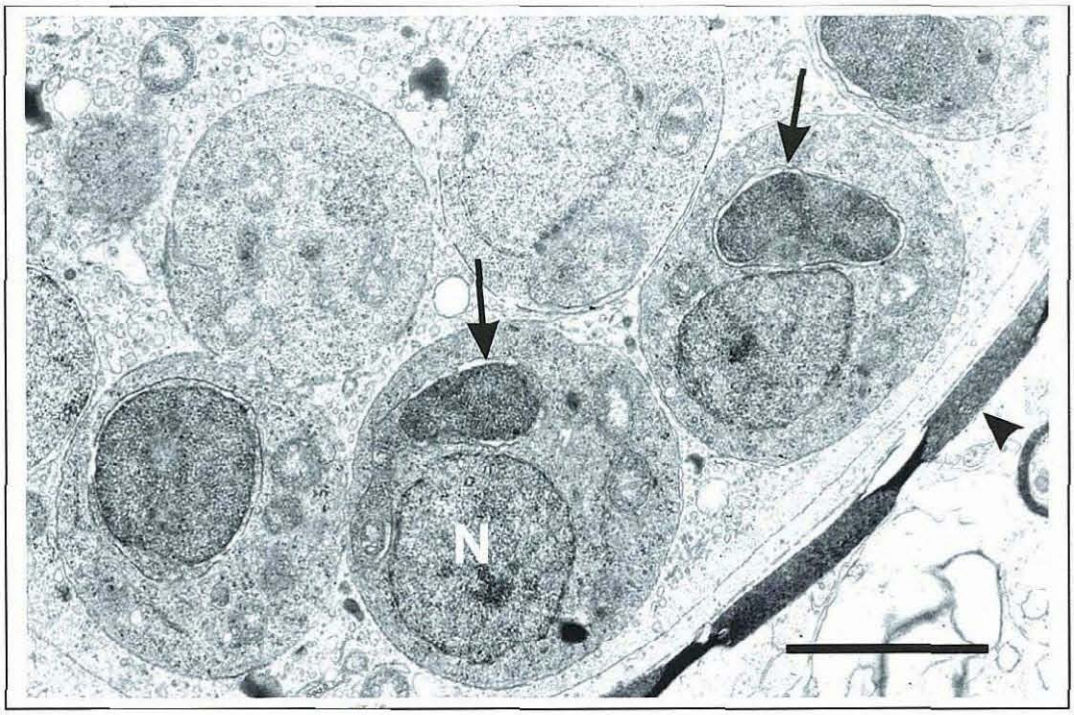


Figure 10.8 Electron micrograph of the protist in the spinal chord of a tadpole of *Litoria aurea* (97 1754/7). The cyst is surrounded by myelin (arrowhead) beyond the cyst membrane. Each cell has a large electron dense membrane bound organelle in the cytoplasm (arrows). N = nucleus. Bar = 1.5 μm.

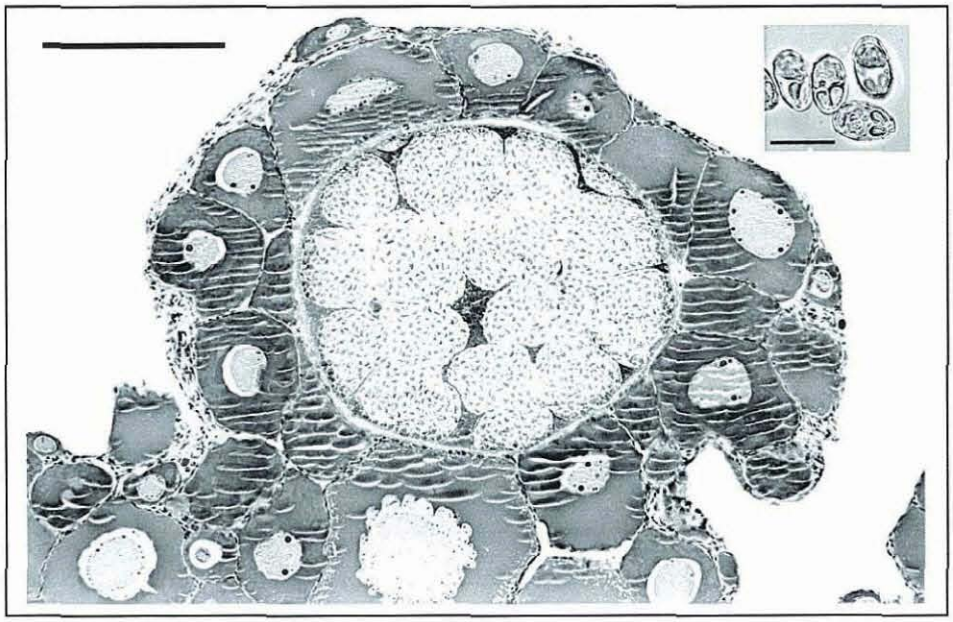


Figure 10.9 Section of ovary of a *Litoria caerulea* (98 1469/13) containing a large cyst containing spores of *Myxobolus hylae*. Bar = 260 μm. Inset: Wet preparation of spores. At the anterior end are two prominent pear-shaped polar capsules that contain long, thin, polar filaments wound in a spiral. Bar = 12 μm.

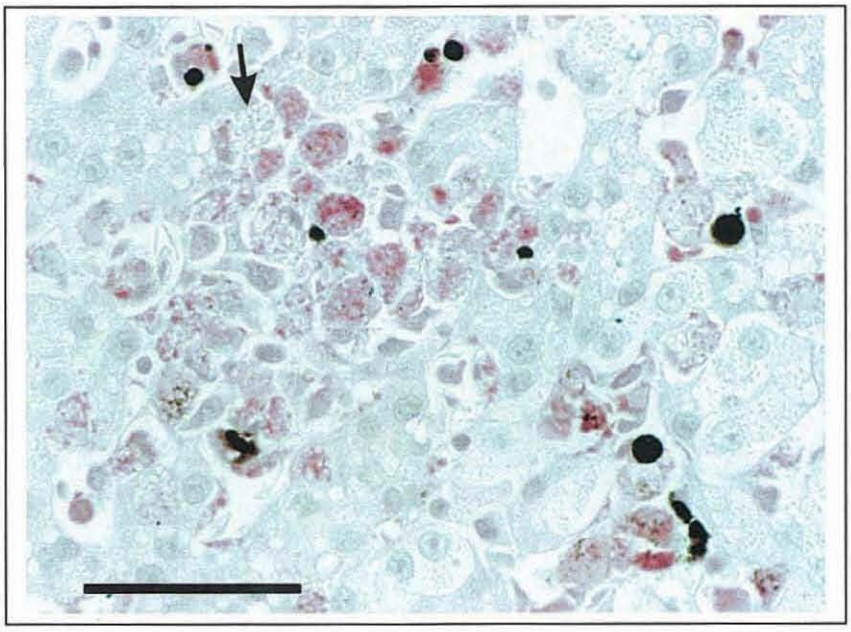


Figure 10.10 Section of liver from a metamorph of *Neobatrachus kunapalari* (96 1431/41) with a focal infection of intracellular refractile spores of *Pleistophora* sp. (arrow). In some spores there is a red stained granule at the anterior end, which is the anchoring disc. PAS. Bar = 60 µm.

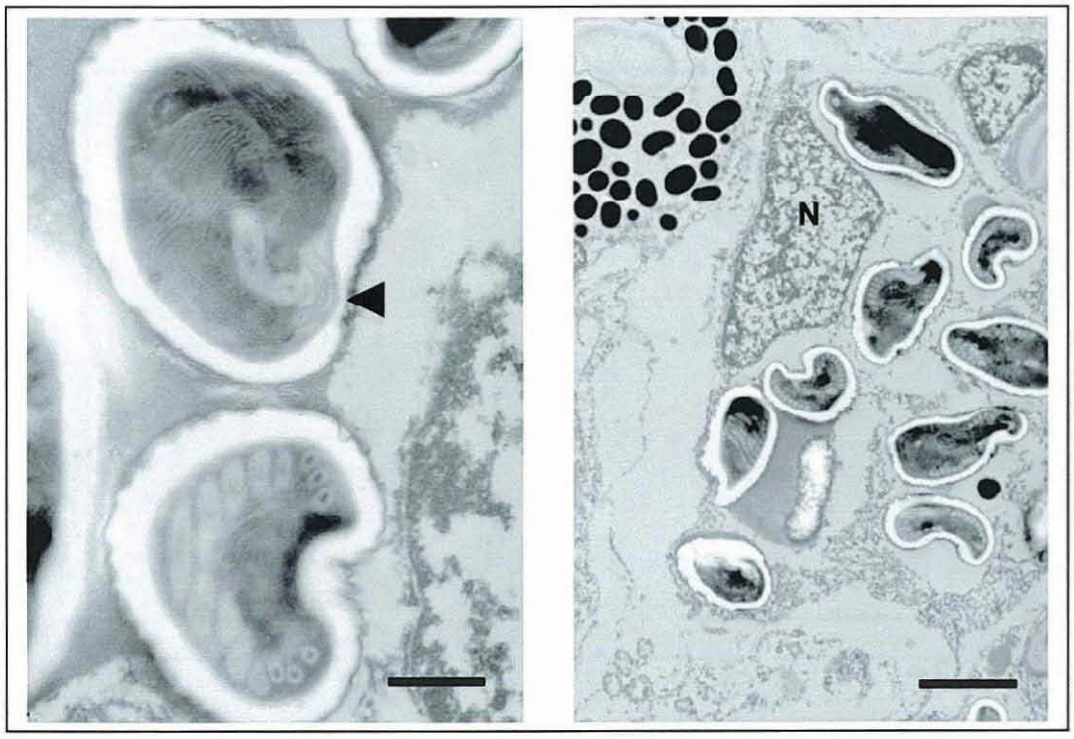


Figure 10.11 Electron micrographs of the microsporidian *Pleistophora* sp. in the liver of a *Neobatrachus kunapalari* (96 1431/41). Left: Spores have a thick capsule. The anchoring disc of the polar tube is visible in the upper spore (arrow head). Bar = 500 nm. Right: Spores occur intracellularly. They contain a single layer of polar tube coils. These are extruded when hatching and are used to inject the spore contents into the host cell. N = cell nucleus. Bar = 1.5 µm.

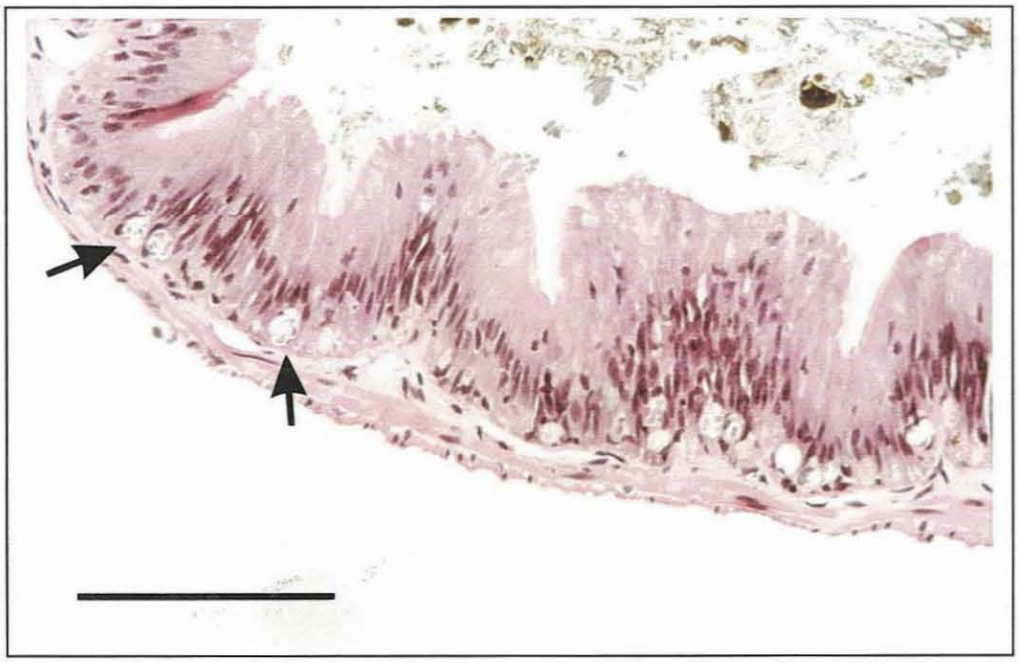


Figure 10.12 Section of intestine from a tadpole of *Mixophyes fasciolatus* (01 95/4) infected with a likely *Goussia* sp. in vacuoles in the basal epithelial cells (arrows). Bar = 140 µm.

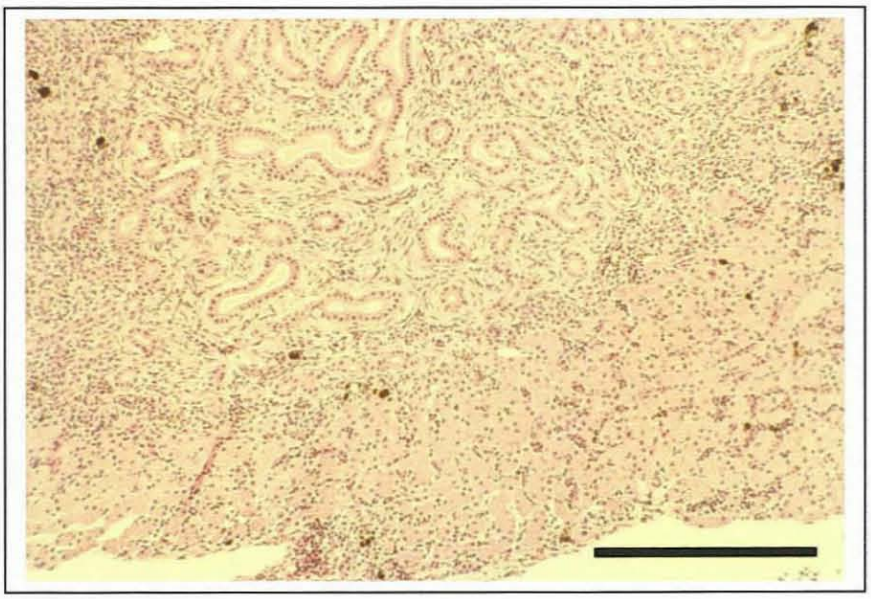


Figure 10.13 Section of liver from a tadpole of *Mixophyes fasciolatus* (01 95/4) with biliary hyperplasia and fibrosis due to an undetermined cause. Bar = 240 µm.

Figure 10.14 Dermatitis in a *Litoria chloris* (97 1754/11) with discolouration, focal erosions and ulcerations. Bar = 18 mm.

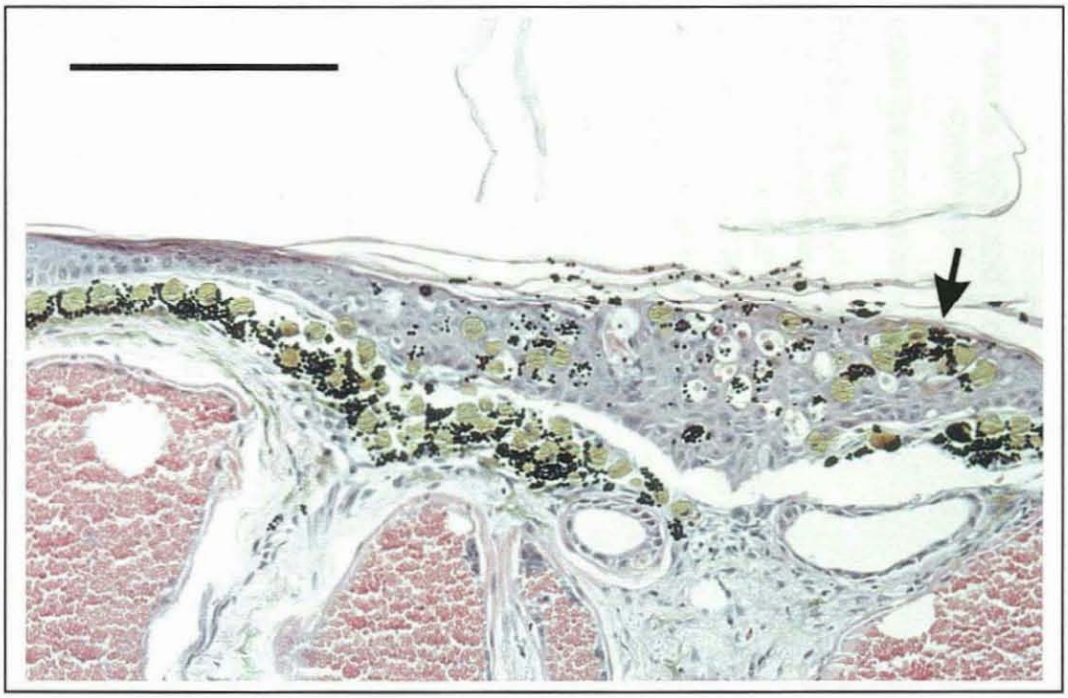
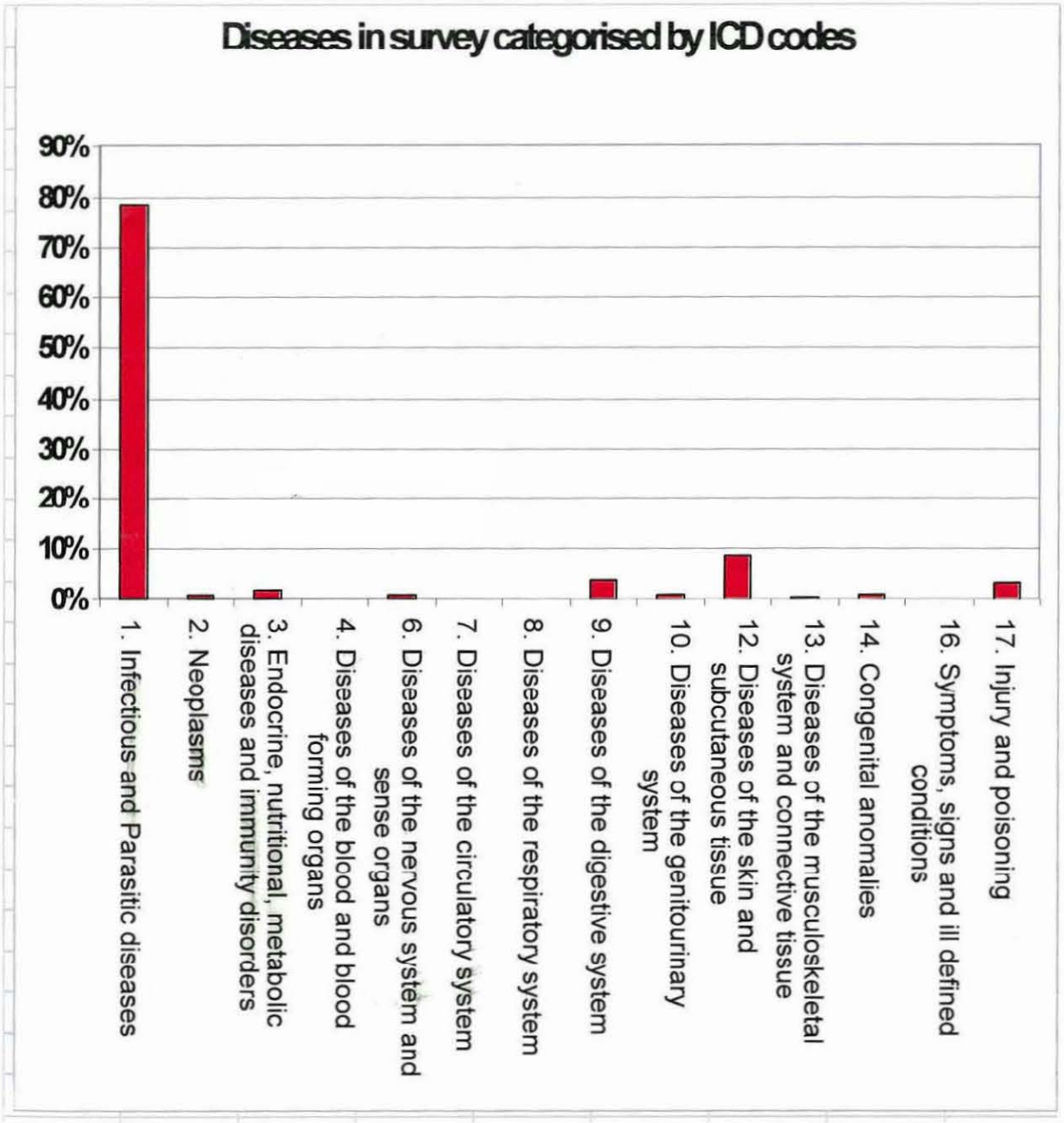


Figure 10.15 A section of skin of *Litoria chloris* (97 1754/11) with vacuolation of epidermal cells, hyperkeratosis and the presence of pigment cells in the epidermis (arrow). Cleavage is occurring with lifting of the entire epidermis. Bar = 160 µm.

Figure 10.16 The number of frogs in each ICD (International Classification of Diseases) category. This graph represents the primary diseases of sick frogs as listed in Appendix 1. This classification system is used in human health to compare populations.



Significant diseases of wild amphibians included chytridiomycosis, sparganosis, mucormycosis, aphanomycetosis, protistan encephalitis, and vacuolating and ulcerative dermatitis. The identification of *Chlamydia pneumoniae* in a case of pneumonia was significant in increasing the number of hosts known to be infected with this human pathogen. Biliary hyperplasia and fibrosis occurred commonly in adults and tadpoles. In adults this was likely to be due to *Myxidium*, as reported by Hill et al. (1997), but the

cause was not determined in tadpoles. The coccidian in tadpole intestine was similar to *Goussia hyperolisi* which occurs in tadpoles in Kenya and this infection expires towards metamorphosis (Paperna et al., 1997). Viruses were not found despite examination of suspicious lesions by electron microscopy, cell culture, ranavirus PCR and ranavirus immunostaining. Reports of pathology due to ranaviruses have usually described acute disease (Daszak et al., 1999) but a recent report demonstrates they can also cause a chronic disease that was only diagnosed by PCR (Cullen and Owens, 2000). Although ranaviral disease was always considered as a differential diagnosis for acute necrotic lesions seen in our survey, chronic ranaviral disease in individuals may have been overlooked as specific ranavirus tests were not done on all specimens.

As this survey was comprised of specimens that were collected opportunistically, it is a highly biased sample and results do not necessarily reflect prevalence of disease in wild frogs. *L. caerulea* are probably over-represented as they are a large, highly visible and easily caught species that live around houses. Intensive monitoring of endangered frogs would have increased the numbers of these frogs found. Although we were interested in diseases effecting and being transmitted by cane toads, few toads were collected despite being obvious and easily caught. This was probably due to a lack of interest by collectors, rather than to low morbidity or mortality.

The list of locations that frogs were collected from also contains significant bias, due to increased searching intensity in areas of monitoring of endangered frogs, near populated areas, and in places where a few enthusiastic collectors were based.

Although chytridiomycosis was obviously the cause of most disease, the true percentage of frogs that died with chytridiomycosis is likely to be even greater. In captivity, often whole collections of frogs died but only a small sample was submitted to confirm chytridiomycosis. In some cases frog keepers became confident in diagnosing this disease themselves and did not submit frogs. Predation and road trauma obviously account for the deaths of a large proportion of free-living frogs, but only a few were included in this survey.

Without results from experimental transmission, it can be difficult to decide when an infective agent is the primary cause of a disease, particularly when dealing with unusual

diseases or concurrent infections. Evidence of pathogenicity is often not demonstrated convincingly by histology, and so conclusions from this survey were based on reasonable assumptions. Although amphibians have immune systems generally similar to mammals (Carey et al., 1999), many examples were seen where heavy, pathogenic infections incited little cellular response, such as with chytridiomycosis, bacterial septicaemia or sparganosis. This lack of host response adds to the difficulties of diagnosis. In other cases, chronic inflammation was suggestive of an infection, but organisms were not detected as they had probably been eliminated by the host. This illustrates a problem with diagnosis of disease in wild animals where only animals in terminal stages are presented, as earlier, subtler clinical signs are not apparent, and since the frogs are not perceived as ill by collectors, they are not removed. There is much opportunity for further investigations into the diseases presented here.