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CHAPTER 15

Unusual anatomy

15.1 Introduction

The Australian frog fauna is unique and different from amphibians in other countries (Cogger, 1992). Most reports on disease or physiology of amphibians concern a few species of large, common anurans from the Ranidae, Pipidae or Bufonidae, families that are not native to Australia. As gross or histological studies on anatomy of Australian frogs have rarely been reported, pathology is sometimes difficult to interpret as information on the normal appearance of organs and tissues is not available. When I began studying frogs, much effort was involved in differentiating pathological lesions from unusual anatomy. One anatomical feature that initially caused some confusion was the presence of an opaque white layer over internal organs in an adult *L. gracilentia*. Investigations showed this to be composed of iridophores, and it was determined to be a normal anatomical structure after being found in all frogs from this species as well as in *L. chloris* and *L. pearsoniana*. This chapter consists of a manuscript describing this feature in Australian frogs as well as in Tanzanian reed frogs.

15.2 My role in the manuscript

I dissected the Australian frogs and examined them by histology and electron microscopy. Rick Speare identified the pigment cells as iridophores from the initial histology. Frank Mutschmann contributed the gross and histological descriptions on the Tanzanian frogs. The manuscript has been prepared for submission to the Journal of Herpetological Medicine and Surgery as:

Berger, L., Mutschmann, F., Speare, R. Internal iridophores in Australian tree frogs and Tanzanian reed frogs.

15.3 Internal iridophores in Australian and Tanzanian frogs

An unusual opaque white layer is present in the serosal surface of various internal organs of red-eyed tree frogs (*Litoria chloris*), Pearson's frog (*L. pearsoniana*) and

dainty green tree frogs (*L. gracilentia*) from Australia, and the pointed nosed reed frog (*Hyperolius nasutus*), water lily reed frog (*H. pusillus*), Argus reed frog (*H. argus*) and green reed frog (*H. viridis*) from Tanzania. Using light and electron microscopy, we identified iridophores as the main cell type in this layer. Iridophores are one of the three chromatophores or “pigment cells” found in the dermis of amphibian skin, the other two being melanophores and xanthophores (Taylor and Bagnara, 1972; Frost-Mason et al., 1994). Iridophores do not contain pigment but have arrays of flat refractile plates composed of a mixture of crystalline purines and pteridines that impart iridescent, blue and white colours to the skin and are involved in physiologic and morphologic colour changes (Frost-Mason et al., 1994).

From Australia, we examined 18 *L. chloris* (3 captive, 15 wild), 8 captive *L. gracilentia*, and 16 wild *L. pearsoniana*. The wild frogs were collected from north-east New South Wales between December and February 1997-1998. These frogs were euthanased with percutaneous MS 222 (tricaine methanesulphonate, Ruth Consolidated Industries, Annandale, Australia), or collected dead. In Tanzania, 10 *H. nasutus*, 8 *H. pusillus*, 6 *H. argus* and 5 *H. viridis* were collected from the wild, exported to Germany and died during air transport or shortly after arrival.

In individuals of *L. chloris*, the bladder and pericardium were opaque and white (Fig. 15.1). The serosal surfaces of other organs, including gastrointestinal tract, peritoneum, lungs and ventral kidney, varied from having fine white speckles to a more continuous opaque layer. The lateral edges of the kidneys were more intensely white. Organs of *L. gracilentia* had a similar appearance but with a more distinct white layer on the liver which varied between individuals from a translucent membrane to a thick white covering. The whiteness was less apparent in adults of *L. pearsoniana*, although bladder and pericardium were usually opaque.

In adults of *H. nasutus* and *H. argus* a continuous opaque white/silver membrane covered the heart, liver and gall bladder (Fig. 15.2). The gastrointestinal tract and urinary bladder were the same colour, while the kidneys and lungs were less intensely coloured. In *H. pusillus* and *H. viridis* the peritoneum, pericardium and the gastrointestinal tract had a white tinge but no white layer was seen in the kidneys, lung and urinary bladder.

There was also considerable individual variation in the extent of the opacity over the various organs within each species.

Organs from all frogs were fixed in 10% formalin, embedded in paraffin and 6 μm sections were stained with haematoxylin and eosin (H&E) by routine methods. Histological examination of the tissue sections revealed masses of plump cells containing granular, khaki green, refractile material in the cytoplasm. These cells were present in the white surfaces noted on gross examination and were identified as iridophores due to their similarity to the iridophores present in the dermis. They occurred within the bladder epithelium resulting in a greatly thickened bladder wall (Fig. 15.3). Accumulations of iridophores were also present in the epicardium and pericardium, with a sparser scattering seen under serosal surfaces of other organs and within the sclera of the eye. Occasionally iridophores were seen extending into the superficial interstitial spaces of the kidney in *L. chloris*, *H. nasutus* and *H. argus* (Fig. 15.4).

For transmission electron microscopy (TEM), pieces of bladder and skin of a captive, healthy *L. chloris* were fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated and embedded in epon resin, then sectioned at 70 nm, stained with 2% uranyl acetate and lead citrate and examined with an Hitachi H7000 electron microscope. The identification of the cells as iridophores was confirmed by the presence of stacks of plates in the cytoplasm (Fig. 15.5). These iridophores appeared similar to those observed in the skin, but the platelets appeared larger and were stacked in a more disordered array in the internal organs. The dermal iridophores observed by TEM were all in a compact, punctate form and comparisons might not be valid, as the size of the cells vary with physiological changes.

It is possible the opaque iridophore layer in these frogs prevents UV radiation damage and dehydration. All species inhabit hot climates. *L. chloris* and *L. gracilentia* are basking frogs, which sleep during the day on leaves in the forest canopy. They usually only descend from the canopy to breed after large rains (H. Hines, pers comm., 2000). *L. chloris* was found to have extremely low evaporative water loss and was observed to voluntarily achieve high body temperatures (Buttemer, 1990). In contrast, *L. pearsoniana* are found close to streams or hiding in crevices or moist leaf litter

(McDonald and Davies, 1990). The hyperolid frogs species inhabit hot dry climates in dense savannas (*H. nasutus*, *H. argus*), open swampy areas (*H. pusillus*) or high grasslands (*H. viridis*) (Schjøtz, 1999). In *H. viridiflavus*, an African arid land species, iridophore numbers in the skin increased during the dry season (Kobelt and Linsenmair, 1986). It was suggested that this may occur as protection from UV radiation by reflecting energy and to provide a barrier against desiccation (Kobelt and Linsenmair, 1986). Nitrogenous wastes produced in this species during periods of water deprivation are deposited in osmotically inert, non-toxic form as guanine in the iridophore platelets (Schmuck and Linsenmair, 1988).

All chromatophores are derived from neural crest cells, and the three types of pigment organelles may be derived from an equipotential organelle in a common stem cell (Bagnara et al., 1979). Pigment cells are believed to become committed to particular cell development at an early stage in neural crest development, such as before or immediately after migration, although the environment may have some effect on the differentiation (Akira and Ide, 1987).

Individual variation in the numbers of internal iridophores could partly be due to metabolic or hydration states, but further work is needed to quantify normal levels, and to correlate with various physiological states. Normal anatomy varies greatly among amphibian species, and this report highlights the need for pathologists to examine control animals when considering an unusual finding in a species not previously examined.

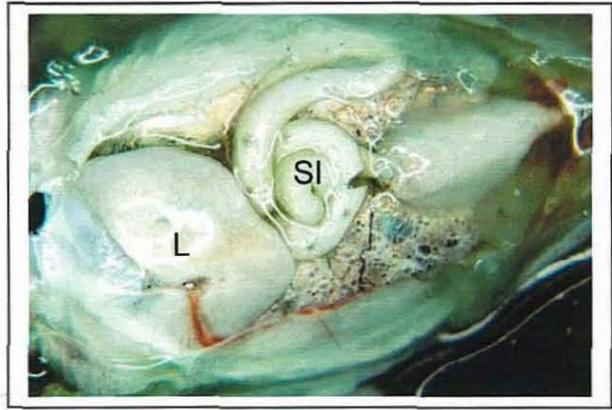
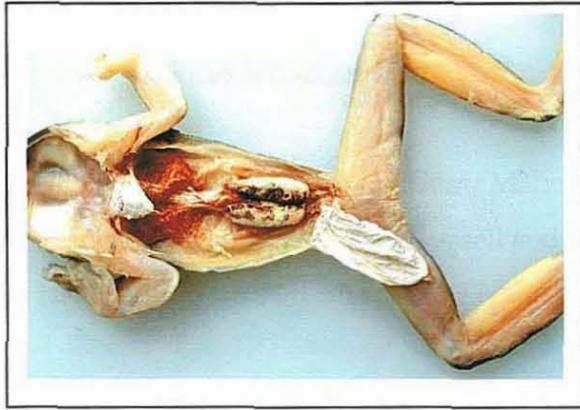


Figure 15.1 Adult of *Litoria chloris* with the liver and intestines removed and the bladder reflected to show the white pericardium and kidneys. The caudal apex of the lungs are also white.

Figure 15.2 Abdomen of an adult of *Hyperolius nasutus* with dense white covering over gastrointestinal tract, liver (L), and heart. SI = small intestine.

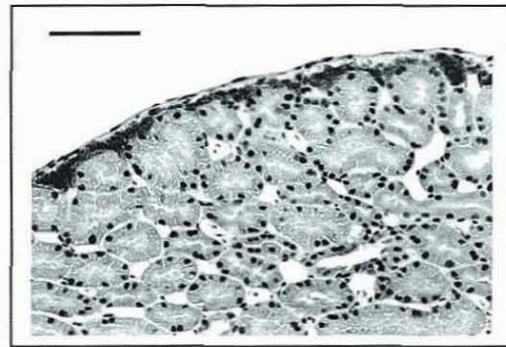
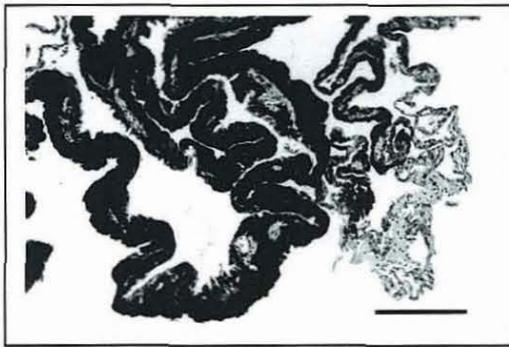


Figure 15.3 Histological section of bladder of a *Litoria chloris*. Note the thickened epithelium of the bladder wall due to the presence of dark staining iridophores. H&E. Bar = 300 μ m.

Figure 15.4 Histological section of kidney of a *Litoria chloris* with iridophores under the serosa extending into the interstitial space between tubules. H&E. Bar = 100 μ m.



Figure 15.5 Transmission electron micrograph of iridophores in the urinary bladder of a *Litoria chloris* with crystalline plates in the cytoplasm. N = nucleus. Bar = 2 μ m.

15.4 Acknowledgements

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15.5 References

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CHAPTER 16

Discussion

In Australia, studies on the role of disease in amphibian population declines were initiated after two decades had been spent obtaining scientific data to prove that declines were occurring. The initial study in 1993 focussed on collapse of the amphibian population in O’Keefe Creek, Big Tableland, north Queensland. This project commenced in 1995, and interest in infectious diseases was stimulated by publication of the infectious disease hypothesis by Laurance et al. (1996). As this project included the first survey of diseases in Australian frogs, it resulted in the discovery of new pathogens and greatly increased the number of amphibian diseases known to occur in Australia (Chap 10). The most common disease was chytridiomycosis (Chap 8), a disease that had not previously been reported. As this disease was present in frogs dying during a mass mortality event in a declining population, it was suspected to be the cause of the declines. Methods for studying chytridiomycosis were developed and the following aspects were investigated: characterisation of the causative agent (*B. dendrobatidis*), pathogenicity, evaluation and improvement of diagnostic tests, epidemiology, and treatment and disinfection. The overall aims were to understand the cause of population declines, to obtain information and tools for management, and to investigate diseases present in Australian frogs.

16.1 Diseases in Australian frogs

The survey relied on collaboration with herpetologists, ecologists, veterinarians and other scientists around Australia for collection of diseased specimens. A broad range of diseases was identified in frogs, with infectious diseases comprising 79% of cases diagnosed. Significant diseases of wild amphibians included chytridiomycosis, sparganosis, mucormycosis, aphanomycetosis, protistan encephalitis, vacuolating and ulcerative dermatitis, chlamydiosis, and neoplasms (Chap 8; Chap 10; Chap 11; Chap 12; Chap 13; Chap 14). Apart from chytridiomycosis, none were considered to be particularly common or suspected to have large impacts on the abundance of wild frogs. Discussions of these other diseases are included in their respective chapters.

16.2 Chytridiomycosis and population declines

The most likely explanation for chytridiomycosis causing such high, unsustainable mortality rates is that *B. dendrobatidis* has been recently introduced to Australia. This suggestion was originally proposed based on epidemiological factors (Laurance et al., 1996; Chap 2) and is now supported by results from examination of skin from archived frogs, with the first positive case appearing in 1978 (Speare et al., 2001). Due to the geographic isolation of amphibian populations worldwide, it is plausible that *B. dendrobatidis* has emerged due to the escape of the organism from a local environment where it existed in balance with a well-adapted host or hosts.

The major results from this thesis that support the theory that *B. dendrobatidis* caused the catastrophic declines and extinctions of frogs in protected montane areas in Queensland are 1) it was present on dead and dying *T. acutirostris* in the last abundant population before they became extinct, 2) it is highly pathogenic to many species, 3) it is widespread in Australia at both high and low altitudes, 4) it is the most common disease of frogs, 5) although tadpoles can be infected they remain healthy carriers, 6) expression of disease is increased under colder conditions, and 7) sporangia and zoospores do not survive drying and require water for dispersal (Chap 5; Chap 7; Chap 8; Chap 9; Berger et al., 1998). These characteristics are consistent with the pattern of the declines, as high altitude populations of stream-dwelling adult and juvenile frogs suddenly disappeared (Fig. 2.1), while tadpoles remained for a few months after the death of adults and juveniles (McDonald and Alford, 1999). Declining frogs are from taxonomically unrelated species. Declines spread from south to north Queensland with a generally regular rate of movement, suggesting the agent was moving naturally through frog populations and would have needed to spread through high and low altitude areas (Laurance et al., 1997).

Although *B. dendrobatidis* has a wide distribution and broad host range, perhaps only some species had the necessary combination of characteristics to render them vulnerable to disease. 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. In addition, a recent experiment comparing the inherent susceptibility of different frog species has demonstrated that extreme variation can exist between species (Katie Ardipraja and Gerry Marantelli, unpub 2001). Other aspects of host biology and complex ecological factors affecting the lifecycle of the chytrid, could also be important in determining the selectivity of the declines. Stream-dwelling frogs may have more chance of exposure to *B. dendrobatidis* than frogs living in isolated or ephemeral water bodies.

As *B. dendrobatidis* can probably exist as a free-living organism in the environment (Longcore et al., 1999; G. Marantelli, unpub 2001), can be carried by healthy tadpoles and has a broad host range (Berger et al., 1999a; Chap 8), this may explain an ability to persist and cause disease even when the density of adults from particular species has been greatly reduced (Daszak et al., 1999).

In some regions of Australia declines have been caused by the introduction of exotic predators, or habitat changes such as by logging, wetland drainage, weed invasion, urban development and agriculture (Tyler, 1997; Gillespie and Hero, 1999; Hines et al., 1999). However, there is no evidence that these factors have been responsible for the catastrophic declines in protected montane areas in Queensland. If frogs were stressed due to environmental changes, we would expect reproductive and nutritional status to be affected before fatal immunosuppression occurs. However, moribund frogs were found which were gravid (Mahony, 1996), and many had adequate fat reserves (Chap 8). Also, with severe immunosuppression a range of opportunistic infections is likely to be involved, rather than a solitary chytrid fungus. The experiments in captivity demonstrate that *B. dendrobatidis* can cause 100% mortality in susceptible species under conditions where uninfected animals remained healthy (Chap 7). There is no evidence that predisposing immunosuppression is necessary for epidemics of chytridiomycosis to occur.

Although most of the frog species that have disappeared from Queensland rainforest since 1979 (e.g. *R. vitellinus*, *R. silus*, *T. diurnus*, *L. lorica* and *L. nyakalensis* (Tyler, 1997)) were not found dying and tested for disease, the epidemiological evidence

suggests that chytridiomycosis caused mass mortalities of these species, as was observed in declining frogs (including *T. acutirostris*) at the Big Tableland site in north Queensland in 1993 (Berger et al., 1998). The significance of mortality due to chytridiomycosis in declining frogs in southern Australia is less clear. Although multifactorial causes appear likely, in many areas the causes of declines have not been identified and there is increasing evidence that the introduction of *B. dendrobatidis* was involved (Rick Speare, unpub 2001).

The episodes of population declines now appear to have passed in many areas. Numbers of some species decreased significantly when other species disappeared, but are now increasing. These species include *T. eungellensis* (McDonald and Alford, 1999), *L. genimaculata* on Big Tableland (Keith McDonald, unpub) and *L. pearsoniana* from southeast Queensland (Hero et al., 1998). As residual populations are increasing after significant declines although chytridiomycosis still occurs (Berger et al., 1998), this may suggest resistance is present in some members of the population. A balance between *B. dendrobatidis* and some species may be developing, and although disease still occurs, it does not devastate the populations. *B. dendrobatidis* may now behave as an endemic pathogen with outbreaks of disease occurring when conditions are optimal. Outbreaks of chytridiomycosis now occur in a seasonal cycle with most deaths occurring in winter (Chap 8). The long-term prognosis may be good for species which have survived and are recovering, as long as remaining habitats are protected to allow damaged populations to re-establish. However, since we currently know little about the interaction between the amphibian chytrid and hosts, and many frog species are currently considered threatened or their status is insufficiently known (Tyler, 1997), we should not be complacent. Also, if uninfected areas still exist in Australia then locally endemic species in those areas may be at high risk. Many species, such as *L. caerulea*, are still highly susceptible, and as chytridiomycosis is such a common disease it would be expected to continue to have a large impact on frog populations. 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. The final balance between *B. dendrobatidis* and various amphibian species cannot be predicted, but the abundance of frogs may be permanently reduced, and additional species could disappear.

Chytridiomycosis has recently been detected in amphibians worldwide and is now known to occur in New Zealand, Germany, Spain, Africa, and North, Central and South America (Mutschmann et al., 2000; Bosch et al., 2001; Speare et al., 2001). Outbreaks were associated with severe population declines in Spain (Bosch et al., 2001) and in Panama (Berger et al., 1998), and these outbreaks appeared to have occurred at the time of first introduction. It seems likely that previous declines in South America were also due to the introduction of *B. dendrobatidis*. Retrospective studies have detected infection as early as 1980 in Ecuador (Ron and Merino-Viteri, 2001), and 1974 in the USA (Speare et al., 2001). The active pet frog trade in the USA and the likely presence of *B. dendrobatidis* in frogs cultured for distribution (Groff et al., 1991) may make interpretation of the epidemiology difficult due to seeding at multiple foci. In South Africa, a high prevalence of infection occurred in a captive group of *Xenopus laevis* without mortality (Rick Speare, Diana Mendez and Michael Cunningham, unpub 2000), raising the suggestion that *B. dendrobatidis* originated in Africa, as African frogs may be well adapted.

Amphibian chytridiomycosis has been occurring at least since the early 1970's, but due to the lack of investigation of diseases of amphibians it was not reported till 1998 (Berger et al., 1998). The known distribution is likely to greatly expand as research on this topic increases.

16.3 Introduced diseases of wildlife

Infectious disease is important in the population biology of wild animals, as it is in humans and domestic animals (May, 1988). A review of infectious disease and animal populations concluded that disease is an important factor affecting survival, reproduction, dispersal, community structure and genetic diversity, and should therefore be considered by ecologists examining host population-dynamics (Scott, 1988). Disease becomes a threatening force when environmental degradation puts pressure on populations, and international trade and smuggling continually threaten to introduce new pathogens to which the native fauna has no resistance (Scott, 1988; Daszak et al., 1999). Exotic diseases can have effects similar to those of feral predators, with susceptible native species facing extinction while the ecosystem readjusts, and are another example of how increased global homogeneity leads to reduced biodiversity.

When an infectious agent is introduced, there are many possible outcomes. Many parasites may not survive due to lack of suitable hosts, vectors or environmental conditions, some may spread into new hosts with little impact, but there is also the possibility of diseases causing unsustainable losses. This may be likely to occur when the new hosts are similar enough to the old hosts to sustain infection, but have been isolated so that immunity is not present.

There are many examples where mass mortality and catastrophic population declines have occurred when infectious diseases have been introduced. Rinderpest was an ancient disease of livestock in Asia and Europe, and was introduced to Africa and spread across the continent between 1889 and 1898 (Scott, 1981; Plowright, 1982). It is fatal to a wide range of hoofed animals, and millions of wild animals died, with carcasses littering the plains. The disease became established in the Serengeti and although some immunity developed, it was a major regulator of buffalo population levels until it disappeared from the Serengeti in the 1960's (Plowright, 1982). The introduction of avian malaria and birdpox is suspected to have been involved in the extinction of low altitude birds in Hawaii (Warner, 1968). As Australia's fauna and flora have evolved in isolation, some introduced diseases have had severe effects. A pathogenic herpesvirus was apparently introduced to Australasian pilchard (*Sardinops sagax neopilchardus*) populations (possibly in imported pilchards fed to tuna) causing a massive epizootic that spread across more than 5000 km of Australian coastline in four months (Whittington et al., 1997). Although many observers suggested the deaths were due to an environmental problem, the dramatic spread of mortalities from a focal origin and the unprecedented losses in a single species over a huge area were consistent with an infectious disease (Whittington et al., 1997). Sarcoptic mange in wombats, caused by the mite *Sarcoptes scabiei* (var. *wombati*), is an horrific introduced disease (Skerratt, 2001). Mange can cause large epidemics, and can also occur endemically with sporadic disease in individual wombats (Skerratt, 2001). There is concern for its effects on small, fragmented wombat populations in particular. *Phytophthora cinnamomi* is an example of a pathogenic, introduced zoosporic fungus with a broad host range that threatens many native Australian plant species (Dawson and Weste, 1985; Wills, 1993).

Quarantine measures are recommended to prevent the invasion of the pathogen into new areas. Some plant species are highly susceptible, whereas others only become diseased

after periods of stress such as a drought (Dawson and Weste, 1985; Wills, 1993). A protozoan parasite of cats (*Toxoplasma gondii*) was probably introduced to Australia with cats, and marsupials are among the most susceptible animals (Reddacliff et al., 1993), although impacts in the wild have not been demonstrated. There are anecdotal reports suggesting that unidentified infectious diseases caused mass die-offs in marsupials in the late 1800's (Low, 1999). In addition, a large proportion of aboriginal Australians died when European settlers brought in measles and small pox (Flannery, 1994).

As many previous declines in wildlife were not investigated at the time, disease can be difficult to implicate as a cause. Studies of the current impacts of infectious diseases in the wild are difficult (Wobeser, 1994). It can also be difficult to prove that a disease has been introduced.

Although the initial >99% mortality rate of myxomatosis was not sufficient to exterminate rabbits from Australia (Fenner and Ratcliffe, 1965), it is plausible that a similar mortality rate could wipe out frog species with limited distributions and relatively infrequent breeding. Even if chytridiomycosis does not cause 100% mortality in the wild, some populations may have been so reduced after the introduction of *B. dendrobatidis* that their vulnerability to stochastic events and breeding failure was greatly increased.

16.4 Management aspects of chytridiomycosis

To prevent further spread of *B. dendrobatidis* within Australia and internationally, protocols regarding quarantine, testing, treatment and movement of amphibians need to be introduced. An important aspect of helping species that are currently endangered will be captive breeding (Marantelli, 1999). By producing large numbers of frogs in captivity, it may be possible to help species to survive and evolve immunity. To improve the chances of populations surviving the impact of chytridiomycosis, other factors that reduce population size, such as habitat destruction, need to be avoided.

Research in this thesis related to practical issues included investigating treatments, disinfectants and diagnosis. Although the compounds tested here for treatment of

chytridiomycosis (Chap 9) were not effective, a further experiment using Lamisil (terbinafine hydrochloride) and raising the temperature to 30°C had promising results (Claire Steel and Gerry Marantelli, unpub 2001). Disinfection was found to be easily achieved using routine disinfectants, heat or drying (Chap 9).

Testing sick frogs with chytridiomycosis by histology or examination of skin scrapings has a high sensitivity, but tests in healthy frogs were much less sensitive (Chap 6). Polyclonal antibodies were produced in rabbits and sheep and used to develop an immunoperoxidase test that was slightly more sensitive and was easier to interpret (Chap 6). The antibodies are also being used to develop an ELISA (Hyatt et al., 2000). The use of PCR for more sensitive diagnosis is being investigated.

At the “Getting the Jump on Amphibian Disease” conference (Cairns, August 2000) delegates discussed management strategies to control amphibian diseases and the resulting recommendations have been published (Speare et al., 2001). The recommendations cover many areas, including management issues and research priorities at national and international levels. Included in the volume are detailed quarantine guidelines for field workers (written by Ross Wellington, NSW NPWS), for captive collections (Lynch, 2001) and for international movement of amphibians (Daszak et al., 2001).

Chytridiomycosis was approved for inclusion in 2001 on the OIE (Office International Des Epizooties) Wildlife List of diseases (Stephanie Haigh, pers comm 2001). This listing provides official recognition of chytridiomycosis as an important disease and international movement of amphibians will now receive greater attention.

Chytridiomycosis has also been nominated as a key threatening process in Australia (Speare et al., 2001), and, if accepted, this will ensure that the government develops a threat abatement plan.

Infrastructure exists to prevent and manage exotic disease outbreaks in domestic animals, but little concern had been shown for wildlife where many diseases are yet to be discovered and understood, and responsibility for monitoring the disease status of populations had not been delegated. The work presented in this thesis has led to practical steps being taken to protect amphibians from exotic disease, and this could

eventually serve as a model for protection of other wildlife species from this neglected threat.

This study has demonstrated a need for broad research into diseases of wildlife. Ongoing research to build a knowledge base, combined with surveillance of wildlife for epidemic disease, is preferable to ignoring this field until a problem occurs.