CHAPTER 1

Aims and scope of thesis

1.1 Overview of the project

The initial aims of this project were to investigate diseases of Australian frogs and to find the infectious cause of the mass mortalities associated with declining frog populations in Queensland. Pathological studies had commenced after a mass die-off was detected in 1993 at Big Tableland, near Cooktown, north Queensland and carcasses were collected for the first time in Australia (Laurance et al., 1996). The large mortality caused a drastic population decline and it appeared that the cause of this event was likely to be the same as in previous population declines that had been occurring since 1979 in mountains to the south in eastern Queensland. The rapid population crashes in previous declines suggested mass mortality of adults was occurring, and the epidemiology suggested the cause was the introduction of an infectious disease that was spreading from south to north Queensland (Laurance et al., 1996; Chap 2). Initial diagnostic investigations involved histopathology, bacteriology and virology but the cause of death was not determined (Speare, 1995). Rick Speare was convinced the deaths were due to a primary infectious agent that was not bacterial. Although attempts were made to isolate ranavirus, no viruses were cultured. A range of opportunistic bacteria was isolated and histopathology showed minor non-specific acute degenerative changes in some organs (Speare, 1995). An unidentified protist occurred in the skin of some of these frogs without causing severe skin lesions, but was not considered to be significant.

This project began with the intention of further investigating the samples from the 1993 die-off. As a virus was suspected to be the cause, but could not be isolated in cell culture, I began work with Alex Hyatt examining lesions in organ samples by transmission electron microscopy. As there had been no studies into frog diseases present in Australia, I also investigated diseases in frogs from around Australia to obtain the basic information on diseases present in non-declining populations that was needed to interpret results from the declining frogs. For this survey a network was established involving universities, government environmental departments and

community interest groups, so that any ill or dead frogs encountered by herpetologists or other scientists would be collected, preserved and forwarded to the Australian Animal Health Laboratory (AAHL) with the appropriate information. Results of diagnostic tests were reported back to the submitters. This collaborative approach worked well with the involvement of many people from diverse locations (Chap 3). A large number of frogs were sent from southeast Queensland, due to the collection and coordination efforts of Harry Hines in Brisbane. A range of diseases was identified, with the more significant ones being studied in detail (Chaps 10-14).

Over half the frogs submitted were infected with the same skin microorganism seen in the initial die-off. Although the epidermal changes were often not severe, this infection appeared to be the only significant finding in these dying frogs. An outbreak at the Amphibian Research Centre (ARC) in 1996 allowed more detailed observations of the disease. Gerry Marantelli was convinced that healthy tadpoles were carrying the disease, although death only occurred after metamorphosis. Examination of tadpoles showed infection with the same skin microroganism occurred only in the keratinised mouthparts, with a rapid redistribution to the skin of the body at metamorphosis (Chap 5).

Using infective material from the outbreak at the ARC, we conducted a preliminary experiment exposing frogs to infected skin scrapings that demonstrated it could cause a fatal disease. Samples were sent to experts for identification by transmission electron microscopy (Peter Daszak) and PCR (Louise Goggin) who identified the organism as a new genus of chytrid fungus. The term "chytrid" is used in this thesis to mean a member of the phylum Chytridiomycota. In 1997 chytridiomycosis was discovered in declining frogs in Panama, and we collaborated with the scientists involved (David E. Green and Karen Lips) to compare the pathogen from both continents. We published a joint paper that described the disease and explained its significance (Berger et al., 1998; Appendix 4). This paper includes the histopathology, the initial transmission experiment, DNA sequencing, and transmission and scanning electron microscopy from the Australian samples, and histopathology and transmission electron microscopy on Panamanian samples. Another group in the USA had been working concurrently on this disease in captive dendrobatid frogs (Pessier et al., 1999). They were able to culture the fungus and named it *Batrachochytrium dendrobatidis* (Longcore et al., 1999). With

Joyce Longcore's instructions, I was able to culture *B. dendrobatidis* from frogs in Australia.

Further animal experiments using cultured zoospores examined the effect of dose, temperature and isolate on pathogenicity, and provided information on incubation times and progression of clinical signs (Chap 7).

Other investigations of chytridiomycosis used histopathology and ultrastructural pathology to investigate the effects on frogs (Chap 5). A variety of electron microscopic techniques were used to study the lifecycle and morphology of *B. dendrobatidis* (Chap 4).

The survey of diseased frogs continued until the end of 2000 and provided information on the host range, incidence, distribution and impacts of chytridiomycosis (Chap 8), as well as resulting in the discovery of additional diseases (Chap 10).

Other areas of work aimed to enhance diagnosis and management of the disease. To improve sensitivity and ease of diagnosis, polyclonal antibodies were generated in rabbits and sheep, characterised, and used to develop an immunoperoxidase test for tissue sections (Chap 6). Chemicals, heat and drying were tested as disinfectants. The efficacy of antifungal drugs was tested on culture and in animal trials (Chap 9).

1.2 Problems with investigating the cause of frog declines in Australia

One of the initial problems in investigating the cause of frog declines was that rainforest populations simply disappeared and no one was present to witness mass mortality occurring. Intensive monitoring of some remaining populations by Keith McDonald led to the discovery of dying frogs in 1993 at O'Keefe Creek, Big Tableland in north Queensland, which could then be examined pathologically. In addition, frogs decompose quickly and when found in the rainforest, appropriate preservation may not be possible. The provision of instructions (Berger and Speare, 1998) to field workers who were dedicated to collection of well-preserved specimens overcame this problem and live or preserved specimens were obtained from many isolated sites. However, even with good specimens interpretation of amphibian pathology is difficult, especially in

Australia due to the lack of knowledge of both diseases and normal anatomy, which varies greatly between frog species. The standard of my pathological interpretation improved as my collection of cases increased and disease patterns were recognised. The finding of a new genus of chytrid fungus infecting the frogs led to new problems. As members of this phylum of fungi had not previously been found causing disease in vertebrates, basic methods for studying *Batrachochytrium* had to be developed. Methods for isolation and maintenance of pure cultures were learnt from Joyce Longcore as they were developed, while I was involved in developing methods for animal infections, diagnosis, antifungal testing and production of polyclonal antibodies. The ability to culture *B. dendrobatidis* allowed us to do new research involving experimental infections and closer study of the pathogen itself, and also made the work easier as we had previously been unable to store live fungus.

1.3 Collaborative approach

Throughout the project, results were shared with scientists, wildlife managers and the public in Australia and overseas. This was necessary as the work has important practical applications for conservation, is a new field of research, and has received wide interest. Many people have begun studying amphibian chytridiomycosis in the few years since we first reported it, and we have made information available though conference and seminar presentations, workshops, and on the Amphibian Disease Home Page (http://www.jcu.edu.au/school/phtm/PHTM/frogs/ampdis.htm), to ensure knowledge in this field could advance rapidly. Cultures, antibodies, photographs and reference material have been sent to numerous researchers.

1.4 Contribution of others to this work

The project has involved the work of many collaborators. The diseased amphibians examined in the pathological survey were collected by over 50 people in the herpetological community including scientists and amateur herpetologists. This enabled us to survey for diseases over large areas of Australia.

As the project involved a range of disciplines, specialised work on some topics (e.g. PCR, parasite morphology) were performed by collaborators (Chap 3). Disease

outbreaks were often investigated by a range of researchers. This thesis covers work where I was the primary investigator, apart from two papers on mucormycosis included in Chap 12. The contributions of others are explained in the preface and at the start of sections that consist of papers by many authors.

1.5 Aims of the thesis

1. To discover and describe diseases in Australian amphibians.
2. To discover the cause of declines of amphibians in pristine locations.
3. To characterise the aetiological microorganism and the pathology of the disease responsible for these declines.
4. To investigate the host range, distribution, incidence and impacts of this pathogen.
5. To develop and evaluate diagnostic tests for this microorganism.
6. To develop disinfection protocols and treatments to cure amphibians of this pathogen.

CHAPTER 2

Literature review

2.1 Amphibian declines in Australia

In Australia, dramatic amphibian population declines have occurred since the 1970's. Of the 214 species of amphibians described from Australia, 15 are classified as endangered, 12 as vulnerable and 4 as extinct (Environment Protection and Biodiversity Conservation Act, 1999). Australia has a diverse amphibian fauna with 28 genera and four families (Cogger, 1992; Tyler, 1997). Declining frogs are from ten genera and most are from Queensland, NSW and Victoria. Lifestyle rather than taxonomy provides the common links between threatened species. They are mostly from high altitude areas (>300 m), 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).

The declines in Queensland have been well documented. Six rainforest species have disappeared and another eight have suffered serious declines. The first declines were noticed in the late 1970's in southeast Queensland and involved the cascade treefrog (*Litoria pearsoniana*), giant barred frog (*Mixophyes iteratus*), Fleay's barred frog (*M. fleayi*), southern dayfrog (*Taudactylus diurnus*) and the southern gastric brooding frog (*Rheobatrachus silus*). The latter two species have not been seen since and were listed as extinct by Environment Australia in 1999. In the mid 1980's, two species in mid-eastern Queensland declined suddenly - Eungella dayfrog (*T. eungellensis)* and the northern gastric brooding frog (*R. vitellinus*). The latter species has not been observed since 1985 and was also listed as extinct in 1999. Declines then occurred in the early 1990's in north Queensland. Three species that only occurred at high altitudes disappeared - the armored mist frog (*L. lorica*), mountain mist frog (*L. nyakalensis*), and the sharp snouted dayfrog (*T. acutirostris*). This was the last remaining population of *T. acutirostris* and this species was also listed as extinct in 1999. In the same area three other species - common mist frog (*L. rheocola*), waterfall frog (*L. nannotis*) and the Australian lacelid (*Nyctimystes dayi*) persist only in the lowland rainforest after the loss

of high altitude populations (McDonald, 1990; Richards et al., 1993; Laurance et al., 1996; Hines et al., 1999).

In some regions of Australia declines have been caused by the introduction of exotic predators, or habitat changes due to logging, wetland drainage, weed invasion, urban development and agriculture (Tyler, 1997; Hines et al., 1999; Gillespie and Hero, 1999). However, habitat disturbance does not explain the rapid disappearance of high-altitude stream-dwelling frogs from many protected areas in Australia (Richards et al., 1993; Mahony, 1996). Measurements of water chemistry, including inorganic ions, heavy metals and pesticide residues failed to identify abnormalities. Declines appear unrelated to mining, logging, low rainfall or unusual weather (Richards et al., 1993; Laurance, 1996). Habitat disturbance caused by pigs may have contributed to declines in some areas (Richards et al., 1993).

Frogs in NSW and Victoria have also declined in the last 20 years. For many examples the cause of the decline appears to be multi-factorial. The introduction of fish, such as brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*) and mosquito fish (*Gambusia holbrooki*), has had severe impacts on some species (Gillespie and Hero, 1999). The spotted treefrog (*L. spenceri*) lives along the Great Dividing Range between Lake Eildon and Mount Kosciusko. It declined in the 1970s and early 1980s. Human impacts on streams appear to have affected populations, and trout are believed to be the cause of major population declines. However, in 1996 a population not in contact with trout suddenly declined (Gillespie and Hines, 1999). The green and golden bell frog (*L. aurea*) disappeared from sites in the Great Dividing Range in NSW in the 1970's but remains in coastal habitats (Mahony, 1999). One of the most endangered frogs is the southern corroboree frog (*Pseudophryne corroboree*) in the Snowy mountains and Brindabella ranges. Breeding occurs in ponds and seepages, and UV-B and climate change are being investigated as causes of the decline (Osborne et al., 1999; Broomhall et al., 2000). Other species are more obviously impacted by habitat changes, for example, the red-crowned toadlet (*P. australis*) is a vulnerable species inhabiting exposed sandstone around Sydney and is threatened by development of its habitat (Thumm and Mahony, 1999). There have not been recent frog extinctions in Western Australia although three species with small, fragmented distributions are considered vulnerable (Roberts et al., 1999).

Several factors in the declines in protected areas of Queensland indicated that a waterborne infectious disease, of high virulence to adults of some species, had been introduced to Australia. These factors were: 1) sudden, severe declines occurred over a few months at individual sites; 2) declines were asynchronous and spread as a front along the east coast of Australia; 3) adults died while tadpoles survived and metamorphs died when they subsequently emerged; 4) no environmental changes were detected; 5) only stream-dwelling frogs disappeared and, 6) in one intensively monitored site, mass mortality was observed at the time of a significant population decline (Trenerry et al., 1994; Laurance et al., 1996). Mass mortality occurred in 1993 at the montane rainforest location of Big Tableland in north Queensland (Fig. 2.1). Sick and dying frogs were collected for pathological examination (Speare, 1995; Mahony et al., 1999). These frogs were of the endangered species *T. acutirostris, L. rheocola* and *L. nannotis*. This was the last time *T. acutirostris* was seen in abundance and there were only a few sightings since (Marshall, 1998). At the time of the declines, healthy tadpoles of *T. acutirostris* were collected to be raised in captivity at the Royal Melbourne Zoo and Taronga Zoo. However, only a few survived past metamorphosis. Wild adults taken to James Cook University all died within weeks of collection (Mahony et al., 1999).

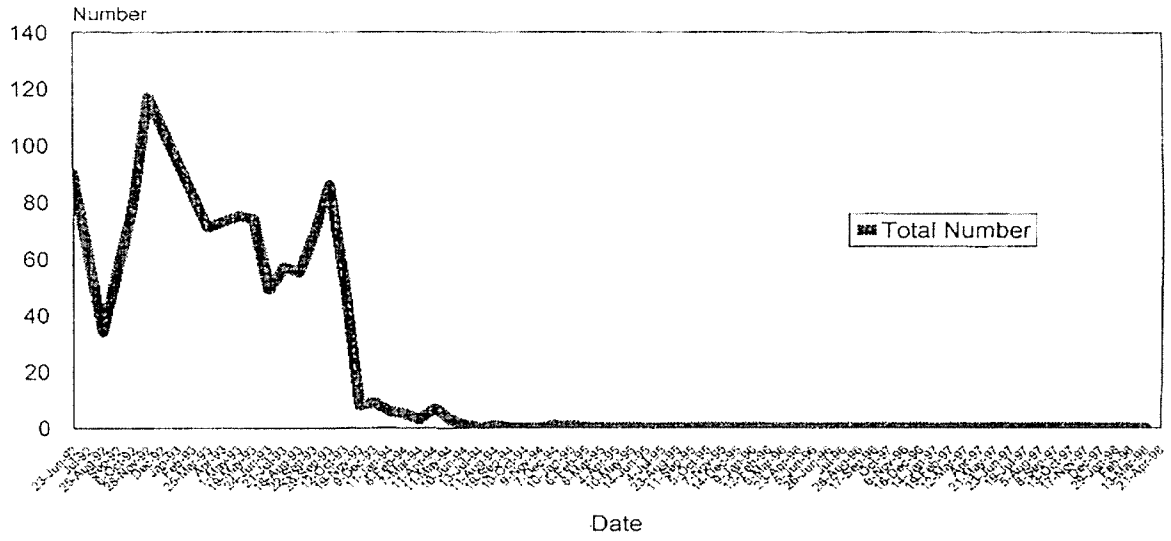

Figure 2.1 Total numbers of four frog species (*Litoria nannotis*, *L. rheocola*, *Taudactylus acutirostris* and *Nyctimystes dayi*) at a site at Big Tableland. Frogs were collected dying from October 1993 to January 1994. (Reproduced from McDonald and Alford [1999], with permission).

It was thought that if the cause of the deaths could be determined, then the cause of the declines would be solved. Initial investigations into the cause of death of wild and captive frogs of the three species from Big Tableland did not result in a diagnosis (Speare, 1995). Clinical signs included abnormal sitting posture, lethargy, loss of righting reflex, and fitting when handled. Small skin ulcers and haemorrhages in skin, muscle or eye were occasionally seen. Acute focal necrosis occurred in range of organs and an unidentified organism, thought to be a protozoan, infected the superficial layer of the skin. Although a range of opportunistic bacteria was isolated including *Aeromonas hydrophila* and *Pseudomonas aeruginosa*, the histopathology was not consistent with bacterial disease. Viruses were not isolated on BF-2 and FHM cells. Inoculation of five juveniles of *Limnodynastes ornatus* with homogenised tissue from sick frogs did not result in disease (Speare, 1995). After consideration of the epidemiology and the pathology, a new infectious agent (possibly a virus) was suspected as a likely cause of the deaths.

There has been debate over the strength of the evidence implicating the introduction of an infectious disease (Alford and Richards, 1997; Hero and Gillespie, 1997) and it was suggested that other causes of declines could be consistent with the epidemiology seen. In particular, the data demonstrating the "extinction wave" pattern was criticised, and the rapidity of the declines was not accepted to mean that mass mortality was the mechanism of declines. Laurance et al. (1997) responded that the wave-like pattern of declines may have moved patchily in some areas, but this does not detract from the general trend showing a spread of declines. An infectious agent may have moved through lowland areas but was only highly virulent at high elevation sites. As declines occurred over a few months, mortality was clearly occurring, not lack of adult recruitment (Laurance et al., 1997). Laurance et al. (1997) accepted that pathological evidence for an infectious disease was lacking, but producing a disease hypothesis based on epidemiology is reasonable. An important part of the infectious disease hypothesis was the lack of evidence for specific alternative theories.

2.2 Amphibian declines in other countries

2.2.1 Americas

In the Americas there have been declines due to environmental degradation (Bonin et al., 1997; Alford and Richards, 1999) but there has also been a similar pattern of declines to Queensland with mysterious, rapid disappearances of montane populations. Dramatic population crashes occurred in stream-dwelling frogs in high altitude rainforest in Brazil, and although mass mortality was not observed, it was suspected to have occurred (Heyer et al., 1988; Weygoldt, 1989). Tadpoles did not appear to survive metamorphosis in the wild and although in 1975 tadpoles were easily raised in captivity, in 1987 most died during metamorphosis (Weygoldt, 1989). Suggested causes included airborne pollution, disease, and climate change secondary to forest loss (Heyer et al., 1988; Weygoldt, 1989). Similarly, the abrupt declines of *Bufo periglenes* and *Atelopus varius* in Costa Rica's Monteverde Cloud Forest suggested high adult mortality rather than just a lack of successful recruitment (Pounds and Crump, 1994). The unusually warm, dry conditions in 1987 caused the frogs to shift in distribution within the habitat in response to desiccating conditions, and one theory was that this shift may have precipitated a disease epidemic (Pounds and Crump, 1994; Pounds et al., 1999). In the early 1990's dead and dying anurans were found before a decline was noticed at Las Tablas, 250 km southeast of Monteverde (Lips, 1998). Skin degeneration in several individuals suggested an environmental or infectious cause (Lips, 1998).

Waves of mass mortalities, described as the "post-metamorphic death syndrome", have been reported in various amphibian populations in western North America (Scott, 1993). Deaths often occurred after cold weather. Ranid species were affected more than Bufonids, and a novel disease that had spread through watersheds was suggested as the cause (Scott, 1993). Examples include *Rana tarahumarae* in Arizona (mid 1980s), *B. canorus* in Sierra Nevada (1976-82), *R. muscosa* (1979- 90), *R. chiricahuensis* in New Mexico and *B. boreas* in Colorado (Kagarise Sherman and Morton, 1993; Scott, 1993). Limited pathological studies were conducted on *B. boreas boreas* and *R. muscosa* found dying during declines in 1974 and 1979 respectively (Bradford, 1991; Carey, 1993). *A. hydrophila* was cultured and proposed as the cause of the deaths, possibly secondary to an unidentified stress (Carey, 1993). In Sierra Nevada, a range of species declined in

relatively undisturbed, protected areas (Drost and Fellers, 1996). Evidence suggested that applications of pesticides and herbicides in California had contaminated these high altitude sites (Fellers, 1997).

Introduced predators (fish and bullfrogs) have had large effects on some lowland populations in the USA (Fisher and Shaffer, 1996), but this does not explain all the declines. The timing of declines does not relate to the period of introduction of fish, and anurans have declined where there are no fish (Hayes and Jennings, 1986; Drost and Fellers, 1996). Unusual egg mortality occurred in a population of *B. boreas* from 1989. *Saprolegnia ferax* was identified on eggs from two days after laying and eventually 95% died. Restocking fish in the lake could have introduced the fungus or stress (from habitat changes or other diseases) may have precipitated the fungal outbreak (Blaustein et al., 1994).

2.2.2 Other continents

Environmental changes such as habitat destruction have obviously caused declines in amphibian populations in many countries (Alford and Richards, 1999). No species have recently become extinct in Europe (Bosch et al., 2001). Declines have mainly occurred due to environmental reasons and cases of inexplicable declines are unusual (Bosch et al., 2001). For example in the UK at least three of the six indigenous amphibians have declined. Frogs declined slightly over most of Britain during the 1940s and 1950s with greater declines in the 1960s. Loss of suitable habitat is the main cause, and acidification is also involved (Cooke, 1972; Beebee et al., 1990). However, recurrent mass deaths in the UK have affected populations and are thought to be due to iridovirus infection (Cunningham et al., 1995; Cunningham et al., 1996a). Mass mortality in the Pyrenean Mountains in Spain was attributed to *A. hydrophila* (Marquez et al., 1995).

In Pakistan, industrialisation, urbanisation, mechanization of agriculture, pesticides, fumigation of granaries, and road kills impact on amphibians (Khan, 1990). In many countries, especially in Asia and Africa, little is known about the indigenous amphibian populations and declines could have easily gone unnoticed.

2.3 Infectious agents with potential to cause epidemics in free-living anurans

This is an historical review of pathogens with potential to cause epidemic deaths and includes papers only up to 1996 when this project commenced. At that time there had been few studies on infectious disease in wild amphibians. Diseases in captive amphibians are included with comments on their potential to be pathogenic in the wild. Many diseases of captive amphibians are related to poor husbandry and are unlikely to cause high mortality rates in the wild. Where cases of mass mortality in the wild were studied, rigorous pathological testing was rarely done. This review describes diseases reported from Australian amphibians in greater detail than those from other countries.

2.3.1 Viruses

2.3.1.1 Iridoviruses

Iridoviruses are significant infectious agents as they can cause large mortalities in free-living amphibian populations (Cunningham et al., 1996a). Iridoviruses causing disease in amphibians are in the genus *Ranavirus*, family Iridoviridae, and are enveloped icosahedral DNA viruses (Hengstberger et al., 1993). Isolates causing disease have been found in Australian, American, and European amphibians (Wolf et al., 1968; Speare and Smith, 1992; Cunningham et al., 1996a).

Bohle iridovirus (BIV) is the only virus isolated from Australian frogs and has been isolated once - from metamorphs of *L. ornatus* that died during metamorphosis in captivity (Speare and Smith, 1992). The frogs had been collected as tadpoles from a temporary pond at Bohle, a suburb of Townsville. BIV has since been experimentally transmitted to adults and tadpoles of *B. marinus*, juveniles and tadpoles of *Limnodynastes terraereginae*, and juveniles of *Litoria latopalmata*, as well as the barramundi (*Lates calcarifer*) (Moody and Owens, 1994; Cullen et al., 1995). Juvenile *L. terraereginae* and *L. latopalmata* were highly susceptible to BIV while larval *L. terraereginae* were less susceptible. Mortality varied with dose and route (Cullen et al., 1995). Typical pathology included renal, pulmonary, hepatic, splenic and haemopoietic necroses and haemorrhages.

Tadpole edema virus (TEV) was isolated from grossly oedematous *R. catesbiana* tadpoles from West Virginia, USA. In August 1965 several metamorphosing *R. catesbiana* tadpoles had oedema and diffuse haemorrhage on ventral surfaces and legs. Acute fatal disease was reproduced with experimental transmission to tadpoles and adults, although pathogenicity varied among species (Wolf et al., 1968). Other isolates of iridoviruses from *R. pipiens* in the USA were found while searching for a viral cause of the Lucké tumour, and were named Frog Virus 3 (FV3). Subsequently, much work was done on the morphology and life cycle of FV3 in the laboratory, and it was shown experimentally to cause deaths with oedema, necrosis and haemorrhage (Granoff, 1989).

An iridovirus was isolated from cases of mass mortality in *R. temporaria* in Britain, which in some areas appeared to reduce local frog populations (Cunningham et al., 1995; Cunningham et al., 1996a). The incidence of deaths peaked in July, August and September. Two main syndromes were identified – one with skin ulceration and the other with systemic haemorrhages. Some frogs had only reddened skin. Histological lesions included epidermal thickening, epidermal necrosis, and necrosis, granulocytic inflammation, congestion and haemorrhage in internal organs (Cunningham et al., 1996a).

An iridovirus-like agent was found in dying captive *R. esculenta* from Croatia that had haemorrhages and skin necrosis (Fijan et al., 1991).

Although iridoviruses have not been linked to the catastrophic amphibian declines, they can cause high mortality rates. Iridoviral disease could have the potential to severely affect some populations, and as they can survive for long periods in the environment (Langdon, 1989) the possibility of introducing iridoviruses is a concern.

2.3.1.2 Erythrocytic viruses

The erythrocytic viruses appear similar to iridoviruses but are larger than the ranaviruses. They have been found in *R. catesbiana*, *R. clamitans* and *R. septentrionalis* from Canada (Gruia-Gray and Desser, 1992), *B. marinus* from Costa Rica (Speare et al., 1991), *Leptodactylus ocellatus* from Brazil (de Sousa and Weigl, 1976) and *Ptychadena anchietae* from South Africa (Alves de Matos and Paperna, 1993).

In *R. catesbiana* in Canada, the prevalence of frog erythrocytic virus (FEV) was higher in juveniles than adults. These viruses appear to be less pathogenic than the ranaviruses, although anaemia was recorded in heavily infected animals (Gruia-Gray and Desser, 1992). Infected animals were slightly less likely to be recaptured suggesting that the virus contributed to the mortality of juvenile bullfrogs.

2.3.1.3 Amphibian leukocyte virus

Polyhedral cytoplasmic DNA virus was found in the cytoplasm white blood cells of a Mexican *R. catesbiana* that was lethargic and had small exudative ulcers (Briggs and Burton, 1973). The large iridovirus found in red blood cells of *B. marinus* in Costa Rica also was found in the cytoplasm of reticular cells in the spleen (Speare et al., 1991).

2.3.1.4 Lucké Herpesvirus

The Lucké tumor herpesvirus induces renal adenocarcinoma in *R. pipiens* in North America. Lucké (1934) gave a comprehensive description of the appearance and histology of this disease. As the tumor was transmissible and had intra-nuclear inclusions, he suggested it was caused by a virus (Lucké, 1938). This hypothesis was later confirmed (McKinnell and Cunningham, 1982; McKinnell and Carlson, 1997). Clinical signs are bloating, lethargy and death, which occur when the tumour is large or has metastasised (Anver and Pond, 1984). Single or multiple white nodules occur in the kidneys and grow into large masses. The tumour is an infiltrating and destructive adenocarcinoma, or less often it is more orderly and adenomatous (Lucké, 1934). Although the gross appearance of the tumour remains relatively unchanged, there are significant seasonal differences in the microscopic appearance. Winter tumours display cytopathic characteristics associated with the presence of virus (enlarged nuclei with eosinophilic inclusions) whereas summer tumours lack virus (McKinnell, 1973). Studies have shown that above 22°C virus replication does not occur and viral particles are not present in the tumour (Anver and Pond, 1984). Surveys of wild *R. pipiens* for the Lucké tumour have found prevalences up to 12.5% (McKinnell, 1969).

2.3.1.5 Herpesvirus-like particles in skin

In Italy, up to 80% of a wild population of *R. dalmatina* had epidermal vesicles associated with a herpes-like virus, but dead frogs were not found (Bennati et al., 1994).

2.3.1.6 Calicivirus

Calicivirus was isolated from two captive *Ceratophrys orata* found dead. Both had pneumonia, while one also had oedema and the other had lymphoid hyperplasia (Smith et al., 1986).

2.3.2 Bacteria

2.3.2.1 Bacterial septicaemia

There are many well-described accounts of large epidemics of bacterial septicaemia in captive amphibians. These are often caused by *A. hydrophila* and other gram negative bacteria or combinations of bacteria, including *Pseudomonas* spp., *Proteus* spp., *Flavobacterium indologenes* and *F. meningosepticum* (Hubbard, 1981; Anver and Pond, 1984; Olson et al., 1992; Taylor et al., 1993). A syndrome attributed to bacterial septicaemia was called "red leg" due to haemorrhages and erythema on hindlimb skin (Emerson and Norris, 1905). This descriptive term appears to have been misinterpreted by some zoologists and the general public to mean that any frog with reddening of the skin of the hind legs has "red leg" and hence bacterial septicaemia.

In captive amphibians outbreaks of bacterial septicaemia have high mortality rates. Clinical signs include pale skin, petechiation, haemorrhagic cutaneous ulcers, lethargy, anorexia, oedema, haemorrhages in internal organs, ascites and pale livers. On histology, there may be degenerative myopathy and multiple foci of coagulative necrosis with clumps of bacteria. Variable results were obtained from transmission experiments - the disease usually required inoculation of the bacteria, or bath exposure and stress (Dusi, 1949; Glorioso et al., 1974; Somsiri et al., 1997). *A. hydrophila* and many of the other bacteria causing infections in captive amphibians can be isolated from healthy animals and from the environment (Carr et al., 1976; Hird et al., 1981) suggesting that disease occurs secondary to stresses caused by poor husbandry such as overcrowding, dirty conditions, trauma, temperature changes, and also after transport (Glorioso et al., 1974; Hubbard, 1981;).

These bacteria can also be cultured from cases of viral disease particularly when frogs are collected dead, and symptoms of "red leg" are similar to those caused by iridoviruses (Cunningham et al., 1996a). Some mass die-offs in the wild have also been attributed to

bacteria due to their presence in dying animals, but these diagnoses are dubious due to a lack of histopathological confirmation and since other agents, particularly viruses, were not looked for. Bacteria were implicated in die-offs in *Alytes obstetricans* in the Pyrenean mountains in Spain (Marquez et al., 1995), in *R. muscosa* in California (Bradford, 1991) and in *B. boreas boreas* in Colorado (Carey, 1993). An epidemic among tadpoles of *Rana sylvatica* in Rhode Island, USA was reported to be caused by *A. hydrophila* (Nyman, 1986).

2.3.2.2 *Streptococcus*

A non-haemolytic group B *Streptococcus* caused an outbreak killing 80% of about 100000 farmed bull frogs (*R. catesbiana*) in Brazil (Amborski et al., 1983). Septicaemia, necrotising splenitis and hepatitis with haemorrhages occurred in frogs. The outbreak was associated with overcrowding and stress.

2.3.2.3 *Salmonella*

Amphibians may carry pathogenic *Salmonella* species, but rarely are frogs reported to be affected (Reichenbach-Klinke and Elkan, 1965; Anver and Pond, 1984). In Australia, *Salmonella* were isolated from 12.7% (19/150) of *B. marinus* collected from the wild and 9 serotypes were identified. All nine had previously been isolated in Australia from humans and livestock (O'Shea et al., 1990).

2.3.2.4 *Chlamydia*

Chlamydial infections have been reported in captive amphibians, causing moderate to high mortality rates in various species including *Xenopus laevis* in the USA, and *Ceratobatrachus guentheri* in Canada (Newcomer et al., 1982; Wilcke et al., 1983; Howerth, 1984; Honeyman et al., 1992). In all cases, the chlamydial species was either unknown or assumed to be *C. psittaci.* Outbreaks of chlamydiosis in captive amphibians resulted in fulminant, multisystemic infections with pyogranulomatus inflammation.

2.3.2.5 Mycobacteria

Mycobacterial infection of amphibians has been reported only in captivity and occurs mainly in immuno-compromised animals. Mortality rates are usually low. Natural resistance to mycobacteria, which are ubiquitous in aquatic environments, is higher than in homeotherms (Reichenbach-Klinke and Elkan, 1965). *Mycobacterium marinum* was

experimentally shown to cause a chronic granulomatous non-lethal disease in immunocompetent leopard frogs (*R. pipiens*) whereas frogs immunocompromised with hydrocortisone developed an acute lethal disease (Ramakrishnan et al., 1997). Infections may primarily involve skin, respiratory tract or intestines. Frogs have been found with single large tumour-like masses or with disseminated nodules throughout internal organs. Organs such as liver, spleen, kidney or testes may become almost completely destroyed by the infection before the animal dies, usually with cachexia (Reichenbach-Klinke and Elkan, 1965). Early granulomas are composed of mostly epithelioid macrophages, which may progress to form encapsulated foci with dry caseous centres. Granulomas typically contain large numbers of acid-fast bacilli.

M. marinum and *M. xenopi* have been isolated from amphibians showing a variety of lesions. *M. chelonei* subsp *abscessus* was isolated from 4/66 *B. marinus* and 2/86 *B. granulosus* in a survey of Amazonian amphibians (Mok and Carvalho, 1984). None of these animals had histopathological lesions, although experimental intraperitoneal inoculation of 29 toads resulted in the death of five animals from mycobacteriosis.

2.3.3 Fungi

2.3.3.1 *Mucor amphibiorum*

Mucor amphibiorum is a zygomycete that causes a fatal disseminated mycosis with fungal sphaerules inciting granuloma formation in most organs. In Australia chronic cases were found in 0.7% of *B. marinus* in the wild (Speare et al., 1994) and mucormycoses has also caused outbreaks in native frogs in captivity (Slocombe et al., 1995). It was first reported as a cause of death in captive anurans in Europe (Frank et al., 1974). It appeared that the original source of the fungus in the European collection may have been specimens of *L. caerulea* from Australia, and *M. amphibiorum* has not been found in wild amphibians outside Australia. In transmission experiments, *R. temporaria, R. esculenta* and *B. bufo* usually died within a month, lizards were mildly infected, and rats, mice and guinea pigs were unaffected (Frank et al., 1974; Frank, 1976). *M. amphibiorum* is also a pathogen of free-living platypus (*Ornithorhynchus anatinus*) in Tasmania (Obendorf et al., 1993). Speare et al. (1994) isolated *M. amphibiorum* from soil samples and were able to grow hyphae on soil. The fungus was also isolated from faeces of infected toads suggesting

cane toads may contaminate the environment. Although outbreaks of mucormycosis occur in captive amphibians, high mortality rates have not been observed in the wild.

2.3.3.2 *Basidiobolus ranarum*

Basidiobolus ranarum is a zygomycete that can be frequently isolated from the intestines of healthy amphibians and lizards (Reichenbach-Klinke and Elkan, 1965). *B. ranarum* was reported to cause an epizootic of cutaneous mycosis in captive *Hymenochirus curtipes* in America (Groff et al., 1991), but the identification of the fungus involved appears doubtful and *B. ranarum* may have been cultured as a contaminant. The morphology of the organism in the skin was different to the appearance of cultured *B. ranarum* since it occurred as a spherical form in the skin with no hyphae typical of *B. ranarum*. Experimental transmission could not be achieved using cultured *B. ranarum*, but the disease was transmitted when healthy frogs were exposed to sick frogs (Groff et al., 1991). Although this section of the literature review is confined to publications until 1996, it should be mentioned that the morphology of the fungus in the skin is consistent with *Batrachochytrium dendrobatidis* (Chaps 5 & 6).

2.3.3.3 *Saprolegnia*

Saprolegnia is listed here under the fungi although recent studies show that Oomycetes are not true fungi. However, in practice they are still referred to as fungi. Hence, I have included them in this Chapter. Saprolegniasis in captive amphibians is similar to the disease in fish with pale tufts of fungus growing on the skin. The disease mainly effects aquatic species and life-stages and usually occurs secondary to epidermal damage. *Saprolegnia ferax* and *S. parasitica* are the most common species isolated (Anver and Pond, 1984). *S. ferax* was found to be responsible for high mortality rates in eggs of *B. boreas* in the wild in northwest USA (Blaustein et al., 1994).

2.3.3.4 Chromomycosis

Chromomycosis refers to infection with a range of pigmented, septate fungi from the phylum Ascomycota. Many reports mention difficulties in identifying fungal species due to a lack of sporulation in tissues and in culture. Pigmented fungi including *Fonsecaea pedrosi, F. dermatitidis, Cladosporium* sp. *Scolecobasidium* sp. and *Phialophora* sp. have been isolated from lesions in a range of captive amphibians including *B. marinus, R. pipiens*, *R. catesbiana, Hyla caerulea, Phyllobates trinitatis,*

Ceratophrys ornata, Rhacophorus sp., *H. septentrionalis* (Cicmanec et al., 1973; Elkan and Philpot, 1973; Rush et al., 1974; Beneke, 1978; Miller et al., 1992) and wild *B. melanostrictus* (Dhaliwal and Griffiths, 1963). These organisms have also been isolated from tanks housing captive frogs. Clinical signs are of chronic debilitating disease, and papules and ulceration may occur. Frogs died 1-6 months after first showing signs of infection. Multiple grey nodules occurred in liver, kidney, heart, lung, skeletal muscle, meninges, bone marrow and other organs. These were fibrous granulomas with mononuclear cells, epithelioid cells and multinucleate giant cells around pigmented, septate fungi or spherical chlamydospores. The granulomas coalesced and replaced much of the parenchyma. Central caseation occurred in very large granulomas. A haematogenous spread was suspected due to the multi-organ infections. Transmission experiments had variable results. Rush et al. (1974) transmitted disease in healthy, unstressed frogs whereas Elkan and Philpot (1973) could not infect healthy frogs by intraperitoneal inoculation. Cicmanec et al. (1973) transmitted the disease by intracoeloemic injection only if toads were stressed by refrigeration, monthly feeding, or limited water.

2.3.3.5 *Dermocystidium* and *Dermosporidium*

Dermocystidium spp. and *Dermosporidium* spp. grow as large spore-filled cysts in subcutaneous tissue or the dermis and can cause inflammation and ulcerations (Broz and Privora, 1951; Jay and Pohley, 1981). Infections have been found in Europe and America, and can occur at high prevalence in a population (Reichenbach-Klinke and Elkan, 1965). Sequencing of small-subunit rRNA genes from *Dermocystidium* spp. showed this genus to be part of a clade of protistan parasites near the animal-fungal divergence (Ragan et al., 1996).

2.3.4 Protozoa

2.3.4.1 Microsporidia

The microsporidian, *Pleistophora myotrophica*, caused high mortality rates in captive *B. bufo* (Canning et al., 1964). This parasite infected all striated muscles resulting in atrophy and emaciation. White streaks between muscle fibres were obvious grossly, and microscopically these were spaces in the muscle fibres packed with microsporidian spores. Muscle regeneration occurred with long chains of sarcoblasts adjacent to

damaged muscle. Experimental infections were achieved by feeding toads infected muscle. Tadpoles did not become infected experimentally but their development was arrested. Only one of 12 experimental *R. temporaria* became infected and had spores in the tongue, whereas 100% of *B. bufo* were infected.

A captive, wild-caught *Phyllomedusa bicolor* was successfully treated for an ulcerative dermatitis that was associated with a variety of infective agents including microsporidia (Graczyk et al., 1996). The microsporidia were not identified.

2.3.4.2 *Myxidium*

Although the myxozoan parasite *Myxidium immersum* has not been associated with mortality, it is included here as it was introduced to Australia with the cane toad. *M. immersum* has low host specificity and has spread into native frog species. It has been recorded in 12 species of *Litoria*, 4 species of *Limnodynastes*, and one each of *Mixophyes*, *Ranidella*, and *Uperoleia*. The large immobile trophozoites (1-5 mm) are found freely floating in bile in the gall bladder (Delvinquier, 1986).

2.3.4.3 Trypanosomes

Over 60 species have been reported in anurans but the taxonomy is confused (Bardsley and Harmsen, 1973). Most infections are non-pathogenic. *Trypanosoma inopinatum* is the only trypanosome of anurans whose pathogenicity has been well studied. Experimental infections with the blood borne flagellate were lethal to European green frogs and caused haemorrhages, swollen lymph glands and anaemia (Brumpt, 1924). Death resulted from destruction of the reticulo endothelial system. The lymph fluid was abundant and contained numerous trypanosomes agglutinated in rosettes with the flagellates in the centre (Brumpt, 1924; Bardsley and Harmsen, 1973). *T. rotatorium* can be pathogenic in tadpoles or in heavy infections, with trypanosomes accumulating in the kidneys (Bardsley and Harmsen, 1973). *T. pipientis* causes spleen enlargement but rarely causes death (Flynn, 1973). Trypanosomes are quite common in frogs from Queensland, but none has been associated with disease (Delvinquier and Freeland, 1989).

2.3.4.4 *Myxobolus hylae*

Myxobolus hylae was found in the reproductive organs of *L. aurea* from Sydney (Johnston and Bancroft, 1918). Infected frogs appeared sickly and emaciated. The testes and vasa efferentia were infected in males and the oviducts were infected in females. High prevalences were observed with infections in 7/7 males and 2/~ 23 females. In cases of heavy infection, the whole testis was swollen and covered with white cysts up to 2-3 mm composed of myriads of spores (Johnston and Bancroft, 1918).

2.3.5 Helminths

Many helminth species infect amphibians, and some cause disease with heavy burdens. The pathogenic effects of trematodes, cestodes and nematodes are reviewed in detail by Flynn (1973). Disease is common in captivity but none has been reported to cause epidemics in the wild. Amphibians have a depauperate helminth fauna with low diversity and low infection levels compared to other vertebrates (Barton and Richards, 1996). This may be due to host specificity of the parasites or to aspects of host biology (Barton and Richards, 1996).

2.3.5.1 *Rhabdias*

These rhabditoid lung-worms are very common parasites of amphibians (Flynn, 1973). Usually they are incidental findings but heavy experimental infections can cause disease (Tinsley, 1995). Experimental transmission of *Rhabdias bufonis* to *B. bufo* resulted in a dose dependent decrease in growth rates, fitness and survival (Tinsley, 1995). *B. marinus* were placed on a culture of infective larvae of *R. sphaerocephala* which rapidly burrowed through the skin and the toads died overnight (Williams, 1960). The skin had numerous tufts of cast nematode skins. Hundreds of larvae were found internally including heart muscle, liver and eye (Williams, 1960). Larvae may reach the lungs indirectly via the blood stream or by direct migration to the lungs. Some larvae do not reach the lungs and encyst in other organs. These aberrant migrating larvae incite granuloma formation that may affect the host (Reichenbach-Klinke and Elkan, 1965). Only a small proportion of wild amphibians have heavy burdens (Tinsley, 1995). In Australia *R. hylae* is the most widespread species (Barton, 1994).

2.3.5.2 *Pseudocapillaroides xenopi*

Pseudocapillaroides xenopi is a capillarioid nematode that burrows in the epidermis. Infection resulted in deaths in captive *X. laevis*. Bacterial and fungal opportunists contributed to the pathogenesis. Clinical signs developed over four months and included ulcers, sloughing of the epidermis, erythema and weight loss (Brayton, 1992; Cunningham et al., 1996b).

2.3.5.3 Filarioids

Foleyella spp. can cause death due to heavy infections with microfiliaria in the blood and adult worms in the lymphatic system (Reichenbach-Klinke and Elkan, 1965). Infections may occur at high prevalence in a population and appear asymptomatic.

2.3.5.4 Spargana

Spargana are the intermediate stage of cestodes (Order Pseudophyllidea) that occur in frogs worldwide (Flynn, 1973). They are potentially pathogenic but their effects in frogs have not been well studied. The adult stage of the cestode, *Spirometra erinacei* (the only species of pseudophyllidean cestode known to occur in Australia), inhabits the small intestine of carnivores such as dog, cat, fox and dingo. The procercoid stage occurs in copepods and the plerocercoid stage (spargana) is found in tadpoles and adult frogs that ingest infected copepods (Sandars, 1953).

In Australian amphibians, spargana have been reported in wild adults of *B. marinus*, *L. aurea*, *L. caerulea, L. nasuta* and *L. rubella* (Sastrawan, 1978; Barton, 1994), and experimental infections were produced in adults of *L. latopalmata* and *Limnodynastes tasmaniensis* and tadpoles of *L. latopalmata, L. caerulea* and *L. tasmaniensis* (Sandars, 1953; Bennett, 1978; Sastrawan, 1978). Sandars (1953) reported that about one quarter of the population of *L. caerulea* in the Brisbane area was infected with spargana. In a group of 1000 *B. marinus* from Ingham, Queensland, 37 (3.7%) were found with infections of spargana provisionally called *S. mansoni* (Bennett, 1978). These toads had light infections, an average of 6.3 spargana per toad with 59% spargana found in thighs. There was a marked local inflammatory response in these toads and over half the spargana were dead. Immunodiffusion and immuno-electrophoretic tests in the toads revealed antibodies were produced to components of the spargana. Attempts to study the reactions in experimental *L. tasmaniensis* failed due to inconsistent infection rates

and frequent deaths of infected frogs and tadpoles, which were thought to be due to the combined stress of parasitism and captive conditions (Bennett, 1978). Growth of experimentally infected *L. latopalmata* tadpoles was inhibited (Sandars, 1953). In a survey of 948 Malaysian frogs, 11.8 % were found infected with spargana, 57% of which had bleeding and/or swelling at infection sites (Mastura et al., 1996).

2.3.5.5 Trematode metacercaria

Metacercariae of various trematode species occur in tadpoles and frogs. The definitive host may be snakes, frogs, birds or mammals (Reichenbach-Klinke and Elkan, 1965). Usually encysted larvae are not pathogenic although infections have been found in vital organs such as eyes, heart, liver, lung and CNS where they may cause disease. Metacercariae of *Neascus* group encysted in the dermis along the lateral line system of captive adults of *X. laevis*, leading to paralysis and death (Elkan and Murray, 1951). *Diplostomulum xenopi* infected the pericardial cavity of *X. laevis* causing pericarditis, respiratory distress and death (Flynn, 1973). Heavy experimental infections with *Cercaria ranae* caused bloat in tadpoles (Cort and Brackett, 1938).

2.3.5.6 Acanthocephala

The spines of acanthocephala inhabiting the stomach and intestine of frogs can cause perforation and death. *Acanthocephalus ranae* is a common species in Europe (Reichenbach-Klinke and Elkan, 1965).

2.3.6 Arthropods

2.3.6.1 Diptera

Various fly species from the families Sarcophagidae, Calliphoridae and Chloropidae have larvae that can develop within amphibians (Reichenbach-Klinke and Elkan, 1965; Crump and Pounds, 1985). The "toad fly" *Bufolucilia bufonivora* lays eggs in the nostrils of toads and the larvae destroy the epithelium and can penetrate deeper into the orbit or brain. Few toads survive an infection (Reichenbach-Klinke and Elkan, 1965). Larvae of *Notochaeta bufonivora* parasitised wild *A. varius* along a stream in Costa Rica during the dry season. Frogs in early stages of myiasis had a single small wound on the posterior surface of one thigh, and all hosts died within four days after they were found. Female frogs were parasitised more often (Crump and Pounds, 1985).

Larvae of *Notochaeta* sp. infected farmed *R. catesbiana* in Brazil. Larvae occurred in the mouth and caused necrotic perforations associated with a range of aerobic and anaerobic bacteria including *Clostridium* spp. (Baldassi et al., 1995).

In Australia the genus *Batrachomyia* contains several species that have been found in 11 frog species (Elkan, 1965). They inhabit the dorsal lymph sac with their posterior spiracles in or close to a hole in the frog's skin. When they are ready to pupate they leave the frog and drop to the ground. The number of maggots (1-5) is much less than seen with *B. bufonivora*, suggesting the eggs are not laid directly on frog skin but are picked up from the soil. Frogs are reported to have survived infection and had little obvious tissue damage, although death can result at the time of larval emergence (Elkan, 1965; Vogelnest, 1994). Especially with small frogs, the hole left in the skin after escape of the maggot would be expected to seriously affect the frog. A *Pseudophryne bibronii* was found with a perforation of the peritoneal wall and the rostral end of the maggot lay within the peritoneal cavity (Elkan, 1965). A *L. caerulea* infected with a larva of *B. mertensis* was in poor body condition and did not eat well until the larva was surgically removed (Vogelnest, 1994).

2.3.6.2 Arachnids

Larval trombiculid mites infect the skin of frogs and toads and cause small vesicles in the skin (Flynn, 1973). Ticks of the genus *Amblyomma* occur on *B. marinus* in Central and South America. They occur on all areas of the body and cause transient focal congestion and haemorrhage (Speare, 1990). Ticks have not been found on *B. marinus* in Australia or on native amphibians.

2.4 Non-infectious agents with potential to cause catastrophic amphibian declines in protected areas

2.4.1 Pollutants

Amphibians have been suggested as useful indicators of environmental contamination due to their permeable skin and because they have aquatic and terrestrial life stages giving them opportunity to be exposed to a range of pollutants. However, it has not been proven that amphibians are more sensitive to contaminants than other animal

groups (Mann and Bidwell, 1999). Toxicological experiments have mostly been conducted on tadpoles rather than adults as tadpoles are more sensitive to contaminants (Mann and Bidwell, 1999). However, typically with amphibian declines in pristine areas, tadpoles have remained for a few months after adults have disappeared (McDonald and Alford, 1999).

Pollutants originate from industrial, agricultural and urban development. Many chemicals, including pesticides, insecticides, herbicides, fertilisers, metals, endocrine disrupting chemicals and salinity, have been shown experimentally to cause chronic or acute disease in amphibians (Mann and Bidwell, 1999). An experiment demonstrated that toads exposed to low doses of malathion had increased disease susceptibility when challenged with bacteria (Taylor et al., 1999d). Few toxicological reports concern Australian amphibians - only 18 reports are summarised in Mann and Bidwell's review (1999). Johnson (1976) tested herbicides on four Australian species and found a reduction in thermal tolerance. Activity of tadpoles was reduced, although older tadpoles were more resistant.

A very commonly used herbicide in Australia containing glyphosate (Roundup) was found to be highly toxic to tadpoles, due largely to the surfactants in the preparation that affect gill respiration (Bidwell and Gorrie, 1995). Adult frogs are less sensitive than tadpoles, but adults of some species are more susceptible than others.

Acute exposure to high levels of a range of commonly used chemicals could potentially cause a mass die-off. Agricultural pesticides and herbicides appear to have contaminated high altitude sites in the Sierra Nevada in USA (Fellers, 1997). However, there is no evidence that this has happened in the mountainous habitats in eastern Australia where frogs have disappeared (Richards et al., 1993; Gillespie and Hines, 1999)

2.4.2 Solar ultraviolet radiation

Increased solar UV-B radiation has been suggested as a cause of the global decline of high altitude amphibian populations. In the Australian Snowy Mountains, embryos and tadpoles of *Litoria vereauxii alpina* (a declining species that lived above the tree-line)

had increased mortality in enclosures unshielded from UV-B (Broomhall et al., 2000). Similar experiments in North America showed variable results (Blaustein et al., 1995; Corn, 1998). Synergistic effects were reported between UV-B and low pH in reducing egg survival (Long et al., 1995). Synergistic effects were also observed experimentally between UV-B and the ability of *Saprolegnia* to infect eggs (Kiesecker and Blaustein, 1995). These experiments were linked to field observations of egg mortality and declines which suggested that increased UV-B may be contributing to some amphibian declines in the Cascade Mountains in Oregon. However, these studies on embryo and tadpole survival do not demonstrate that increased UV-B is likely to be the cause of the acute epidemics in adult frogs in Queensland rainforest, and there are difficulties in using experimental data to predict population effects (Alford and Richards, 1999). Most importantly, there was no correlation between frog population declines and changes in ground level solar UV-B radiation in Queensland (Aurel Moise, unpub data).

2.4.3 Introduced fish

There are examples in Australia and America where introduced fish and frogs do not co-exist (Fisher and Shaffer, 1996; Gillespie and Hero, 1999). Predation on tadpoles by introduced fish such as brown trout (*S. trutta*), rainbow trout (*O. mykiss*) and mosquito fish (*G. holbrooki*) has had severe impacts on some endangered species in Australia (Gillespie and Hero, 1999). Some populations are restricted to areas where fish have not colonised. Predation on adults is considered likely as frogs make good bait for trout and perch, but predation of tadpoles is considered to be the most important effect, and experimental data show tadpoles are palatable to these fish (Gillespie and Hero, 1999). As introduced fish are absent from the Wet Tropics, fish have not been implicated in the declines of rainforest frogs. There have been suggestions that imported aquarium fish may have introduced a disease into Australia (Laurance et al., 1996) and a ranavirus originally isolated from frogs, Bohle iridovirus, can infect fish (Moody and Owens, 1994). However there is no specific evidence to support the theory that fish have introduced an amphibian disease.

2.4.4 Introduced amphibians

The effect of predation by the introduced bullfrog (*R. catesbiana*) in western USA has been implicated in some declines (Fisher and Shaffer, 1996) but there are doubts over the significance of these effects (Hayes and Jennings, 1986). *B. marinus* were introduced to Australia in 1935 and occupy different habitats to rainforest frogs (Barton, 1997), so they are not linked to the catastrophic declines. As well as the potential effects of predation and competition, introduced amphibians could affect native amphibians if they brought in an exotic pathogen. From surveys of past and present amphibian parasites, it was concluded that cane toads introduced four species of protozoa and three of these now occur in native frogs, but it appears that helminths were not introduced (Barton, 1997). *Myxidium immersum* is one of the toad protozoans that have infected native Australian frogs, and a *Myxidium* sp. has recently been found associated with biliary hyperplasia, inflammation and fibrosis in the livers of thin *L. caerulea* (Hill et al., 1997). Antibodies to ranaviruses were found in cane toads from widespread locations throughout their distribution (Zupanovic et al., 1998), but viruses have not been isolated. The identity of the virus(es) and whether they are introduced or endemic is unknown. The introduction of other infectious organisms (e.g. bacteria, fungi) with the toads has not been investigated, but no diseases have been suggested for examination. The reasons so few parasites were introduced may be because only 101 toads were imported, and that suitable vectors were not present (Barton, 1997).

2.5 Directions of this thesis

The main aim of this project was to diagnose the cause of mass die-offs associated with declines in Queensland's rainforest frogs. The timing of frog population declines in Australia has been well documented compared to other countries. In Queensland rainforest there was no evidence for environmental change as the direct cause, and the epidemiology was consistent with an introduced infectious disease (Laurance et al., 1996). In North America and Europe, the pattern was not so well defined and multifactorial causes appeared likely, although declines in Central and South America had many similarities to those in Queensland.

When investigations of mass die-offs began in Australia, there had been only four infectious diseases reported in Australian frogs. These were diseases caused by *M. hylae*, *Batrachomyia* spp., Bohle iridovirus and *M. amphibiorum* (Johnston and Bancroft, 1918; Elkan, 1965; Speare and Smith, 1992; Speare et al., 1994; Vogelnest, 1994). Bohle iridovirus caused acute death in captive metamorphs, but the other diseases were less pathogenic. Outside Australia, the diseases associated with significant epidemics in free-living amphibians were thought to be caused by iridoviruses (Cunningham et al., 1996a), and there were less convincing reports of epidemics associated with bacterial septicaemia (Nyman, 1986; Bradford, 1991; Carey, 1993).

Due to the lack of knowledge on amphibian diseases, the progress of the work was divided into three sections, which tended to overlap in time towards the end of the project. The first part focussed on the cause of the mass die-off in frogs from Big Tableland, north Queensland, and related declines in Queensland. As the pathogenicity of iridoviruses had been demonstrated previously, an iridovirus was considered a possible cause and so I started work at AAHL, a centre for iridovirology and the investigation of novel pathogens. The second part involved surveying frogs from around Australia for disease, with the aim of increasing the scant knowledge available on this subject. Information on diseases of frogs from stable populations and non-declining species was needed to interpret pathology seen in the declining frogs. The third part began when we realised that the amphibian chytrid fungus was a highly significant pathogen of amphibians, and commenced studies to test this hypothesis and its implications.

CHAPTER 3

General materials and methods

3.1 Collection of specimens for the disease survey

A request for diseased amphibians was made to herpetologists, ecologists, veterinarians and other scientists around Australia. Diseased frogs were obtained through several avenues - during monitoring of wild populations by university, environmental protection agency/parks and wildlife staff or by amateur herpetologists; after members of the public notified agencies of the presence of sick frogs in residential areas; and from captive breeding programs or private collections. Healthy specimens were received from archived collections, and from captive breeding programs during screening of frogs before groups were released. Results of diagnostic tests were reported to submitters and often these results were important in the management of wild populations and captive collections. Instructions for recognising ill frogs, preservation of carcasses, and recording observations were prepared and distributed (see Berger and Speare, 1998). Sick frogs were identified by their unusual behaviour (e.g. lethargy, sitting unprotected during the day) or by the presence of lesions (e.g. reddened areas of skin, ulcers, lumps). Dead frogs were immediately preserved by the collector by freezing, by placing in 10% buffered neutral formalin or 70% ethanol after slitting open skin over the ventral abdomen or injecting fixative. Sick frogs were couriered overnight to AAHL, or were euthanased and preserved as for dead frogs. The network of people involved in submitting specimens is represented in Figure 3.1. The collector identified the species of most specimens.

I also went on four field trips to collect specimens. This enabled the observation of ill frogs in the wild and allowed post mortems to be done on freshly collected specimens. Two trips were to locations where unusual mortality had recently occurred: Craig Williams and Mike Tyler (University of Adelaide) organised a trip to Paralana Springs in the Flinders Ranges, South Australia in May 1995, and Harry Hines (Queensland Parks and Wildlife Service) organised a trip to Goomburra and Cunningham's Gap in south east Queensland in December 1996. I volunteered for two other monitoring surveys in Queensland - with Jean-Marc Hero (James Cook University) at Python Creek

near Tully in November 1995 and with John Clarke (Queensland Parks and Wildlife Service) on FrogSearch 2000 to Kroombit Tops and to Eungella National Park in November/December 2000.

The earliest and most significant specimens were collected by Rick Speare, Kelly Field and Keith McDonald during the catastrophic decline and mass mortality that occurred in O'Keefe Creek, Big Tableland in late 1993. Rick Speare necropsied these frogs, and preliminary results from histopathology, bacteriology and virology are reported in Speare (1995). The work in this thesis included further examination of these specimens by histology and electron microscopy.

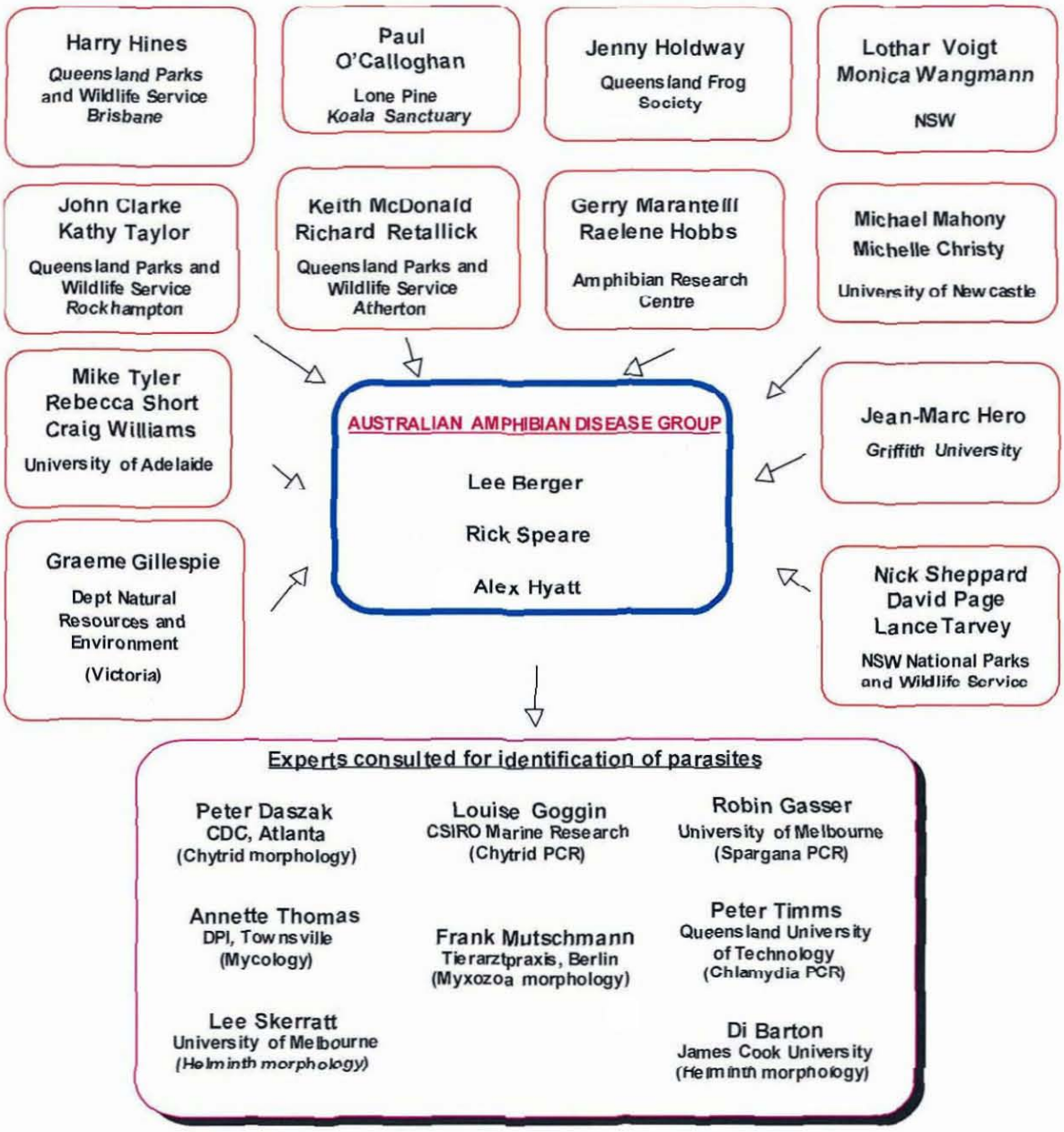

Figure 3.1 Flowchart representing the network for collection and diagnosis of ill or dead frogs.

3.2 Post mortem technique

Each frog was assigned an AAHL accession number that labelled every sample taken, and these are used throughout the thesis to refer to specimens. The sender's identification number was also recorded. Observations on clinical signs and pathology were recorded on the "Amphibian PM Form" (Appendix 3). The post mortem technique varied depending if frogs were received live, dead or preserved. Described below is the method used when frogs were received live, while sampling of other specimens was less comprehensive.

The behaviour of live frogs was observed, especially their posture and presence of righting reflex (Speare, 1989).

Frogs were examined for external lesions and these were photographed. Powder-free disposable gloves were used for handling frogs, which were contacted only by sterile instruments or new disposable materials.

Frogs were euthanased by bathing in an approximately 1 cm deep solution of 0.2% MS 222 (tricaine methane sulphonate) (Ruth Consolidated Industries, Annandale, NSW) at neutral pH. In preparing the solution, sodium bicarbonate was added to neutralise the pH, which was checked using indicator paper. After frogs were placed in this solution (in a plastic bag or in their transport container), deep anaesthesia with loss of pain reflex usually occurred within five minutes. Deep anaesthesia was confirmed by a lack of withdrawal reflex when toes were pinched, and lack of corneal reflex. Post mortems were begun at this time, although the heart often continued beating, and did so even after removal from the body. Animals were weighed and snout-vent and snout-urostyle length measured.

Skin scrapings were examined for the presence of *B. dendrobatidis* with a compound microscope after placing on a slide with a drop of water and a coverslip (see Chap 6). If culture of *B. dendrobatidis* was planned, skin scrapings were collected before euthanasia to avoid exposing the fungus to chemicals. For PCR, skin scrapings were collected into 80% ethanol using a sterile disposable scalpel and then stored at -20°C.

Frogs were pinned in dorsal recumbency on to a plastic cutting board covered in plastic coated bench paper. Small frogs were autopsied under a dissecting microscope. The ventral skin was cleaned by wiping with 70% ethanol. With sterile instruments a midline incision was made through the skin and the skin was separated from underlying muscle and reflected. The instruments were flamed again before abdominal muscles were opened with a midline incision and pinned back. The sternum was cut and reflected. The abdominal contents were examined for abnormalities, and the size of the fat bodies and stage of vitellinogenesis were assessed.

Blood was withdrawn from the heart with a heparinised 23G or 25G needle. Blood smears were made by placing a small drop of blood on a microscope slide and placing a second slide over the drop and drawing the two slides apart. Smears were air dried then fixed in 100% ethanol or methanol, and stained with Romanowski Giemsa. Whole blood was collected into an Eppendorf tube and the serum was collected. In large frogs, blood was also collected into 1 ml heparin tubes and sent to the Clinical Pathology Laboratory, University of Melbourne Veterinary Clinic, for biochemical and haematological analyses.

Samples of liver, kidney, lung, spleen, heart, intestines, fat bodies, muscle and skin were taken using aseptic technique and stored in separate 2 ml plastic screw top tubes (Nunc or Sarstedt) and frozen at -80°C. Intestines were examined for parasites. The remainder of each organ, as well as eyes, spinal chord, brain and the carcass were placed in 10% buffered neutral formalin for histopathology. For electron microscopy, selected organs were cut into 1 mm thick slices and placed in 2.5% glutaraldehyde for 24 hours, then into 0.1 M phosphate buffered saline and stored at 4°C until processed.

Nematodes were preserved in hot formalin or glacial acetic acid and cestodes were relaxed in water then fixed in 70% ethanol. Some worms were also frozen at -80°C.

Small frogs or tadpoles (i.e. <15 mm) were not dissected but were cut in half and preserved in formalin and frozen. Within a group, some were preserved or frozen whole.

A variety of diagnostic tests to determine the causes of illness and to identify any parasites were conducted as required, and are described below. Many frogs were received whole in formalin or ethanol which precluded culture and identification of infectious agents, and frozen frogs were not always examined histologically. Towards the end of the project, chytridiomycosis could be diagnosed confidently by examination of unstained skin scrapings and not all frogs with chytridiomycosis were examined histologically, although their internal organs were examined for gross lesions. Specialised tests, including immunostaining, virus isolation on cell culture and 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 (Fig. 3.1).

Cases were entered on a frog disease database in Microsoft Access.

3.3 Histopathology

3.3.1 Preparation and processing

Organs were held in 10% buffered formalin for at least two days. Representative tissue samples were placed between sponge biopsy pads (Edward Keller, Hallam, Victoria) in cassettes to prevent loss of small tissues. Between one and three cassettes were used per frog. Feet were placed ventral side down. A transverse section of spinal cord was cut at the 4th vertebrae. Tadpoles and small frogs or metamorphs (i.e.<15 mm), were cut sagitally in the midline and embedded with the cut side down. Bony tissues were placed in cassettes before decalcifying in 5.5% Ca EDTA in neutral buffered formalin for at least two days at 37°C. Specimens were dehydrated and infiltrated with paraffin wax (Paraplast, Oxford Labware) under vacuum by standard procedures using an automatic processor (Shandon Hypercentre). To ensure correct placement of small lesions, tissues were carefully embedded in blocks, using a magnifying glass if necessary. Sections were cut at 5 μm thick and stained with routine Lillie-Mayer haematoxylin and eosin, dehydrated and mounted with depex. Special stains included periodic acid-Schiff (PAS), Ziehl-Neelsen acid fast stain, Gram's stain, Grocott-Gomori methenamine silver, Giemsa, Ayoub-Shklar, and Perl's stain for iron (Luna, 1968).

Slides were examined with a Nikon light microscope and photographed using a Polyvar-Leica microscope on Ektachrome 160 colour film or Techpan 64 black and white film. Measurements were made using a calibrated eyepiece micrometer.

All photomicrographs presented as figures in this thesis are stained with haematoxylin and eosin unless otherwise stated.

3.4 Immunoperoxidase staining for ranavirus

For indirect immunoperoxidase staining, paraffin sections were dewaxed and taken to distilled water, then incubated for 20 minutes with 0.1% trypsin in 0.1% aqueous $CaCl_2$ at 37°C for antigen unmasking. Slides were rinsed in distilled water and PBSA, loaded into Sequenza cassettes and incubated with 200 μl of rabbit anti-EHNV antisera ("Flopsy") at 1:800 in 0.1% skim milk powder/PBSA for 1hr at 37°C. After a 5 minute rinse with PBSA, slides were incubated with biotinylated porcine anti-rabbit, anti-goat immunoglobulin, (Dako large volume DAKO LSAB kit, DAKO Corp., CA, USA) for 20 minutes at 37°C. Slides were rinsed with PBS then incubated with 3% H_2O_2 in distilled water for 20 minutes at room temperature to block endogenous peroxidase activity. After rinsing in PBS slides were incubated with streptavidin peroxidase conjugate (DAKO LSAB kit) for 20 minutes at 37°C, rinsed again then removed from the Sequenza cassettes. The antigenic complex was visualised using an AEC (3-amino-9-ethyl carbasole)(Sigma) chromogen system. The substrate and chromogen (freshly made AEC solution (2 mg AEC powder in 200 μl DMF (dimethyl formamide) added to 10 ml 0.05 M acetate buffer with 5 μl 30% hydrogen peroxide) were added and incubated at room temperature 10-15 minutes. After washing, slides were counterstained in Lillie's modified Haemalum, rinsed in tap water and Scott's tap water and mounted in an aqueous mounting medium.

3.5 Mycological techniques for *B. dendrobatidis*

3.5.1 Isolation from frogs

Isolation was only attempted from skin samples from live or freshly dead frogs with infections with *B. dendrobatidis* confirmed by direct microscopy (see section 6.4 that describes diagnosis by examination of skin scrapings). Culture was not used as a diagnostic technique. A method of isolation developed by Joyce Longcore was adopted (Longcore et al., 1999; Longcore, 2000). Small pieces of shedding skin (<1 mm) were removed from infected frogs and placed on plates of mTGhL agar with antibiotics (8 gm tryptone, 2 gm gelatin hydrolysate, 4 gm lactose, 10 gm agar, 1 L distilled water, with 400 mg streptomycin sulphate, 200 mg penicillin G and 1 mg ciprofloxacillin added after autoclaving). Skin pieces were wiped through the agar with a sterile needle to remove fungal and bacterial contaminants, while observing through a dissecting microscope. Cleaned skin pieces were placed on a new agar plate. About five plates were prepared with approximately ten skin pieces per plate. These were sealed with Parafilm® and incubated at room temperature (20-25°C). Plates were observed daily by inverting the plates under a compound light microscope. Skin contaminated with hyphal fungi or bacteria were cut out of the plate inside a Class II cabinet. Chytrid colonies were observed as clusters of spherical fungi surrounded by a rim of motile zoospores. After approximately 10 days, robust colonies were transferred to TGhL plates without antibiotics (16 gm tryptone, 4 gm gelatin hydrolysate, 2 gm lactose, 10 gm agar, 1 L distilled water). An alternative method involves culturing from whole pieces of skin rather than from sloughing skin (Joyce Longcore, pers comm 2000).

3.5.2 Maintenance of cultures

For maintenance of cultures, chunks of agar were dropped into 100 ml TGhL broth cultures in glass bottles, left at room temperature to grow for 1 week then placed at 4°C. Refrigerated stock cultures were passaged into fresh broth every 2 - 3 months, whereas cultures kept at room temperature were passaged every 2 - 3 weeks. Cultures were also maintained for periods in agar plates at room temperature that were passaged every 2 - 3 weeks. Broth cultures were grown in plastic tissue culture flasks (Corning) when

regular observation was required, as these can be examined on an inverted microscope. Cultures were passaged in a Class II cabinet that was disinfected and left running for at least 10 minutes between use of different isolates.

3.5.3 Collection of zoospores

Zoospores used for animal infections, antifungal testing or electron microscopy were harvested by placing about 3 ml of distilled water on agar cultures for 5 minutes, then pipetting off the water at a site distant to colonies of sporangia. Zoospores were counted using a Neubauer haemocytometer, and could be concentrated by centrifuging at 1800 *g* for 2 minutes. When zoospores collected into distilled water were moved to broth they usually died, although they survived being placed in half strength broth. Zoospores put onto agar plates did not grow unless they were highly concentrated. Most zoospores remained motile for over 12 hours in distilled water at 4°C, whereas less than 30% survived more than 24 hours.

3.6 Virus isolation in tissue culture

I attempted virus isolations on samples collected from 1995 to 1997, and Donna Boyle tested samples from late 1997 till 2000. Some variations in the method are described.

3.6.1 Cells and media

Bluegill fry cells (BF-2, ATCC CCL 91) and chinook salmon embryo cells (CHSE-214, ATCC CCL 1681) were grown at 22°C in Eagle's minimum essential medium (EMEM) supplemented with 10% FCS (FCS) and 10 mM HEPES. Bullfrog tongue cells (FT, ATCC CL 41) were grown at 24°C in EMEM supplemented with 10% FCS and 20% water. South African clawed toad kidney cells (A6, ATCC CCL 102) were grown at 24°C in RPMI supplemented with 15% distilled water and 10% FCS. Turtle heart cells (TH-1, ATCC CCL 50) were grown at 22°C in basal medium of Eagle (BME) supplemented with 10% FCS. Vero cells were grown at 37°C in EMEM supplemented with 10% FCS, 5 mM HEPES and glutamine. Maintenance media for virus isolations contained 1% or 2% FCS.

3.6.2 Cell inoculations

Tissue samples kept at -80°C were defrosted and homogenised by mortar and pestle (at room temperature or at 0°C), or using glass beads. Samples were diluted in 20 times their weight of PBSA and centrifuged at 3500 rpm for 5 minutes. The supernatants from the tadpole and skin homogenates were filtered through a 0.45 μm filter (Sartorius Minisart).

0.1 - 0.2 ml of supernatant (of final dilutions of 1:20 and 1:100 in PBSA) was added to each well in duplicate to the near-confluent cell monolayers in 24 well cluster plates (Nunc). Control wells with PBSA alone were included. After adsorbing for 1 hour wells were topped up to 2 ml with the appropriate maintenance media containing penicillin and streptomycin. For samples collected after 1997, plates were rocked while adsorbing, and were incubated at 26°C. Amphotericin (Fungizone) was added when contamination was considered likely, for example with skin or gut samples, or with whole tadpoles. Cultures were passaged twice after 7-10 days. To pass cultures, the cell monolayers were scraped and 0.3 ml of cells and media adsorbed to new monolayers then topped up with fresh media. For passage of samples collected after 1997, plates were frozen and thawed, 0.5 ml from duplicate wells were pooled, spun briefly in the microfuge, and 0.1 ml of supernatant was added to new monolayers. Wells were examined each day for cytopathic effects and the results recorded.

3.7 Electron microscopy

3.7.1 Transmission electron microscopy

3.7.1.1 Conventional processing

Samples of fresh or formalin-fixed tissue (~1 mm thick) were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 6.8, 300 mOs/kg) for 1 hour, washed in buffer (3 x 20 minutes), post-fixed in 1% (w/v) osmium tetroxide in 0.1 M cacodylate buffer for 1 hour followed by washing (3 x 5 minutes) in reverse osmosis water. Samples were then dehydrated in graded alcohols (70-100%), infiltrated with Spurrs resin by placing them in 50/50 Spurrs/100% ethanol and then in two changes of 100% Spurrs followed by embedding in Spurrs epoxy resin at 65°C (overnight). However,

hard tissues such as skin were easier to section when embedded in Epon resin.

Fixation of cultures of *B. dendrobatidis*, differed to that above in that glutaraldehyde was added to the culture medium (TGhL broth) to a final concentration of 2.5%.

Other samples were resurrected from paraffin blocks. Paraffin sections were cut at 10 μm and dried on round plastic 13 mm coverslips (Thermanox). The coverslip sections were deparaffinised then processed as for tissue samples and baked on top of the resin blocks. Immediately after removal from the oven, coverslips were removed from the hardened resin leaving the section of tissue on the surface of the block.

3.7.1.2 High pressure freezing and freeze substitution

To obtain high resolution images without artifacts of fixation, samples of *B. dendrobatidis* culture and of infected frog skin were rapidly frozen under high pressure at the Electron Microscopy Facility located at the Botany Department, University of Melbourne. Brass planchets were soaked in a lecithin-chloroform solution and allowed to dry, before packing with agar cultures or pieces of toe skin. A range of embedding media was used - TGhL broth, 2.3 M sucrose, hexadecene and hexene. Planchets were rapidly frozen at 2000 bars within the Leica high-pressure freezer. The frozen planchets were transported to AAHL in liquid nitrogen and kept in a liquid nitrogen storage dewar until processing. Substitution was initiated by placing planchets and samples within perforated beam capsules in 1% (w/v) osmium tetroxide/2.5% (v/v) glutaraldehyde in 100% acetone at -90°C in the presence of a molecular sieve within a "Leica CS auto" for 8 days. The temperature was raised to -60°C at 2.5°C/hr and kept at 60°C for 2 days. The temperature was then raised to -18°C at 2.5°C /hour then up to 0°C at 10°C/hour and the media replaced with 100% acetone. Samples were then warmed to room temperature (24°C) in an hour. The substitution medium was replaced with 1:1 propylene oxide (PO)/acetone, 100% PO, 1:1 PO/epon, 100% epon x 2 and embedded at 60°C for 24 hours. All media used in planchets resulted in excellent preservation of samples.

3.7.1.3 Cutting and examination of samples

Thick sections were examined with methylene blue stain by light microscopy to select the lesion or organisms. Ultra-thin sections (70 nm) were cut on a Leica-Reichert - Jung Ultracut E microtome, floated and adhered onto a grid, double stained in uranyl acetate and lead citrate and examined with a Hitachi H7000 or Philips CM 120 transmission electron microscope at 75 or 100 kV.

3.7.2 Scanning electron microscopy

3.7.2.1 Critical point drying

Tissues were fixed in phosphate buffered 2.5% glutaraldehyde for an hour, washed in phosphate buffer (pH 6.8, 300 mOs/kg) for 15 minutes, post-fixed in buffered 1% (w/v) osmium tetroxide for 3 hours, and rinsed in the same buffer (x10). Samples were then placed in saturated aqueous filtered thiocarbohydrazide (1% w/v) for 10 minutes, rinsed in distilled water (x10), then placed in 1% (w/v) aqueous osmium tetroxide at 0°C (30 minutes), rinsed in reverse osmosis water and sequentially dehydrated in graded alcohol (70 to100%). Following dehydration, tissues were critically point dried from liquid carbon dioxide, mounted on a stub with carbon dag, and sputter coated with gold. Samples were viewed with a JEOL JSM 840 at 5-15 kV with a working distance of between 16 and 22 mm.

3.7.2.2 Bulk-frozen hydrated samples - Latrobe University

To obtain high resolution images of fungal sporangia representative of the live state, samples were examined at the Zoology Department at Latrobe University using a JEOL 6340F field emission scanning electron microscope fitted with an Oxford 1500 cryo system.

Five day old agar cultures were examined as bulk-frozen hydrated samples. Agar was adhered to stubs with OCT compound cryo adhesive (Tissue Tek, Gurr) and then plunged into melting nitrogen and transferred under vacuum to the microscope at liquid nitrogen temperature. Samples were etched at -95°C for 60 seconds, then coated with gold/palladium in the transfer chamber. Samples were examined at 2 kV and a working distance of 20 mm.

3.7.2.3 Bulk-frozen hydrated samples - AAHL

To obtain a cleaner view of the base of sporangia, cultures were grown on sterile round plastic 13 mm coverslips (Thermanox), by placing coverslips in petri dishes of active TGhL broth culture. At between 6 -10 days, broth was removed by rinsing the coverslips through three changes of distilled water. Excess water was removed by touching the edges with filter paper, and leaving to dry for about one minute. To reduce charging within the microscope, coverslips were cut into quarters before they were adhered to the stub with carbon dag. Stubs were plunged into melting nitrogen then transferred under vacuum at liquid nitrogen temperature to the Hexland chamber and into the JEOL JSM-840 scanning electron microscope at -170°C. Extended drying at -80°C was necessary to sublimate ice, however good results were obtained on a sample that was desiccated overnight and examined as a freeze-dried sample, although some sporangia were crumpled. Samples were gold coated within the anti chamber of the microscope. Samples were examined at 3 -5 kV and a working distance of 20 mm.

3.7.2.4 Freeze fracture

Freeze fracture preparations were produced and examined by scanning electron microscopy at the Centre of Electron Microscopy and Microanalysis of South Australia (CEMMSA) at the University of Adelaide and at AAHL.

At CEMMSA, a Philips XL30 FEG microscope was used. Five to six day old agar cultures were packed into rivets that were joined upright. After plunging in melting nitrogen and placing in the transfer chamber of the microscope, the top rivet was knocked off, exposing the fractured surface of the culture. The sample was etched at -96°C for 2 minutes to remove about 1200 nm water, and coated with platinum. The sample was etched again at -100°C for 2 minutes. Samples were examined at 6 kV with a working distance of 20 mm.

At AAHL, a similar method was employed using the JEOL JSM-840 scanning electron microscope. The differences were that two week old agar cultures were used, etching was done at -85°C and samples were coated with gold. The operating conditions were similar to those described previously.

3.8 Other diagnostic tests

Ranavirus PCR was performed at AAHL by Jacqui Kattenbelt, using a method developed at AAHL to amplify coding regions for the major coat protein (Gould et al., 1995).

Bacterial cultures and identification were performed at AAHL by Julia Hammond and Tam Chamberlain, and at JCU by Joy Koehler. Samples included blocks of tissue and swabs from the peritoneal cavity. Bacteriological studies were performed on frogs from Big Tableland and specimens in which bacterial infection was detected on histopathology. In the latter case frozen archived specimens were used. Samples were streaked on MacConkey agar and horse blood agar and incubated at 20°C and 37°C. Bacterial isolates were identified by Gram stain and API 20E systems (BioMerieux, Charbonnier les Bain, France).