CHAPTER 5

Pathology of chytridiomycosis

5.1 Chytridiomycosis in post-metamorphic frogs

5.1.1 Introduction

There are few publications on amphibian pathology, and the most comprehensive work was produced in England, mainly on European, North American and laboratory species (Reichenbach-Klinke and Elkan, 1965). A few mycotic dermatoses have been reported from amphibians. These include saprolegniasis in aquatic amphibians, which appears as tufts of mycelia and causes focal ulceration (Anver and Pond, 1984). An *Aphanomyces* sp. caused focal ulceration with distension and inflammation in the dermis of tadpoles of *B. marinus* (Berger et al., 2001; Chap 12). *Dermocystidium* and *Dermosporidium* are protistans that grow as large spore-filled cysts in subcutaneous tissue or dermis and can cause inflammation and ulcerations (Broz and Privora, 1951; Reichenbach-Klinke and Elkan, 1965). Focal mucormycotic dermatitis with hyphae in the epidermis and no significant inflammation occurred in captive *B. baxteri* (Taylor et al., 1999b). Chromomycosis, due to brown-pigmented fungi, occurs as systemic and cutaneous infections and is typically chronic with granulomatous inflammation (Rush et al., 1974; Anver and Pond, 1984).

The first descriptions of amphibian chytridiomycosis include brief descriptions of the histopathology of this disease (Berger et al., 1998; Pessier et al., 1999). A fatal mycotic dermatitis thought to be due to *Basidiobolus ranarum* was reported in *Hymenochirus curtipes* (Groff et al., 1991) and in *B. baxteri* and *B. hemiophrys* (Taylor et al., 1999a,). However, the histopathology they present appears identical to lesions of chytridiomycosis with typical zoosporangia in the markedly thickened *stratum corneum.*

This chapter reports the effect of infection with *B. dendrobatidis* on individual frogs. General descriptions of clinical signs, gross pathology and histopathology from examination of many frogs are included. Ultrastructural changes in a few individuals are described, and the distribution of infection and pathology in the skin of *L. caerulea*

is quantified. Specimens examined pathologically were obtained during the disease survey of frogs from around Australia and from experimental infections.

5.1.1.1 Structure and function of amphibian skin

The epidermis in frogs is composed of about 5 - 7 epithelial cells in thickness (Fig. 5.5) and is divided into four layers - 1) the outer keratinised *stratum corneum*, 2) the *stratum granulosum*, 3) the *stratum spinosum* and 4) the innermost *stratum basale* (Fox, 1994). Cells of the germinative *stratum basale* divide and move distally, and their cytoplasmic contents become more complex. The *stratum corneum* is composed of one or two layers of dead cells filled with keratin. Keratinised cells are squamous cells with flat nuclei and autolysed organelles. Keratin has not been studied in detail in amphibians, but in mammals it is formed when the inner surface of the plasma membrane thickens and material including microfilaments, keratohyaline and lysed material are deposited into an amorphous-filamentous complex (Fox, 1994). In mammals, some keratin is present in all layers of the epidermis, but there are many different types encoded by a large family of genes (Alberts et al., 1983). As cells mature, different keratins are expressed.

Sloughing in amphibians occurs periodically. Junctions connecting the keratinised layer to the underlying replacement layer loosen and the cell monolayer is shed. Shedding is thought to be due to an autonomous cycle influenced by temperature and endocrine hormones (Fox, 1994).

Other specialised cells types, such as merkel cells (for mechanoreception) and flask cells (secretions of which are possibly involved in moulting) are scattered among the epithelial cells (Fox, 1994; Warburg et al., 1994).

The dermis is composed of two layers of connective tissue (Fox, 1994). The thicker *stratum spongiosum* contains large glands, pigment cells (chromatophores), capillaries and nerves. The deeper *stratum compactum* contains dense collagen and connective tissue. Between these two layers, some species (including some Australian Hylids) have a basophilic granular layer composed of calcium and polysaccharides (Elkan, 1968). This is known as the Eberth - Kastchenko layer or *substantia amorpha* and probably functions to prevent dehydration. It is more abundant on the exposed dorsal skin (Elkan, 1968).

The smaller basophilic mucous glands in the dermis secrete mucopolysaccharides that cover the skin surface and keep the skin moist (Duellman and Trueb, 1986). The large serous or granular glands in the dermis are filled with eosinophilic droplets containing an array of bioactive compounds. About 150 peptides have been isolated from skin secretions of 25 Australian amphibians in the genera *Litoria*, *Uperoleia* and *Limnodynastes* (Bowie et al., 1999). These include wide-spectrum and narrow-spectrum antibacterial peptides such as the caerin group. The peptides kill bacteria by forming ion channels in cell membranes. The large parotoid glands that occur on dorsal skin behind the tympanum in many species are composed of multiple massive granular glands. Some frog species have additional types of gland such as lipid glands and breeding glands (Duellman and Trueb, 1986).

The layer beneath the dermis is the *tela subcutanea* which forms the outer lining of the subcutaneous lymphatic space and contains blood vessels, nerves and elastic fibres (Elkan, 1968).

Amphibian skin has important functions that do not occur in mammalian skin. Frogs do not drink water but absorb it through their skin, and water flow in *R. catesbiana* was found to be highest in the pelvic area (Parsons and Mobin, 1991). Amphibian skin is involved in ion uptake and has a major role in osmoregulation (Katz and Nagel, 1994) as well as pH regulation (Stiffler, 1994). Amphibians also breathe through their skin. In most amphibians, cutaneous gas exchange accounts for about 3/4 of total carbon dioxide elimination and 1/3 total oxygen uptake while resting (Malvin, 1994). Gas exchange is affected by epidermal thickness (Malvin, 1994).

5.1.2 Methods

5.1.2.1 Clinical signs and pathology

Details of methods for collection of specimens, post-mortems, histopathology, and transmission and scanning electron microscopy are included in Chapter 3. Specimens were obtained during the disease survey of frogs from around Australia (Chap 8) and from experimental infections (Chap 7). Clinical signs were reported by herpetologists who collected frogs, and were observed in frogs submitted live, experimental animals and during two field trips. Routine post-mortems were performed at the laboratory.

Formalin-fixed tissues were processed into paraffin blocks and 5 μm histological sections were examined with H&E staining.

The focus of this chapter is on the pathology of frogs with severe chytridiomycosis, and the photographs are of frogs in terminal stages of disease unless stated otherwise.

5.1.2.2 Electron microscopy

For examination of ultrastructural pathology in skin using transmission electron microscopy, high pressure freezing and freeze substitution was used to prepare samples as high resolution images without artefacts of fixation are obtained by this method (Chap 3). Samples from two infected *L. gracilenta* were examined. A toe-clip sample was taken from a healthy infected frog with a light infection, and from a frog that had recently died with severe chytridiomycosis. Samples were kept on ice for about two hours before processing as described in Chapter 3. Selected electron micrographs were sent to Norman Cheville (Iowa State University) for assistance with interpretation of pathology.

For scanning electron microscopy, feet from three freshly dead or euthanased infected frogs (*L. caerulea*, *L. lesueuri* and *M. fasciolatus*) and one uninfected frog (*L. caerulea*) were fixed in 2.5% glutaraldehyde and processed by critical point drying (Chap 3).

5.1.2.3 Distribution of infection and pathology

The distribution of infection was studied in ten sick wild *L. caerulea* with severe chytridiomycosis (accession nos. 96 961/15, 18, 19, 97 845/11, 13 & 14, 98 871/6, 99 1385/1 & 12, 00 1645/3). This species was chosen as large, freshly preserved specimens were available, as they are a highly susceptible species and are commonly found dying with chytridiomycosis in the wild. These frogs died in Queensland or northern NSW in winter. Nine female frogs and one male frog that were euthanased or were observed to die naturally were subsequently preserved in 10% buffered neutral formalin. They had a mean snout-vent-length (s-v-l) of 78.2 mm +/- 20.0 (range 43 - 109 mm) and were all in reasonable body condition. Typical signs of terminal chytridiomycosis occurred in all frogs such as erythema of ventral skin and congestion of internal organs. One frog had four 3 x 1 mm ulcers on the dorsal tibia, one had a small ulcer (1 x 1 mm) on the thigh, and one had multiple ~3 mm ulcers on the back, urostyle and tibio-tarsal joints. Internal

organs were cut in for histology. Strips of skin (3 x 10 mm) from six sites on the body (Fig. 5.20) were processed for histology and embedded longitudinally and perpendicularly so sections were transverse. A toe from each foot was cut at the start of the webbing and embedded upright. A calibrated eyepiece micrometer was used to make a variety of measurements on 2.5 mm (10 micrometer lengths) long sections at the centre of each strip, including number of sporangia, presence of pathology, thickness of skin, and number of serous and mucous glands (Table 5.1). Due to ulceration, erosions and hyperplasia, thickness of the epidermis varied greatly, so mean thickness is not included in the table. However, the range in thickness and number of cells was noted. For some toes less than 2.5 mm of epidermal length was present, but at least 1 mm of skin was counted for each toe. Counts of glands and measurements of skin thickness on toe skin are not included in table 5.1 due to difficulties in obtaining consistent transverse sections of ventral or dorsal skin. Ten sites on two healthy control adult frogs were also examined, a female (s-v-l 96 mm) and a male (s-v-l 83 mm). Sites were compared for significant differences using the Student's paired and non-paired two sample *t*-test two tailed or an ANOVA single factor test using Microsoft Excel. The correlation between number of sporangia and thickness of *stratum corneum* was performed using Pearson's coefficient of product moment correlation on SPSS® for Windows 6.1 (SPSS Inc., Chicago, Illinois, USA). Differences were regarded as significant when $P \leq 0.05$.

5.1.3 Results

5.1.3.1 Clinical signs and gross pathology

Typical clinical signs in Australian frogs with chytridiomycosis were lethargy, inappetence, and sitting unprotected during the day with hind legs slightly adducted (Fig. 5.1) (Berger et al., 1999a). Frogs appeared unable to perform their normal behaviour - burrowing frogs were found uncovered, and arboreal frogs were seen sitting on the ground. Frogs in early stages of becoming symptomatic displayed some escape activity and had a fairly rapid righting reflex, but if turned over two or three times they rapidly became tired and responded slowly. Frogs became moribund within two to five days of showing clinical signs and soon died. Frogs found dead often appeared to be sitting in normal postures as if frozen in place, or were also commonly found floating in water (Harry Hines and Gerry Marantelli, pers comm. 1997). Skin lesions ranged from

subtle to more obvious changes, and included darkening and patchy discolouration of skin, presence of excessive sloughed skin, erosions, and ulcerations less commonly (Figs. 5.1- 5.4). Some notable species variation in the clinical signs was observed. The rainforest frogs from Big Tableland, northern Queensland predominantly showed neurological signs; most commonly abnormal sitting posture with hind legs adducted, lethargy, and slow response to tactile stimuli (Speare, 1995). When handled, these frogs (*L. rheocola, L. nannotis, T. acutirostris*) became rigid and trembled with extension of the hindlimbs and flexion of the forelimbs. Many of these frogs appeared anaemic, with pale muscles and internal organs. In contrast, individuals of *L. caerulea* often became intensely hyperaemic on the belly, legs and feet (Fig. 5.1). Moribund or dead adults of *Mixophyes fleayi* often had swollen thighs (Fig. 5.4) (Harry Hines, pers comm. 1997).

Although disease induced experimentally had a moderately long incubation period (typically 18 - 48 days) (Chap 7), frogs were active and ate normally until they suddenly declined and died within a few days. Frogs with a heavy infection were able to appear and behave normally until some threshold was reached where signs of disease appeared. Frogs with obvious clinical signs invariably died.

In captivity, high mortality rates in some groups of animals but not in neighbouring tubs were often indicative of chytridiomycosis. Death of all in-contact 2-3 week old metamorphs was also a typical scenario.

Most diseased frogs received during the disease survey were in reasonable body condition and 81 out of 110 were considered to have moderate or large fat bodies. Many females were gravid. Gross pathology of internal organs was generally unremarkable. Congestion and reddening of organs occurred in *L. caerulea*.

5.1.3.2 Histopathology

Skin

On histologic examination, sporangia of *B. dendrobatidis* were seen in the superficial epidermis and occurred in the *stratum granulosum* and *stratum corneum*. Immature, dark sporangia occurred in the more viable cells while mature zoosporangia and old empty stages were more prevalent in the outer keratinised layers, including layers sloughing from the surface (Figs. 4.26 & 6.1).

Skin lesions were often mild with hyperkeratosis, over an intact epidermis, being the most obvious change. In hyperkeratotic areas, many keratinised cell layers built up to form a thick *stratum corneum*. Cell junctions to underlying skin appeared to break down intermittently and then lifting of the thickened, infected *stratum corneum* occurred. Large numbers of sporangia were removed when skin was shed, so the intensity of infection varied depending on the stage in this cycle (Fig. 6.6). The shedding layers often remained on the skin of debilitated, terminal frogs and were seen grossly. Bacteria often colonised the layers of sloughing keratin and grew within depleted sporangia.

More severe lesions included irregular multifocal hyperplasia, disordered epidermal cell layers, spongiosis, erosions and occasional ulcerations of the skin (Figs. 5.6-11) (Berger et al., 1998; Pessier et al., 1999). The usually smooth surface became roughened and irregular. Width of the epidermis could be highly variable with diffuse or focal thickening in some areas, as well as large areas of thinning. In some frogs the epidermis appeared to be digested away and only a thin layer of one or two cells remained. Clusters of sporangia sometimes occurred in deep pockets of missing epithelium (Fig. 5.12). Hypertrophic epidermal cells were evident in some frogs.

Individual epidermal cell necrosis was commonly seen in scattered cells in the *stratum basale* or *stratum spinosum*. These cells had pyknotic nuclei and pale swollen cytoplasms (Fig. 5.8). Occasionally vacuolated degenerate cells appeared to coalesce into vesicles that resulted in lifting of the epidermis and ulceration (Figs. 5.9-11). This ballooning degeneration and cleavage occurred in the suprabasilar layer or between the dermis and epidermis. Ulceration was seen more often in *L. caerulea* than in other species. A few frogs had extensive ulceration leaving the basement membrane exposed. In these frogs, infection was lost with the epidermis and only a few sporangia could be found. Two experimentally infected juveniles of *M. fasciolatus* displayed this type of pathology (Chap 7).

There was only a mild inflammatory response in the skin. This usually occurred as a slight increase in mononuclear cells in the dermis. Foci of lymphocytes, macrophages and a few neutrophils sometimes occurred in the superficial dermis, particularly in areas of ulceration. Occasional inflammatory cells occurred in the epidermis. Intense inflammation was never seen.

Progression of the pathology appeared to be related to intensity of infection. Examination of toe-clips from early infections in experimental animals showed that initially a few sporangia were present with no obvious effects on the epidermis. As the scattered foci became larger, focal hyperkeratosis occurred, sometimes with mild degeneration of underlying epidermal cells (Fig. 5.13). In heavy infections, the fungi occurred diffusely over ventral surfaces with hyperplasia and more extensive degenerative changes as described above. Infections seen in early summer, from examination of toe-clips from healthy frogs, seemed to show infections that were resolving with light infections of old empty stages (Diana Mendez, unpub 2000).

Other organs

There were few specific internal lesions in sick frogs, suggesting that the ultimate cause of death was metabolic or toxic. Necrosis, vacuolation, or cloudy swelling was sometimes apparent in a range of internal organs, including focal or diffuse acute necrosis in renal tubules, and possible oedema and vacuolation in the brain.

Histologic examination of organs involved in immunity – i.e. spleen and bone marrow, revealed no evidence of immunosuppression, apart from that observed in two frogs. Tests (including viral and bacterial culture, electron microscopy and haematology) for infectious organisms did not detect any other significant pathogens in most frogs. Twenty-five (12.4 %) out of the 201 frogs with severe chytridiomycosis had concurrent diseases (Chap 8). The contribution of these diseases to the pathogenesis in these frogs is unknown, although in most frogs chytridiomycosis appeared to be the major disease process.

5.1.3.3 Ultrastructural pathology

Transmission electron microscopy

Transmission electron microscopy of skin from the lightly infected *L. gracilenta* showed the *stratum corneum* was focally thickened due to a build up in the layers of infected keratinised cells in areas of infected epidermis. Up to four additional layers of infected, keratinised cells made up the *stratum corneum*. The keratin was denser than in non-infected areas (Fig. 5.14).

There was a zone of condensed fibrillar cytoplasm, up to 2.5 μm thick, around some sporangia that appeared to be mainly fibrils in a roughly lamellar arrangement with no organelles (Fig. 5.15). The more superficial epidermal cells contained larger sporangia and the cell nuclei and organelles such as mitochondria were pushed to one side of the cell. Near the skin surface the epidermal cell cytoplasm condensed to a thin layer around the fungi and organelles were lost, in a similar process to normal epidermal cell maturation. Cell nuclei became dark and condensed but were not as flattened as in normal *stratum corneum*. Keratinisation may have occurred prematurely in dark cells below the skin surface (Fig. 5.14). The cell junctions of infected cells appeared to be normal (Fig. 5.15).

A more diffuse change in the epidermis was acute cell swelling of infected and uninfected cells in the surrounding foci of infection, although mitochondria and other organelles in these cells were intact. Nuclei of some infected cells in the *stratum granulosum* were shrunken and chromatolytic. Pathology in the deeper epidermal cells included focal shrinkage, increased intercellular spaces, and dissolution of the cytoplasm (Fig. 5.14).

The *L. gracilenta* that died from chytridiomycosis had severe epidermal lesions (Fig. 5.16). Although some intracellular changes were due to autolysis, major structural changes were evident. Maturation of epidermal cells was disrupted, and shedding cells were often pale and incompletely keratinised with overly plump nuclei. There were areas where cells had sloughed to expose a non-keratinised surface. Many epidermal cells were necrotic with swollen, degenerate nuclei and mitochondria. Some necrotic infected cells appeared to lack intercellular junctions. Bacteria were common between cells and colonising old depleted sporangia.

Scanning electron microscopy

Examination of diseased skin by scanning electron microscopy demonstrated the extensive peeling and degeneration of the hyperkeratotic surface (Fig. 5.18), whereas skin from the healthy control frog was smooth and intact (Fig. 5.17). Fungal discharge tubes emerging through the surface of the skin were seen at higher magnification (Fig. 5.19). Tubes protruded through a hole in the epidermal cell membrane, but the edge of the skin closely apposed the tube and could not usually be discerned from the fungal

elements. The presence of sporangia within cells caused the cells to bulge. Many spherical bodies were seen on the skin surface, some of which may have been sporangia free of skin cells, but lack of distinguishing features hampered their identification.

5.1.3.4 Distribution of infection and pathology

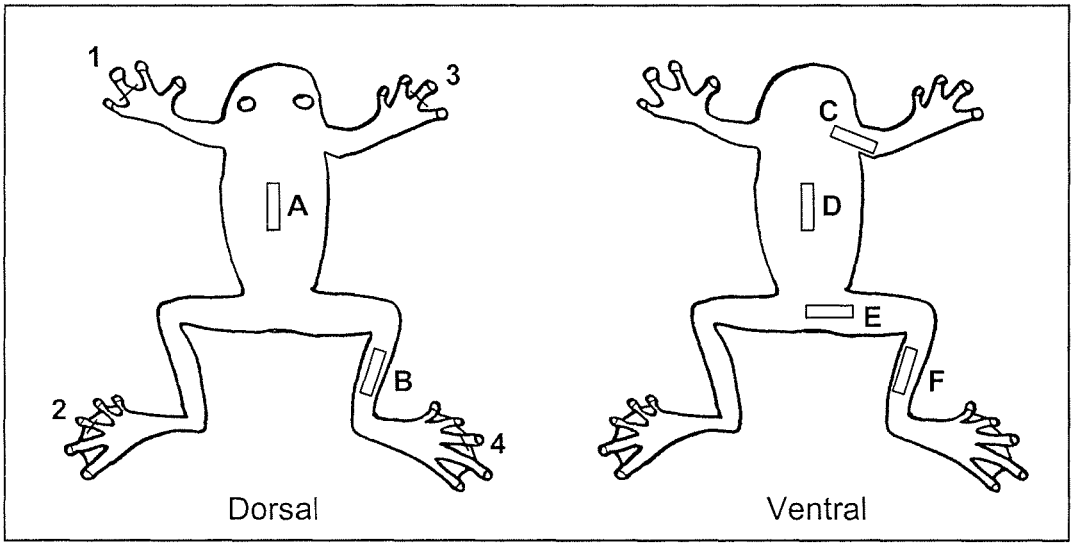

Figure 5.20 Sites on dorsal and ventral body and toes that were sampled to determine the distribution of infection, presence of pathology and number of skin glands.

Examination of skin from different sites of the body (Fig 5.20) of ten *L. caerulea* revealed that very few sporangia occurred on dorsal sites (Table 5.1). Sporangia occurred in only one frog on the back (site A), but occurred in four frogs on the dorsal skin over the hindlimb (site B). Heavy infections of sporangia occurred in all frogs at other sites, but numbers were highly variable and no significant differences were noted in the numbers of sporangia on ventral surfaces (sites C, D, E), and on toes ($P > 0.05$). Site F on the medial hindlimb had a lower mean number of sporangia compared to ventral sites, and the difference was significant when compared to sites C and E but not to D. Few sporangia remained in ulcerated areas in ventral sites, and this added to the variability of the results.

Pathological changes were less common on back skin (site A), although ulceration was present in 30% of frogs, occurring even in the absence of infection. Ulceration and erosions were seen in 30 - 90% and 60 -100% of frogs at other sites. Congestion was

not seen on the skin of the back but occurred in 60-100% of frogs at other sites that had dilated capillaries in the superficial dermis and *tela subcutanea*.

Inflammation occurred in the dermis at 10% to 40% of sites. This usually occurred as a slight but diffuse increase in mononuclear cells in the dermis, or as small foci of cells in the dermis below areas of ulceration. Thickness of the *stratum corneum* was highly correlated with numbers of sporangia ($r = 0.62$; $P < 0.001$). Ulceration, erosions, hyperkeratosis, congestion and inflammation were not seen in uninfected control frogs.

Epidermal thickness was highly variable ranging from a complete absence of epidermis in ulcerated areas, to a thin layer two or three cells thick, to relatively normal areas about five cells thick, to hyperplastic areas up to 125 μm thick consisting of up to 13 layers of cells. In control frogs the epidermis was smooth and even, ranging from 25 to 60 μm and was always between 4 and 7 cells thick.

Thickness of the dermis was not significantly different from the controls ($P > 0.05$). The dermis was thicker on dorsal skin, and was thinner in smaller frogs. Greater numbers of serous glands occurred on the two dorsal sites ($P < 0.05$), with more on the hindlimb than the back ($P < 0.05$). However the size of serous glands varied greatly, with the largest ones occurring on the back, and this was not measured. Serous glands also occurred at all ventral sites and no significant differences were detected between sites ($P > 0.05$). Serous glands occurred even in areas of heavy infection with sporangia (Fig. 5.21). Serous glands were abundant in dorsal skin of the toes but not ventrally. Mucous glands were more evenly distributed over the body and no significant differences between sites were detected ($P > 0.05$). Some glands could not be identified.

The distribution of the *substantia amorpha* was not quantified, but it was noted to occur more continuously and abundantly on dorsal skin, and was present on ventral skin of the belly and legs in a thinner and less consistent layer. It occurred on dorsal skin of the toes but not ventrally. Skin over the dorsal radio-ulnar appeared similar to that of the hindlimb, with few sporangia.

Table 5.1 Mean numbers of sporangia of *Batrachochytrium dendrobatidis* per 1 mm skin section, number of serous and mucous glands per 1 mm skin section, presence of pathology, and thickness of skin at ten sites on the body on ten wild *Litoria caerulea* naturally infected with severe chytridiomycosis. (See figure 5.20 for position of site on body). SD = standard deviation.

Site	A	B	C	D	E	F	Toe 1	Toe 2	Toe 3	Toe 4
% frogs with sporangia	10	40	100	100	100	100	100	100	100	100
Mean no. sporangia per infected frog (SD)	15	12.2 (10.4)	98.4 (74.04)	92.4 (98.80)	95.2 (63.92)	49.6 (27.88)	96.8 (51.00)	113.2 (69.52)	118.0 (101.32)	90.8 (74.48)
% frogs with hyperkeratosis	10	20	100	90	80	80	100	100	100	90
% frogs with congestion	0	60	100	60	70	60	80	100	100	90
% frogs with ulceration	30	50	80	90	60	30	70	90	90	90
% frogs with erosions	30	60	100	100	80	60	90	100	100	100
% frogs with inflammation	10	20	20	20	30	10	40	20	40	30
Thickness of *st. corneum* (μm) (SD)	3.25 (1.68)	2.75 (0.80)	12.0 (8.00)	10.0 (6.75)	11.0 (8.00)	9.25 (9.50)				
Thickness of dermis (μm) (SD)	363.75 (98.78)	381.25 (127.05)	245.75 (62.45)	291.5 (91.43)	223.75 (105.55)	195.25 (55.78)				
No. serous glands (SD)	2.24 (0.88)	4.4 (1.64)	0.88 (0.76)	1.12 (1.2)	0.52 (0.68)	0.84 (1.28)				
No. mucous glands (SD)	5.08 (1.72)	5.6 (0.8)	4.52 (1.96)	5.92 (1.8)	5.24 (1.64)	4.64 (1.36)				

There were no consistent changes observed on examination of the internal organs of these ten frogs. Six frogs were considered to have large fat bodies, three had moderate sized fat bodies and one had small fat bodies. Three frogs had concurrent chronic diseases: one had biliary fibrosis and was infected with *Myxidium* sp. in the gall bladder and two frogs had localised infections with *Mucor amphibiorum* - in the small intestine in one frog and in the liver and large intestine of the other. In addition, the latter frog had cysts of *Myxobolus hylae* in the ovaries. Three frogs had acute changes consisting of necrotic changes in the kidney with fluid in the tubule lumens and degenerate tubules, and one of these frogs was also septicaemic with bacterial rods in blood vessels throughout the body.

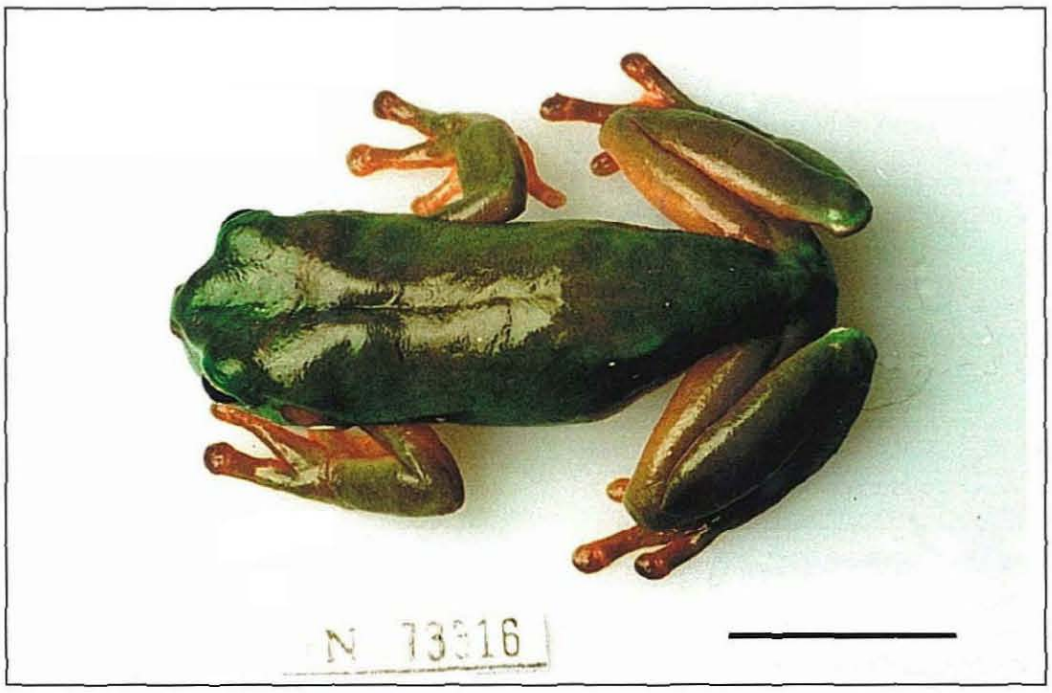

Figure 5.1 Live adult of *Litoria caerulea* (97 845/4) in the terminal stages of chytridiomycosis. The frog is weak and is sitting with legs adducted, and the skin is severely reddened due to congestion. Bar = 25 mm.

Figure 5.2 Captive metamorph of *Mixophyes fasciolatus* (96 1431) with terminal chytridiomycosis. Note depressed attitude, partially closed eyes and accumulations of sloughed skin over the body (arrow head). Bar = 5 mm.

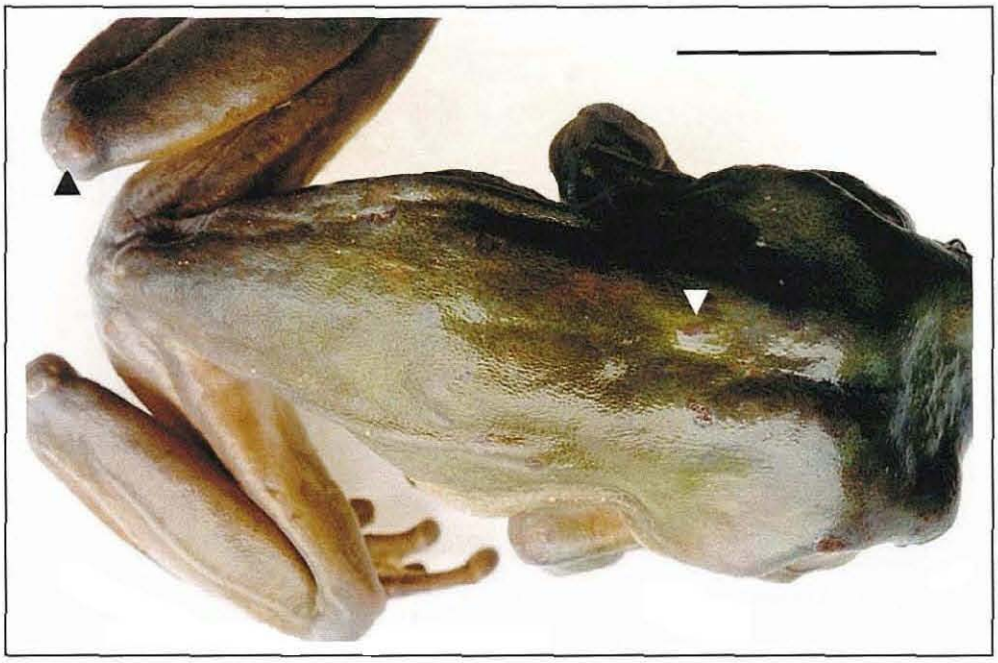

Figure 5.3 Formalin-fixed adult of *Litoria caerulea* (99 1385/12) with discolouration and ulcers on dorsal skin (arrow heads). Bar = 25 mm.

Figure 5.4 Dead adult of *Mixophyes fleayi* (96 962/4) with chytridiomycosis. The thighs are swollen and there is reddening and dilation of blood vessels in the ventral skin of the hindlimbs. Bar = 24 mm. (Photograph by Harry Hines)

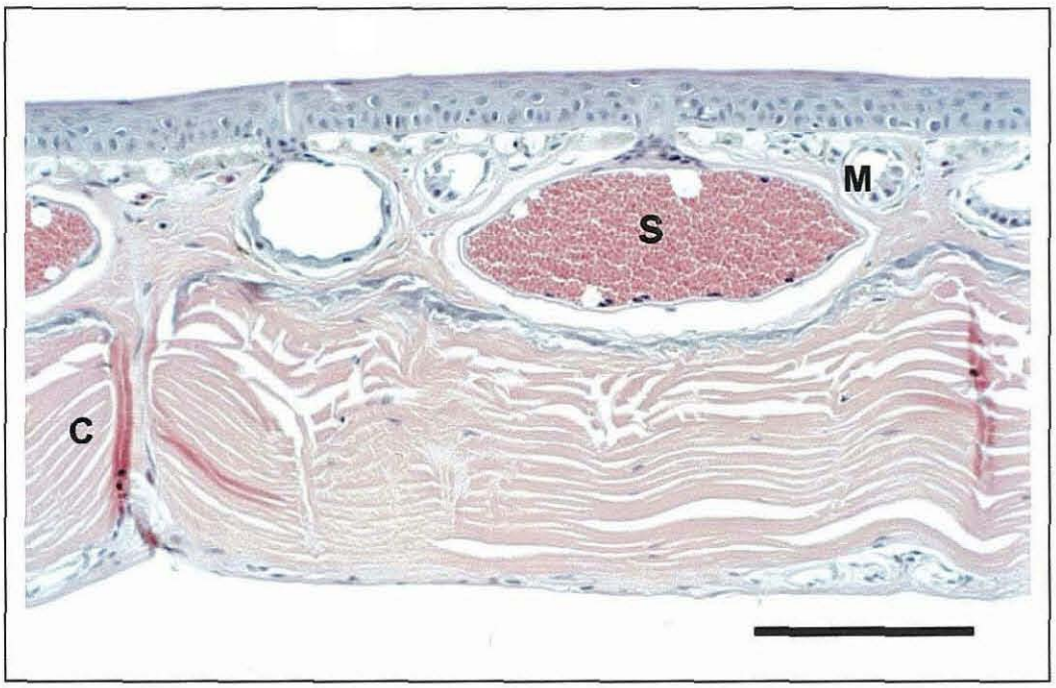

Figure 5.5 Histological section of healthy dorsal skin from an adult *Litoria caerulea*. The epidermis is smooth and of even thickness. Pigment cells occur beneath the basement membrane. The large serous glands (S) and smaller mucous glands (M) occur in the dermis and their ducts penetrate the epidermis. Beneath the glands is the basophilic *substantia amorpha* that occurs between the *stratum spongiosum* and deeper *stratum compactum*. C – connecting pillar. Bar – 150 μm.

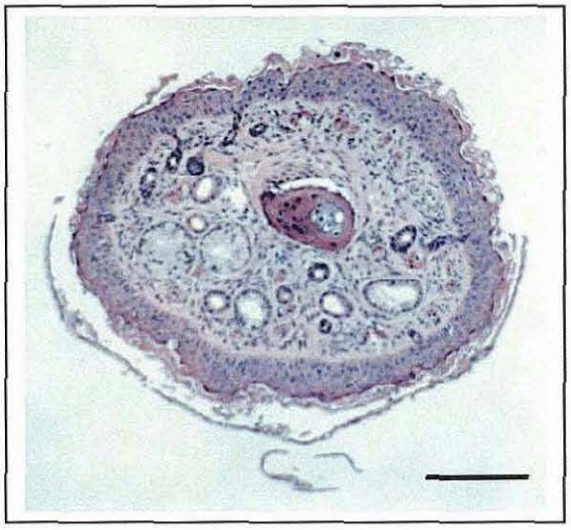

Figure 5.6 Transverse section of a toe from a metamorph of *Mixophyes fasciolatus* (96 1431) with a heavy infection of *Batrachochytrium dendrobatidis*. There is severe hyperkeratosis with shedding of some layers of the thick, infected *stratum corneum*. These layers of shedding skin are visible grossly (see fig 5.2). The epidermis is of uneven thickness due to irregular hyperplasia and erosions. Bar = 100 μm.

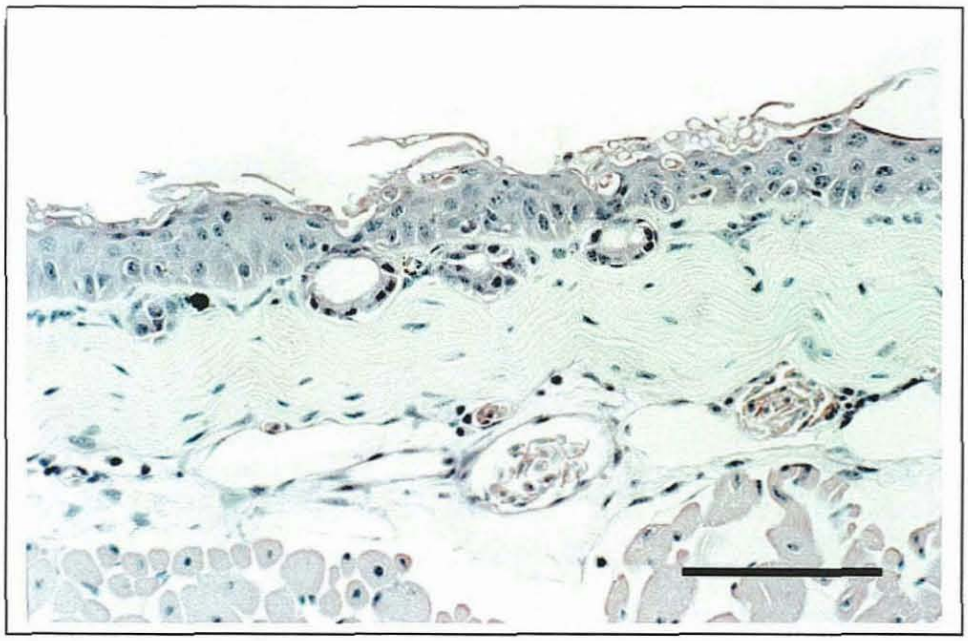

Figure 5.7 Section of infected skin from a captive metamorph of *Mixophyes fasciolatus* (96 1431/7) with mild epidermal lesions. The surface is eroded and there are some pyknotic cells in the basal layer. There is negligible inflammation, but capillaries in the *tela subcutanea* are congested. Bar = 80 μm.

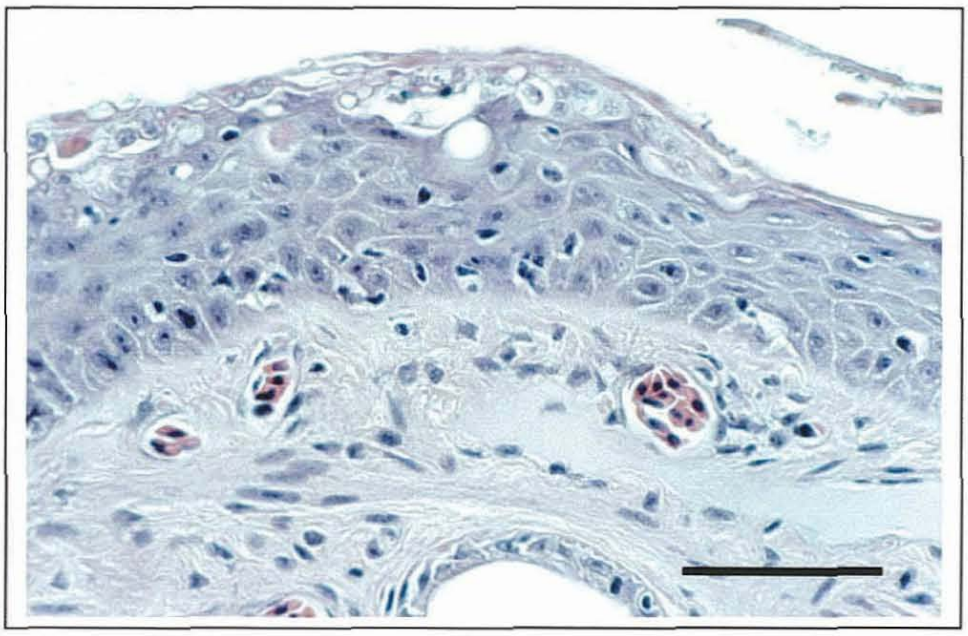

Figure 5.8 Infected skin from the toe of an adult of *Litoria chloris* (01 95/2). The epidermis is hyperplastic and there is loss of normal stratification. Many epidermal cell nuclei are pyknotic. Bar = 60 μm.

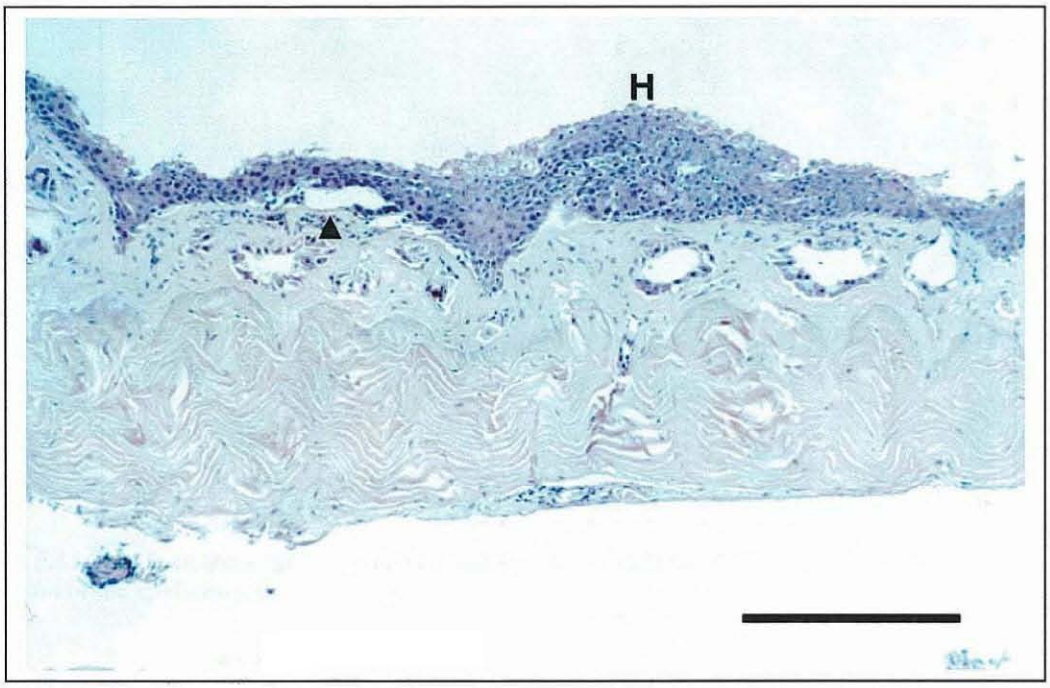

Figure 5.9 Skin section from the ventral abdomen of an adult of *Litoria caerulea* (00 1645/3) with more severe lesions. There is a focus of epidermal hyperplasia (H) and an area of cleavage and vesicle formation above the basement membrane (arrowhead). There is a diffuse increase in inflammatory cells in the dermis. Bar = 200 μm.

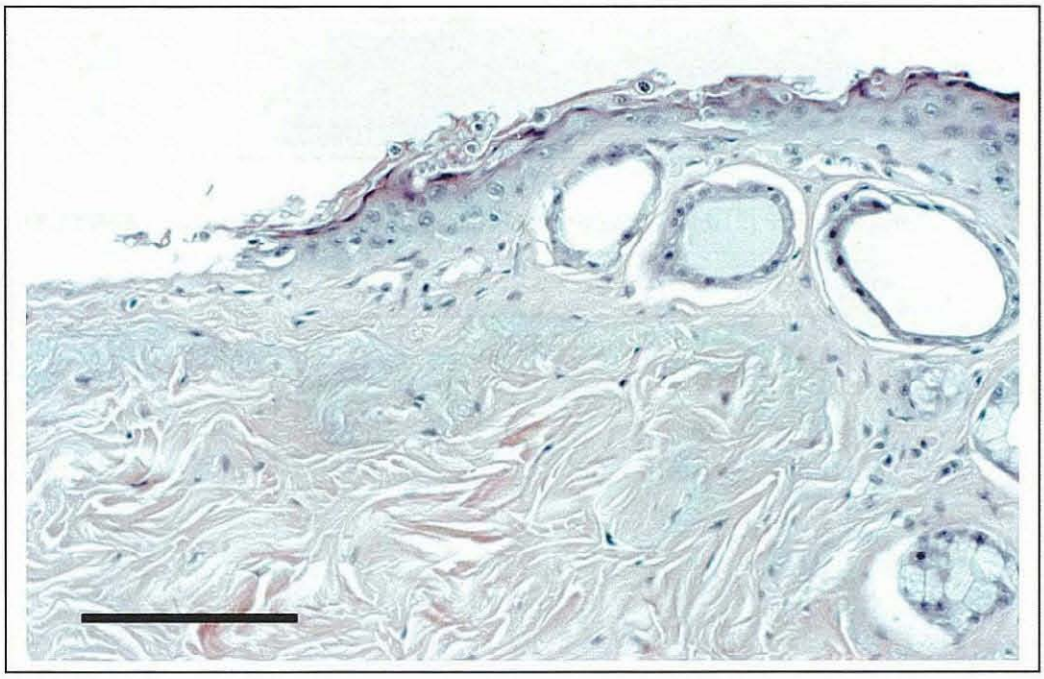

Figure 5.10 Edge of an ulcer from the *Litoria caerulea* in figure 5.3 (99 1385/12). The remaining epidermis is thin (2-5 cells thick). Bar = 90 μm.

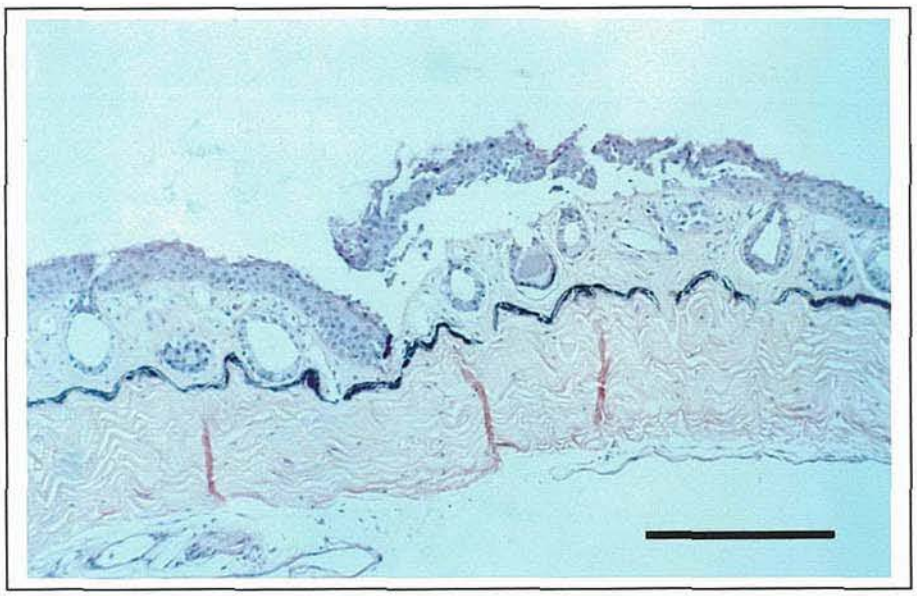

Figure 5.11 Skin from the thigh of an infected adult of *Litoria caerulea* (99 1385/12) with ulceration of the epidermis. Bar = 220 μm.

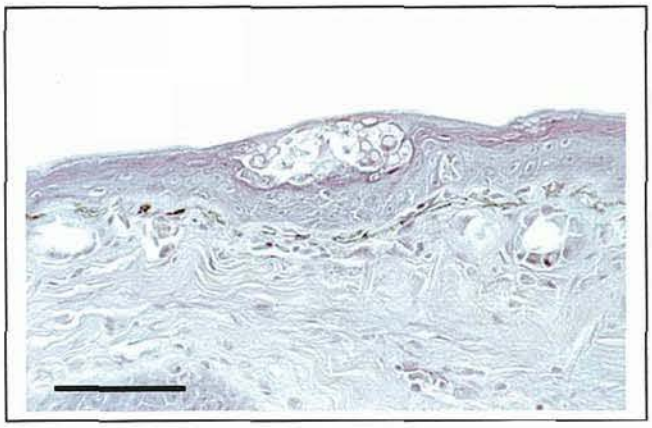

Figure 5.12 Pocket of infection in an adult of *Litoria caerulea* (97 845/12). Bar = 70 μm.

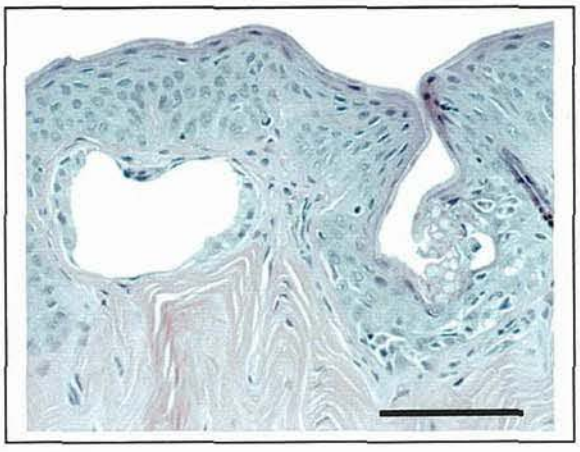

Figure 5.13 Foci of infection and hyperkeratosis in skin over the thighs from a very lightly infected *Litoria caerulea* (97 854/7). There is degeneration of the epidermis adjacent to the cluster of sporangia. Bar = 90 μm.

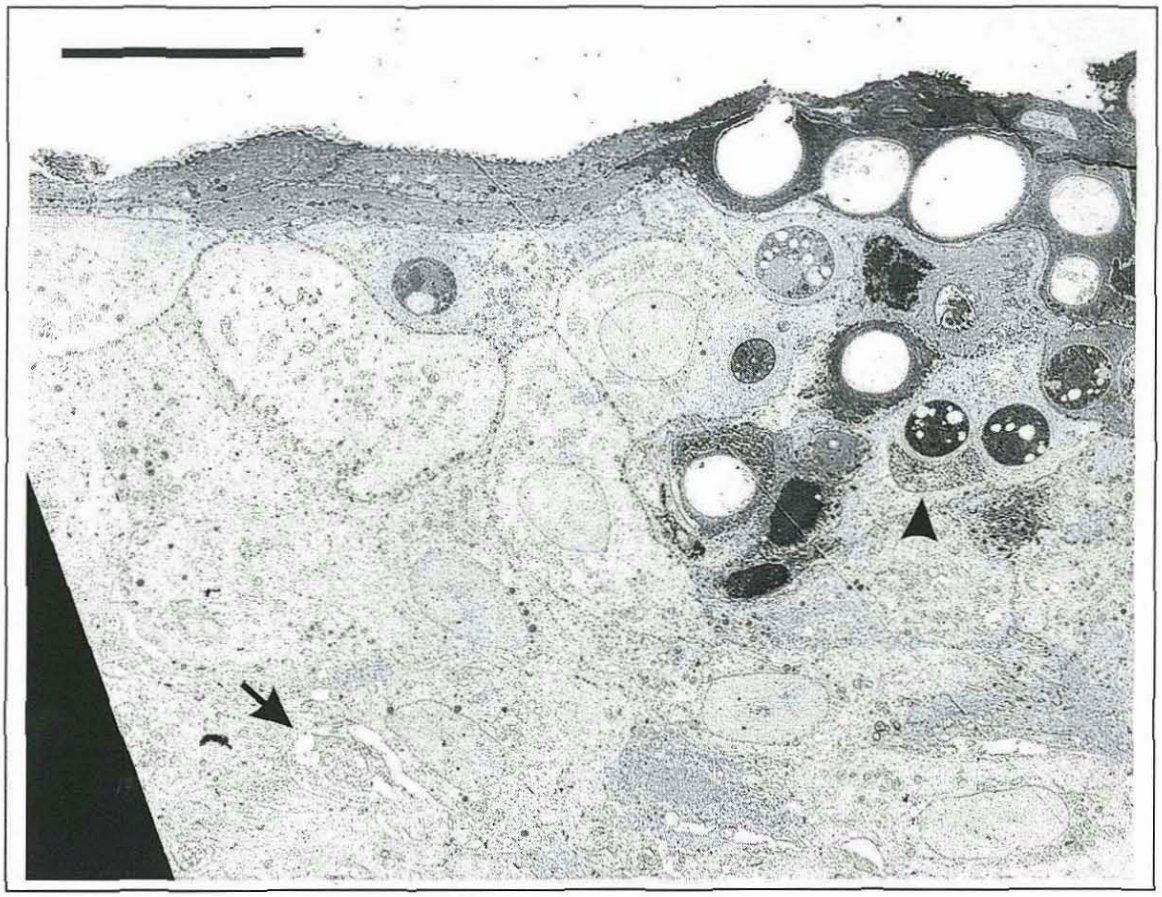

Figure 5.14 *Transmission electron micrograph (TEM) of infected epidermis in an adult of Litoria gracilenta* without clinical signs. There are multiple layers of dark infected keratinised cells, whereas the *stratum corneum* is one cell thick away from the cluster of sporangia. Some nuclei of infected cells are degenerate and chromatolytic (arrow head), and there is necrosis and dissolution of cells in the deeper epidermis (arrow). Bar = 18 μm.

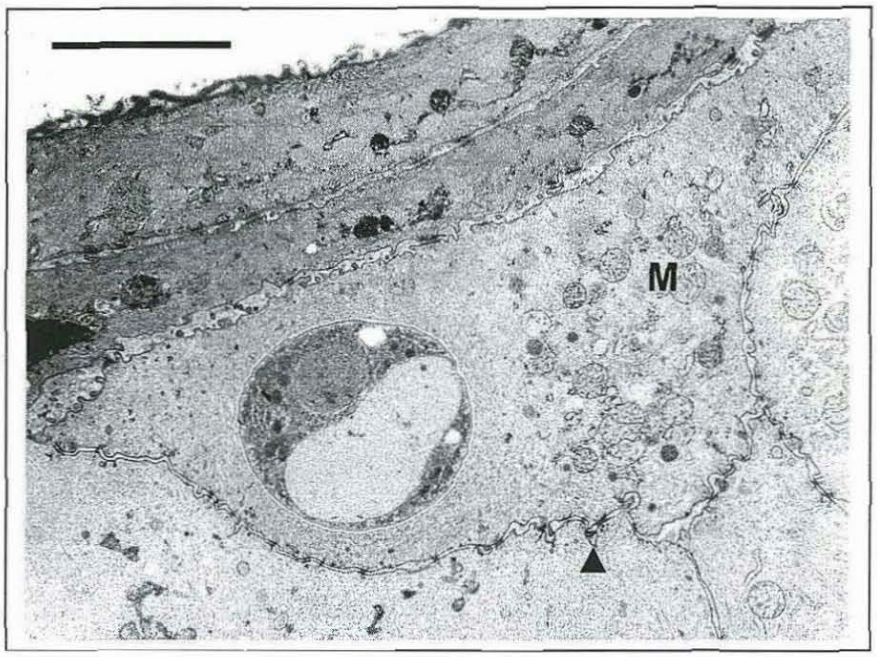

Figure 5.15 Higher magnification of an infected cell from figure 5.14. There is a zone around the sporangium that contains no organelles. Mitochondria (M) and apparently normal cell junctions (arrowhead) are present. Bar = 4 μm.

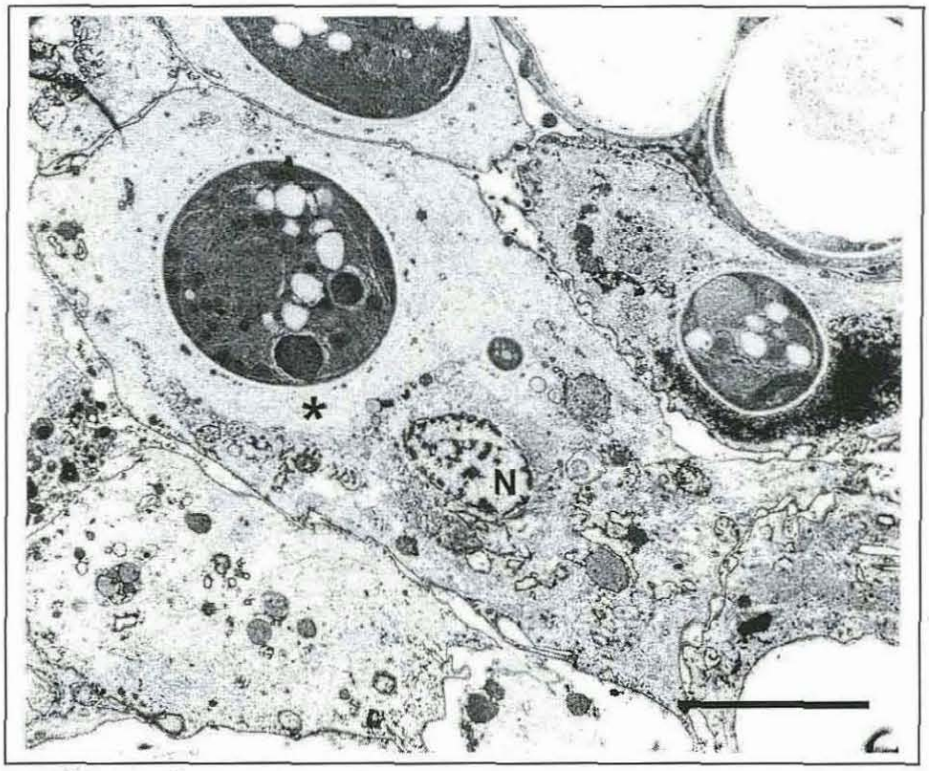

Figure 5.16 TEM of superficial epidermal cells from an adult of *Litoria gracilenta* that died with chytridiomycosis. There is a clear fibrillar zone in the cytoplasm around the sporangium (*) and the chromatolytic nucleus (N) and necrotic organelles have been displaced. Bar = 6 μm.

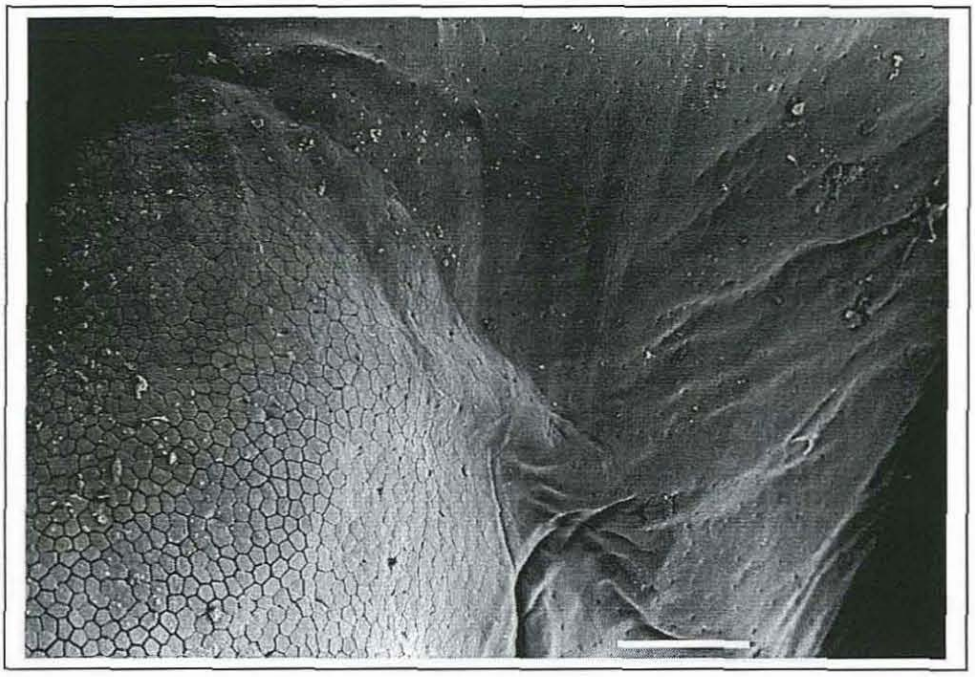

Figure 5.17 Scanning electron micrograph of smooth intact healthy skin from a *Litoria caerulea.* The toe pad is in the left part of the image. Bar = 100 μm.

Figure 5.18 SEM of the surface of skin from the foot of an infected adult of *Litoria lesueuri* (97 574/1), showing the extensive degeneration and peeling of the superficial epidermis. Bar = 100 μm.

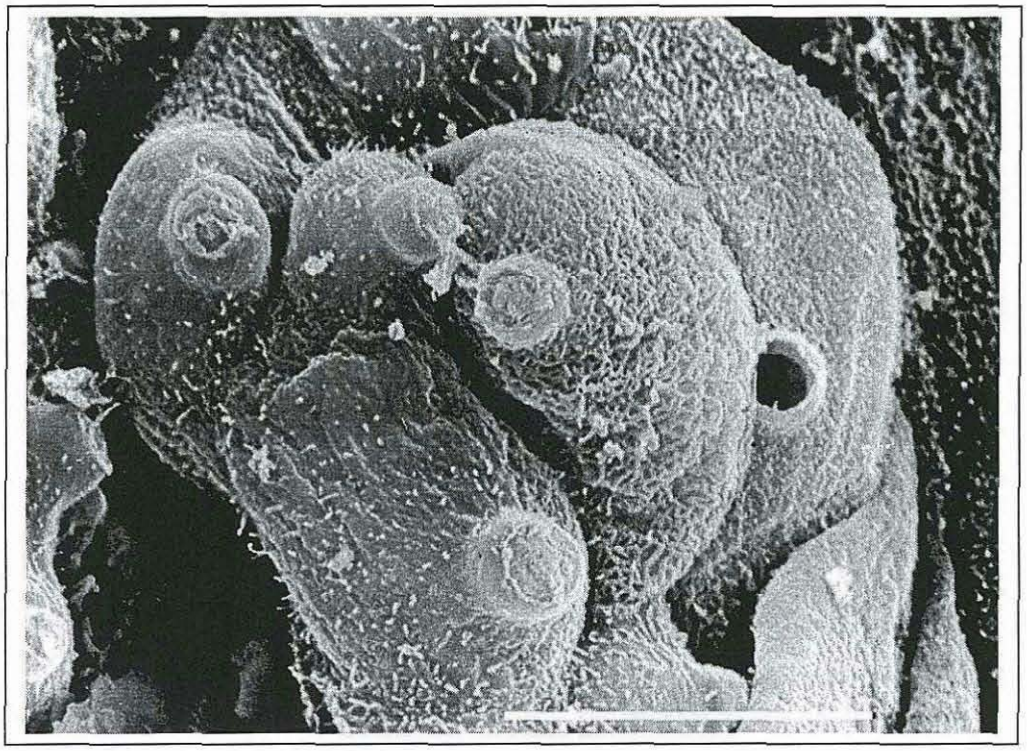

Figure 5.19 SEM of infected epidermal cells on the surface of skin from an adult of *Litoria lesueuri* (97 574/1). Discharge tubes from the intracellular sporangia are protruding through the cell membranes. Bar = 10 μm.

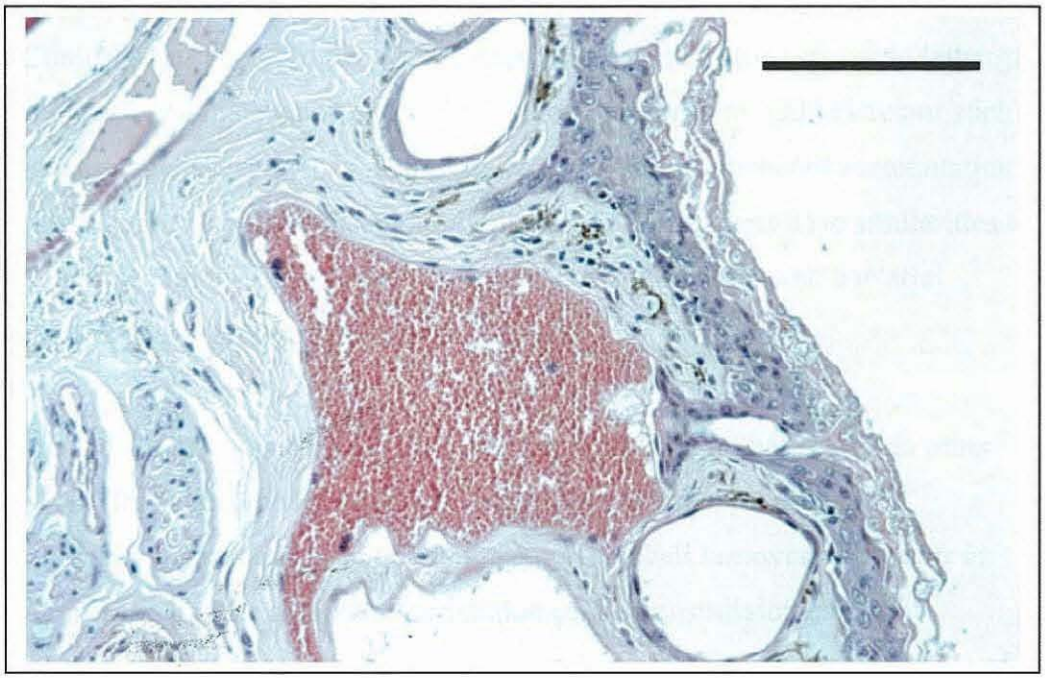

Figure 5.21 Section of infected toe skin from an adult of *Litoria caerulea* (00 1645/3). There is a heavy infection with *Batrachochytrium dendrobatidis* in the thickened *stratum corneum*, and growth of sporangia does not appear to be affected by the presence of a duct from a serous gland. Bar = 90 μm.

5.1.4 Discussion

The clinical signs of frogs with chytridiomycosis were non-specific and varied between species. Clinical signs occurred two to five days before death. Frogs appeared lethargic and became weak and inappetant, and were unable to perform normal behaviour such as burrowing, climbing, escaping or seeking shelter. Skin lesions included accumulation of shedding skin and reddening or discolouration. These clinical signs have similarities to those seen in other amphibian diseases, such as iridoviral infection and bacterial septicaemia ("red leg") (Cunningham et al., 1996a).

The presence of shedding skin on the body is a nonspecific sign that is seen in other diseases where frogs become lethargic and fail to wipe themselves, but with chytridiomycosis, an increased rate of keratinisation and cell turnover may occur in response to infection of the skin. On examination of the transmission electron micrographs, the hyperkeratosis appeared to be partly due to an increased turnover of epidermal cells, and the swelling of epidermal cells surrounding foci of infection suggested a hyperplastic response. Stimulation of the *stratum basale* leading to hyperplasia is a common response to epidermal injury and occurs with other epidermal infections such as papilloma viruses in mammals (Cheville, 1996). The layers of skin in infected frogs were uneven and maturation of epidermal cells appeared disrupted. The cycle of cleavage and sloughing of keratinised cells was also disrupted with multiple layers of keratinised cells building up before being shed. Effects on infected cells included altered fibrils in the cytoplasm and displacement of the nucleus by sporangia. As infected cells are keratinising, the impact of infection on individual cells was difficult to determine, but sporangia may have initiated early cell death and keratinisation. The presence of erosions and the appearance of missing epidermis suggested digestion of epidermal cells had occurred. Thinning of the epidermis may occur when the germination of epidermal cells does not keep up with the increased rate of sloughing from the surface. Extensive disruption of the keratin layer may be significant to the frog. Necrosis of scattered cells in the deeper epidermis occurred commonly and the actively dividing basal cells were often affected. Necrotic cells occurred in infected regions but were not necessarily adjacent to infected cells. A few frogs had widespread degeneration of the epidermis with ulceration.

The lack of consistent inflammation in the skin could be due to a lack of stimulation of the host immune system - perhaps due to the superficial site of infection, or the chytrid may have low inherent antigenicity. As sporangia occur in dense keratinising cells, perhaps this provides some protection from being detected. In mammals, antibodies are not generated in response to dermatomycoses (ringworm) as the superficial epidermal infections do not stimulate antibody producing cells as occurs with systemic fungal infections (Carter, 1986). With dermatomycoses, mild lymphocytic infiltration may occur, along with hyperkeratosis, vesicles and subepidermal hyperemia (Anderson and Kissane, 1977; Jubb et al., 1992). With chytridiomycosis, focal inflammation in the dermis was often associated with areas of ulceration, and may have been stimulated by the damage or the presence of bacteria.

In the study of distribution of infection in severely infected *L. caerulea*, large numbers of sporangia occurred in all areas of ventral skin examined, and no significant differences were detected between these sites. Numbers of sporangia were highly variable and this may be related to the stage in the cycle of sloughing of the *stratum corneum*, and because sporangia are not present in ulcerated areas. Very few or no sporangia occurred on two sites of dorsal skin. Large parotoid glands occur on dorsal skin behind the tympanum, and antifungal secretions from these glands or the smaller dispersed serous glands (which were more abundant in dorsal skin) may inhibit infection. However, sporangia were seen growing at the opening of ducts of serous glands, suggesting secretions are not effective in stopping infection. Three peptides (including synthetic caerin 1.1) found in dermal glands in Australian tree frogs that were tested on *B. dendrobatidis* inhibited zoospore encystment and growth at concentrations of 12.5 μg/ml (Chap 9), but the concentration at which they occur naturally on skin is not known. Caerin 1.1 is the major peptide isolated from *L. caerulea* and has a broad antibacterial spectrum of activity (Bowie et al., 1999). A total of 40 peptides have so far been identified from skin secretions in *L. caerulea* (Bowie et al., 1999). Although these host defences are obviously not effective in protecting most frogs from chytridiomycosis, they may be involved in the different susceptibility of some individuals and of some species. There are major differences between frog species in the type and amounts of peptide produced. Consideration of the susceptibility of unusual species, such as *L. rubella* which produces no antibacterial peptides (Bowie et

al., 1999), may lead to better understanding of the effect of skin secretions on chytridiomycosis.

As with other species that have a *substantia amorpha* (Elkan, 1968), the *L. caerulea* examined here also had a thicker layer in dorsal skin. The dermis of exposed dorsal skin is thicker, and the skin surface is presumably drier than the ventral skin that is used for water uptake. *L. caerulea* is a large xeric-adapted frog that can withstand dry conditions in arid Australia (Warburg et al., 2000). This species loses little water through evaporation (which may be partly due to lipid in the skin secretions) (Warburg et al., 2000) and so the backs may remain dry. As zoospores of *B. dendrobatidis* require water for dispersal, the dryness of dorsal skin could explain the distribution of infection. There are also other differences between dorsal and ventral skin that could affect the establishment of infection. The epidermal infection of *B. baxteri* with *Mucor* sp. also occurred on ventral skin (Taylor et al., 1999b).

The pathology in *L. caerulea* does not appear representative of all frog species and differs in at least three aspects. Severe congestion consistently occurred in skin and internal organs, ulceration appeared more commonly than in other species, and some sporangia were seen on the back of heavily infected frogs from other species, although they were less abundant than ventrally. Wetter skin in species that are more associated with water may enable infection to occur dorsally. Reasons for the congestion and ulceration were not evident. In a recent experiment comparing the susceptibility of different frog species to chytridiomycosis, *L. caerulea* were found to be highly susceptible (Katie Ardipraja and Gerry Marantelli, unpub 2001).

The study on distribution of sporangia needs to be repeated in healthy frogs with light infections. Greater differences between sites may be detected before infection becomes overwhelming, and knowledge of initial sites of predilection would be useful in diagnosis of low level infections and would increase our understanding of chytridiomycosis.

Why do frogs die?

Two theories have been proposed to explain how a fungus restricted to the superficial epidermis could kill frogs (Berger et al., 1998; Pessier et al., 1999). The first hypothesis is that the chytrid releases proteolytic enzymes or other active compounds that are absorbed through the permeable skin of the frog. Although necrosis in the skin was associated with the presence of sporangia, it is not known if the necrosis that was seen in some internal organs was a secondary terminal change or was due to direct effects of the fungal infection. The second hypothesis is that damage to skin function results in disturbance of water or electrolyte balance resulting in death. The epidermal damage caused by chytridiomycosis does not appear severe enough to result in the major changes in water and electrolyte balance required to cause death, although there may be metabolic effects on the skin unable to be detected by histology. Amphibians can withstand severe dehydration and increased osmotic concentrations, for example *L. caerulea* survived losing a water equivalent of 45% of its body weight (Bentley, 1966). The proliferation of bacteria in layers of keratin and empty sporangia in the terminal stages may contribute to the pathogenesis.

The mechanism by which *B. dendrobatidis* causes death remains one of the most important aspects yet to be understood about this pathogen. Studies on enzymes produced by *B. dendrobatidis*, effects of unpurified fungal secretions on the health of frogs, and more detailed studies on pathology and clinical pathology of infected frogs may provide some answers. As a preliminary investigation into haematological and biochemical changes, the following parameters were measured in five infected *L. caerulea* collected from Queensland in winter - packed cell volume (PCV), estimated leucocyte count, leukocyte differential, alkaline phosphatase (AP), aspartate aminotransferase, total protein and creatinine. Results were variable and the only findings that appeared consistently outside normal ranges were that few leucocytes were seen in smears (four frogs) and AP was low (Lee Berger and Bruce Parry, unpub 1997). Mainly mononuclear inflammatory cells were seen in the blood of three frogs, in the other two neutrophils predominated. Serum electrolytes and osmolality were not measured. These preliminary studies provided no explanation for death, and more detailed studies are required.

5.2 *Batrachochytrium* in tadpoles: association with keratin and changes in distribution during development of tadpoles of *Mixophyes* spp.

5.2.1 Introduction

In many of the intensively monitored population declines in Queensland, tadpoles were seen for months after juvenile and adult frogs disappeared (McDonald and Alford, 1999). When remaining tadpoles of *T. acutirostris* were collected to be raised in captivity, only one survived for a significant time after metamorphosis (Mahony et al., 1999; G. Marantelli, pers comm, 2000). Therefore, it appeared that the cause of death of the adults was not affecting tadpoles, but was killing them after metamorphosis. One of the first outbreaks of chytridiomycosis in captivity that I investigated occurred at the Amphibian Research Centre in metamorphs of *M. fasciolatus* from a single spawning group. Gerry Marantelli's husbandry records indicated that infection of tadpoles probably occurred within two weeks of hatching. All eggs had been removed from the spawning tank and kept in a separate tank for hatching, except for a few eggs that were left behind in the spawning tank. Hatchlings were moved to a common early raising tank for up to two weeks and fed on leaf mat collected from the wild. The tadpoles were then moved into 14 isolated 70 litre aquariums. They grew well and almost all survived to metamorphose 8-12 months later. As they began metamorphosis, tadpoles were moved into frog boxes containing no more than 10 animals. Between 10 and 25 days after metamorphosing, 100% mortality occurred in all these tubs, and over 500 metamorphs died. However, about 12 tadpoles that hatched from eggs left in the original spawning tank survived till metamorphosis in this tank, were moved directly to frog boxes and survived to adulthood. The epidemiological links between the boxes of dying frogs were the hatching tank and the early raising tank (Fig. 5.22). Contamination of these tanks could have occurred through a lapse in quarantine, or the source of infection may have been the leaf mats collected from the Lerderderg River, Bacchus Marsh. Healthy groups of tadpoles (or their substrates) remained infected for the 8-12 months till metamorphosis when death occurred (G. Marantelli, unpub). This instance (and a similar finding in captive *B. marinus* tadpoles) led to the discovery that healthy tadpoles can carry *Batrachochytrium* in their mouthparts (Berger et al., 1998). The explanation suggested for this localised distribution was that the mouthparts contain the only keratinised epithelium in tadpoles (Berger et al., 1998). The study reported here

investigated the development of keratinisation in tadpoles, the distribution of *B. dendrobatidis* in tadpoles throughout development and the relationship between the two.

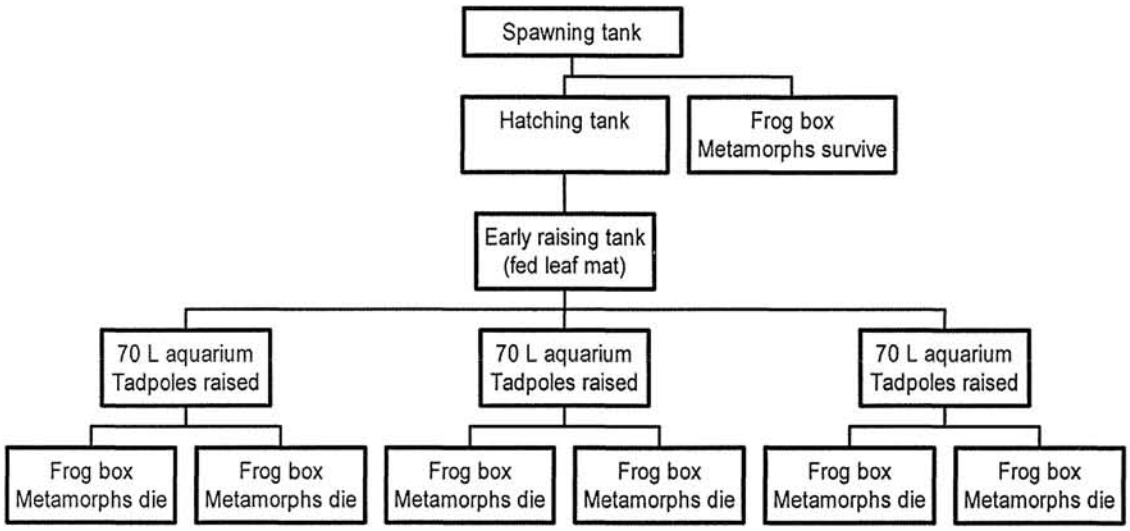

Figure 5.22 Diagram explaining the husbandry of the tadpoles that died after metamorphosis. See text for explanation. Diagram adapted from presentation by Gerry Marantelli (2000).

5.2.1.1 Development of tadpoles

The time from hatching to metamorphosis varies between species of amphibians and is also affected by environmental conditions. Although the period of development can be between two weeks and two years, the stages of development are common between anuran species and can be identified by Gosner stages (Gosner, 1960). Gosner (1960) identified 46 stages from spawning to completion of metamorphosis. Stages 1 to 25 are embryonic or prefeeding stages with hatching occurring around stage 25. Keratinised beaks first appear at stage 24 (Kaung, 1975). Development of the hind limbs is used to identify stages up to 40. The marked changes of metamorphosis occur between stages 41 and 46. Forelimbs appear at stage 42 as the mouthparts are shed and the ciliated epithelium of adults forms in the mouth. Tadpole skin is not keratinised and consists of 2-3 layers of epidermal cells. The mouthparts are the anatomical structures in the tadpoles that are keratinised until shortly before metamorphosis. At metamorphosis the tail becomes necrotic at the tip, degenerates proximally and is rapidly resorbed and epidermis of the tail becomes keratinised. Skin over the body rapidly changes for terrestrial life into a stratified, keratinised epidermis in the few days it takes to complete metamorphosis (Gosner, 1960; Kaung, 1975; Fox, 1994).

The mouthparts of tadpoles consist of upper and lower horny beaks (or jaws) in the centre of rows of lips (or labial fringes) that consist of rows of individual labial teeth (Fig. 5.25). The beaks and lips are covered by cornifying epidermal cells that are continually growing and transforming and eventually slough from the surface.

5.2.2 Methods

Twenty-eight (15 infected and 13 uninfected) tadpoles and metamorphs of *M. fasciolatus* and *M. fleayi* previously submitted from various sources (mostly captive) and preserved in formalin were assigned Gosner stages (Gosner, 1960) then processed for histology. Specimens were sectioned sagitally, with the cut surface downwards. Feet were placed ventral side down if later than stage 33. The Ayoub-Shklar stain for keratin (Luna, 1968) was selected as the acid fuchsin stains keratin a distinct, brilliant red, and *B. dendrobatidis* is stained by the aniline blue. Histological sections were examined for the presence of *B. dendrobatidis* and keratin using the compound light microscope.

5.2.3 Results

5.2.3.1 Development of keratinised epidermis

The distribution of keratinisation varied with Gosner stages (Table 5.2, Figs. 5.23 & 24). Until late in development, keratinised epidermis occurred only on the beaks and caudal surface of labial fringes (Fig. 26). Keratin also extended a short distance caudally from the beak along the surfaces of the buccal cavity. The ventral surface of the feet became keratinised shortly before metamorphosis.

Keratin was present in the mouthparts at stage 25, including an eight day old tadpole which was the youngest specimen examined. Small amounts of keratin were first seen on the ventral surface of the hind feet at stage 39. The ventral fore feet were slightly keratinised when they first emerged. By stage 42 the keratinised mouthparts were shed (Fig. 5.27), although one tadpole had remnants of keratin remaining. At stage 43 keratin appeared on the body, legs and tail and the mouth had a ciliated epithelium as in adults. As the tail resorbed, keratin became established over the tail and body. As the tails shrunk and were resorbed, deep infoldings occurred in the degenerating epidermis lined by thick accumulations of sloughing keratin.

Table 5.2 The number of tadpoles examined and the presence of keratin and *Batrachochytrium* at different stages of development.

Gosner Stage	No. examined		No. (%) with keratin			No. with *Batrachochytrium*		
	Infected	Un-Infected	Mouth	Hind feet	Tail	Mouth	Hind feet	Tail
25	3	5	8 (100%)	nf	0	3	nf	0
29	0	1	1 (100%)	nf	0	-	-	-
33	0	1	1 (100%)	0	0	-	-	-
37	0	1	1 (100%)	0	0	-	-	-
38	1	0	1 (100%)	0	0	1	0	0
39	1	1	2 (100%)	1 (50%)	0	1	0	0
40	2	0	2 (100%)	1 (50%)	0	2	0	0
41	1	0	1 (100%)	1 (100%)	0	1	0	0
42	1	2	1 (33%)	3 (100%)	0	1	1	0
43	0	2	0	2 (100%)	2 (100%)	-	-	-
44	2	0	0	2 (100%)	2 (100%)	0	2	2
45	4	0	0	4 (100%)	4 (100%)	0	4	4

Key: "nf" = not formed; "-"= no infected tadpoles available for this stage.

5.2.3.2 Occurrence of *B. dendrobatidis* in tadpoles

Healthy tadpoles were infected with *B. dendrobatidis* in the surface epithelium of the mouthparts and the same lifecycle stages occurred as in adults (Figs. 5.28 & 6.4). With heavier infections there was hyperkeratosis and sloughing of infected *stratum corneum*. In most cases, no significant pathology was seen grossly or histologically.

B. dendrobatidis often occurred on all surfaces of the horny beaks but were usually only present on the caudal aspect of lips (Fig. 6.12). Sporangia were more consistently seen on the beaks. Infection with *B. dendrobatidis* also extended caudally from the mouth a short way along the surfaces of the anterior buccal cavity (Fig. 5.28).

The feet were first seen with a light infection with *B. dendrobatidis* at stage 42. As the tail resorbed, sporangia became established over the body and a thick infection developed by stage 45. The resorbing tails and tail stumps of infected animals contained extremely heavy infections with *B. dendrobatidis*.

5.4.3.3 Relationship between keratinised epidermis and *B. dendrobatidis*

The distribution of *B. dendrobatidis* throughout development followed the distribution of keratin, and sporangia were not found in epithelium that did not stain positively for keratin (Table 5.2).

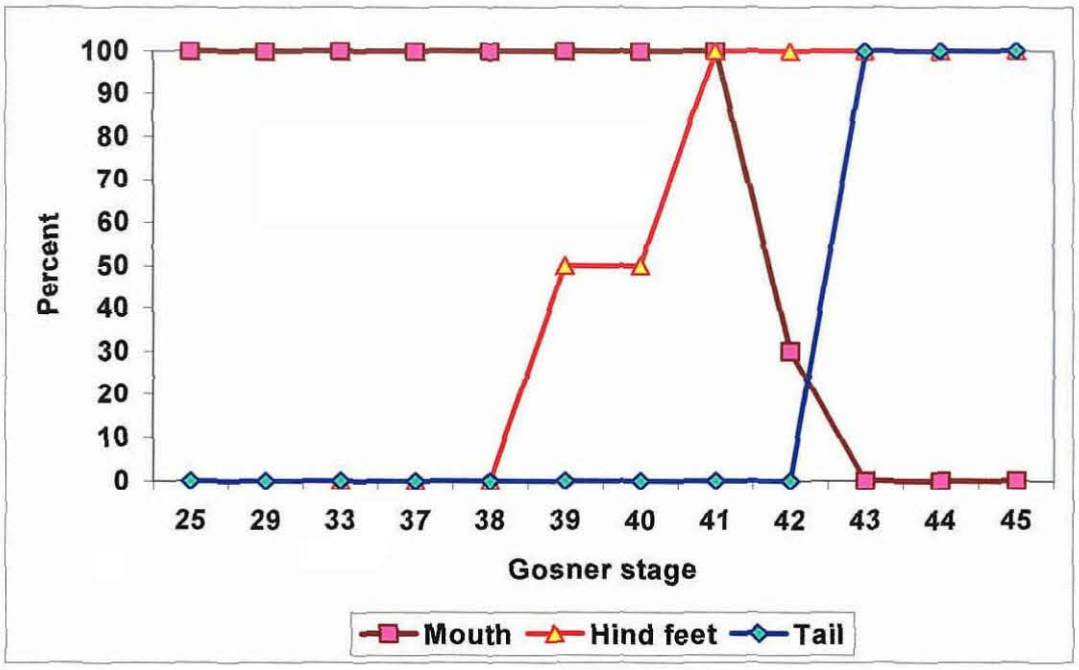

Figure 5.23 Percent of tadpoles with keratin in mouth, hind feet and tail during development.

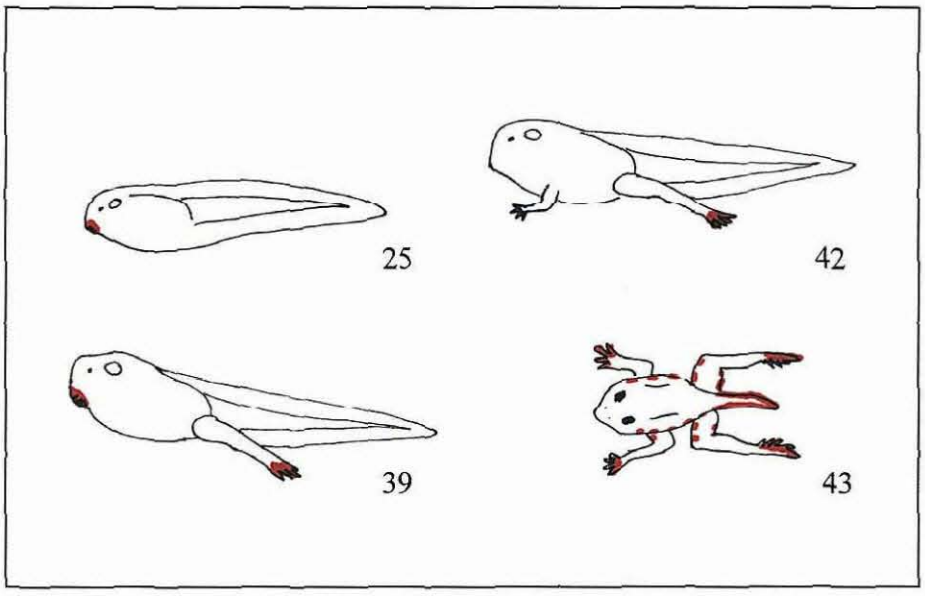

Figure 5.24 Diagram of the distribution of keratin at crucial stages in tadpole development. The numbers refer to Gosner stages.

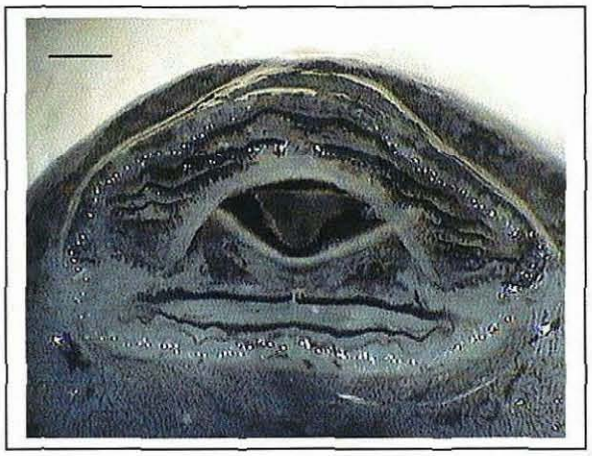

Figure 5.25 Oral disc of a normal tadpole of *Mixophyes fasciolatus* with the dark pigmented horny beaks in the centre surrounded by the pigmented upper and lower labial fringes. Bar – 0.5 mm.
(Photograph by Ed Meyer.)

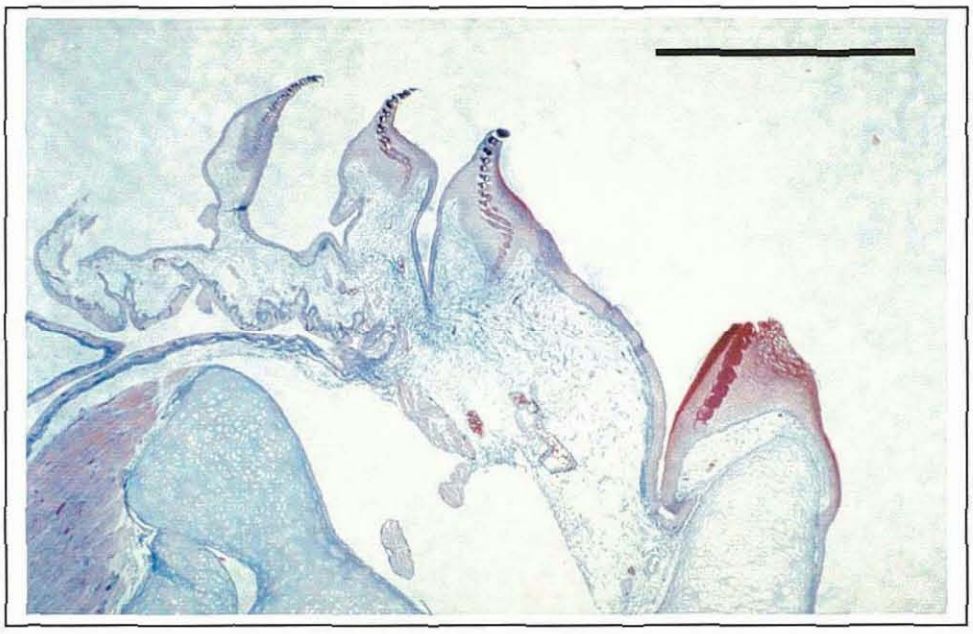

Figure 5.26 Ayoub-Shklar stained section through the mouthparts of an uninfected tadpole of *Mixophyes fasciolatus* demonstrating the presence of red stained keratin on all surfaces of the horny beak (far right), but only the caudal surfaces of the lips. Bar = 700 μm.

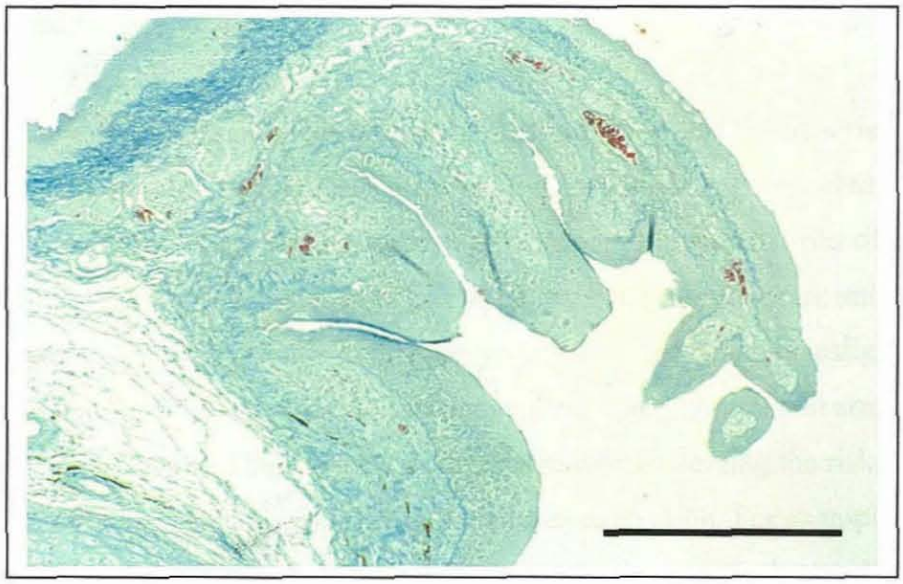

Figure 5.27 Ayoub-Shklar stained section through the mouthparts of a metamorphosing tadpole of *Mixophyes fasciolatus* at stage 42. The keratinised and pigmented surfaces of the mouthparts have recently been shed. Bar = 350 µm.

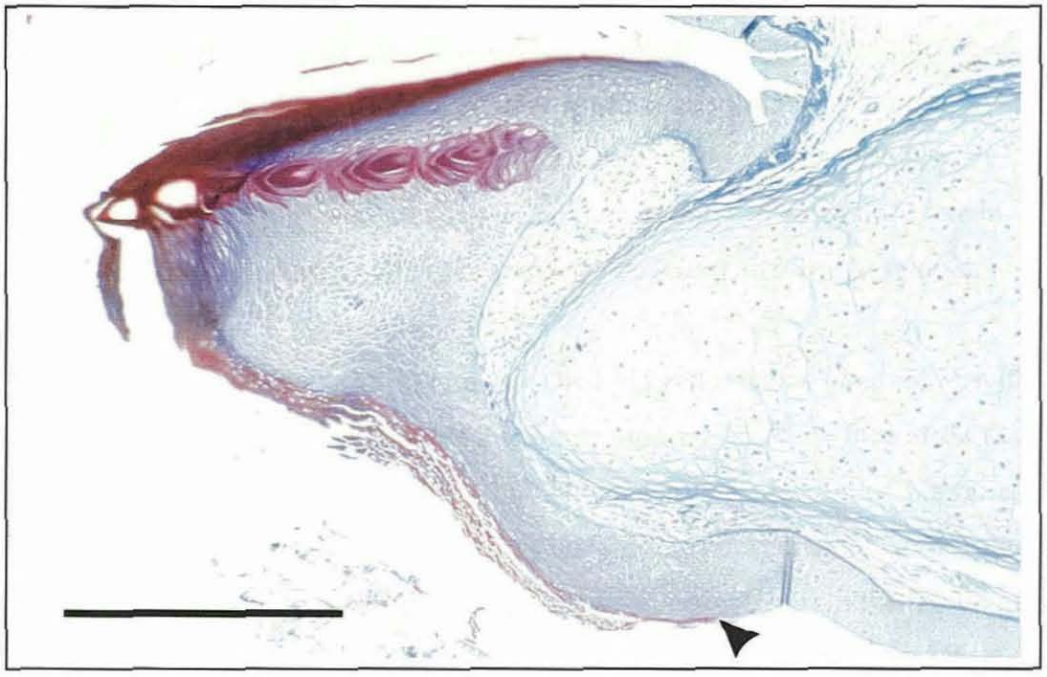

Figure 5.28 Ayoub-Shklar stained section through the horny beak of an infected tadpole of *Mixophyes fasciolatus.* Keratin extends caudally towards the mouth (arrow) and sporangia occur only within the keratinised area. There is severe hyperkeratosis in the infected region. Bar = 250 µm.

5.2.4 Discussion

B. dendrobatidis was found only in keratinised epithelium. Tadpoles in this series were infected at stage 25, usually the first stage after hatching. This finding supported the epidemiological evidence of the original captive outbreak among metamorphs of *M. fasciolatus* described in Section 5.2.1. Infection of embryonic stages appears unlikely, but the potential of *Batrachochytrium* to infect egg capsules needs to be investigated by experimental infection. Other chytridiomycetes have been found growing on amphibian eggs (Czeczuga et al., 1998). These issues are important when assessing the risks of moving eggs or tadpoles, which is commonly done for conservation. For example, enhancement of tadpole survival in endangered frog species has been achieved by rearing tadpoles in captivity after collection of eggs from the wild (Hunter et al., 1999). Samples of southern corroboree frog (*P. corroboree*) tadpoles raised in this way were sacrificed for testing before the remainder of the group were released. All 26 tadpoles tested from 3 release events were negative for *Batrachochytrium* by histology (Chap 8).

For diagnosis of chytridiomycosis, the examination of the horny beaks is ideal as these are more heavily infected than the lips. However, a histological sagittal section which contains beaks and lips can be difficult to achieve (particularly with small tadpoles) and so obtaining a section that contains the pigmented fringes of jaws or lips is adequate. The feet are not useful for diagnosis as they are not consistently infected until late in metamorphosis. Tail stumps in metamorphs appear to be a sensitive site to sample.

The keratinised surface of the beaks and lips are shed between stage 41 and 42, resulting in the loss of the main foci of infection. At this time only the ventral surface of the feet is keratinised. This surface may be infected, but the intensity of infection in this series was light. The feet therefore become infected by waterborne zoospores released from distant sporangia and may not necessarily be infected by zoospores from the mouth of the same tadpole. The stage when the mouthparts are shed is the period of lowest intensity of infection and may be a window of opportunity for use of antifungals. Bath treatment commenced before the feet become keratinised and continued until beaks are shed (stages 38 to 42) may prevent the redistribution of infection. The period of infection limited to the feet lasts a short time as keratin appears on the body at stage 43.

In North and Central America, deformed mouthparts and loss of pigmented lips have been indirectly associated with chytrid infections in a range of anuran species (Lips, 1999; G. Fellers, pers comm 2001). However, as yet *B. dendrobatidis* has not been shown to be responsible for these lesions. In Australia, gross lesions in tadpole mouthparts have not been seen.

This study demonstrated that tadpoles can be infected from any time after hatching. Future studies should examine the natural history of infection in individual tadpoles. This will require diagnostic tests that don't involve sacrifice of the tadpole.

The presence of *Batrachochytrium* in wild tadpoles from stable populations is reported in Chapter 8, which discusses the epidemiology of chytridiomycosis.