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CHAPTER 6
Diagnosis of chytridiomycosis

6.1 Introduction

As chytridiomycosis is a newly discovered disease, methods of diagnosis were developed and evaluated during the project. Most of the research included in this thesis relied on light microscopy of H&E stained histology sections and examination of unstained skin scrapings. Later we produced polyclonal antibodies and developed an immunoperoxidase test for improved examination of skin sections. More sophisticated diagnostic tests, such as indirect ELISAs for use in the laboratory, and field tests, are being developed by other workers at AAHL using these polyclonal antibodies and new monoclonal antibodies.

Diagnosis of chytridiomycosis is important in managing the disease in wild populations, in maintaining captive collections, and in research on amphibian declines. Since amphibian chytridiomycosis has been listed by the OIE (Office International des Epizooties) as a significant disease of wildlife (Stephanie Haigh, pers comm 2001), diagnostic tests may need to become standardised for use in veterinary pathology laboratories.

This chapter consists of 1) a published report on histological diagnosis, 2) a section describing diagnosis by examination of skin scrapings, 3) a section comparing histology and skin scrapings, 4) a submitted manuscript on production of polyclonal antibodies and their use in an immunoperoxidase stain, and 5) a discussion on these three tests.

6.2 My role in the papers

The paper on histological diagnosis was written with Rick Speare and Andrew Kent based on shared observations of the best methods and diagnostic features of chytridiomycosis.

Many people contributed to the paper on polyclonal antibody production. I produced the antibodies with advice from Alex Hyatt and help with the animals from Sandy

Matheson and Susanne Wilson. I developed and optimised the immunoperoxidase test, then examined cross-reactivity with other fungal species and evaluated the test for diagnosis using staining largely performed by Veronica Olsen. The immuno-electron microscopy was done by Sandra Hengstberger and Alex Hyatt. Donna Boyle developed the fluorescence stain. Fungi used for testing cross-reactivity were isolated by Joyce Longcore and Kaye Humphreys. Gerry Marantelli provided toe-clips from infected frogs that were used to evaluate the stain for diagnosis.

6.3 Histological diagnosis of chytridiomycosis

This paper was written as a practical guide to diagnosis, for people with or without experience in histopathology.

Berger, L., Speare, R., Kent, A. 2000. Diagnosis of chytridiomycosis in amphibians by histologic examination. Zoos' Print Journal. 15: 184-190.

6.3.1 Abstract

Chytridiomycosis is a fatal disease of post-metamorphic frogs and can be carried by healthy tadpoles. Here we describe methods for preparing and interpreting histologic sections from adults and tadpoles for the diagnosis of chytridiomycosis.

6.3.2 Introduction

A newly identified chytrid fungus in the genus *Batrachochytrium* (Longcore et al., 1999) that infects amphibians and can cause death has been found in Australia, South America, Central America and the USA (Berger et al., 1998; Daszak et al., 1999; Pessier et al., 1999;). The chytrid fungus is the most common cause of death in Australian frogs and has also been found in a small proportion of apparently healthy frogs and tadpoles (Berger et al., 1999). *B. dendrobatidis* can be detected by routine histology of skin specimens preserved in formalin or ethanol. Examination of unstained skin scrapings is a quick method, but requires greater expertise in identifying organisms. Chytrid culture from fresh specimens requires specialised methods (Longcore et al., 1999) and is difficult; so most diagnoses are made using histology. Diagnosis using

histology requires skills in microscopy, knowledge of the morphology of *B. dendrobatidis*, knowledge of normal histology of the skin, and an ability to differentiate the chytrid from other structures in the skin. The structure of normal amphibian skin has been described (Fox, 1994). Pessier et al. (1999) describe the histological appearance of the amphibian chytrid, but this paper provides more detailed criteria so investigators unfamiliar with histopathology can make a diagnosis of chytrid infection. This technique may be used for surveying toe-clips from wild and captive amphibians, surveying archived specimens, to test animals before translocation, and to determine the cause of mortality in the wild and in captivity so that appropriate management can be implemented.

Using histological techniques *B. dendrobatidis* has been found in 31 species of amphibian in Australia, 10 species in USA, 7 species in Panama and 3 species in Ecuador (Nichols et al., 1998; Berger et al., 1999; Carey et al., 1999). In Australia the technique has been used to start mapping the distribution of the chytrid and has identified three geographic foci: east coast, Adelaide and environs and southwest Western Australia (Berger et al., 1999).

Healthy tadpoles can carry *B. dendrobatidis* for months. Tadpole skin does not become infected as it is not keratinised, but chytrids may infect the keratinised mouthparts. Healthy infected tadpoles may be highly prevalent in a population and so sampling tadpoles is a sensitive way of assessing a location. This paper refers to diagnosis in post metamorphic amphibians except where tadpoles are specifically mentioned.

6.3.3 Methods

The following description is based on histological sections prepared from tissue preserved in 10% formalin or 70% ethanol, dehydrated, embedded in paraffin, sectioned at 5 μm, and stained with haematoxylin and eosin (Drury and Wallington, 1980). Strips of skin from the pelvic region were embedded to obtain the maximum length of *stratum corneum* from the specimen available with the best plane for interpretation being a vertical section through the skin. Digits were examined by 1) sectioning a whole foot ventral side down or 2) sectioning a single toe (when testing live animals or when minimal mutilation of the carcass is required). For toes the maximum length of *stratum*

corneum was obtained from a longitudinal section rather than a cross section. Larger digits, for example from amphibians with a snout-vent length > 60 mm, were degloved by removing skin from the underlying phalanx and sectioning the skin without bone. Smaller toes were usually sectioned without decalcification. Decalcification was required for larger toes if bone was not removed. Decalcification was performed by placing toes in EDTA for 48 hours at 37°C or in 10% formic acid for 3-5 days prior to processing. For population surveys, or for smaller specimens, about three serial sections were usually placed on the same slide to increase the length of skin available for examination. To prevent loss of toes during processing, sponge biopsy pads (Edward Keller, Hallam, Victoria) were used within cassettes. Skin was obtained from naturally and experimentally infected frogs and toads from Australia. Measurements were made with a calibrated eyepiece graticule.

6.3.3.1 Specimens

B. dendrobatidis only invades the *stratum corneum* and *stratum granulosum*, in particular the subsurface layer (Fig. 6.1). Therefore, skin is the only organ required for diagnosis. Since the keratinised surface layer of infected epidermis may slough and it contains organisms, sloughed epithelium can also be used for diagnosis.

6.3.3.2 Site

The fungus is not evenly distributed on the surface of the body. More sporangia occur in *stratum corneum* of the digits and ventral surfaces, and less occur on dorsal surfaces (Berger et al., 1998; Pessier et al., 1999). In severe infections chytrids may be detected on the dorsal surface. In light infections the feet and the thighs are the sites of choice. Examination of toe-clips has been used to conduct prevalence surveys of clinically normal frogs, as toe-clips can be retained for examination and the amphibians released.

6.3.3.3 Tadpoles

Diagnosis in tadpoles can only be done by sacrificing the animal. Bathing in MS 222 (tricaine methanesulphonate, Ruth Consolidated Industries, Annandale) is a suitable method for euthanasia. A section through the mouthparts which includes the dark brown keratinised "teeth" is required for diagnosis. Large tadpoles are cut longitudinally through the midline with a scalpel and embedded with the cut surface downwards. Small tadpoles are best embedded whole on their side in the paraffin block,

and then serially sectioned to reach the mouthparts. The size of the mouth varies between species, which affects the ease of obtaining a suitable section.

6.3.4 Morphology of *B. dendrobatidis*

6.3.4.1 Shape

In the *stratum corneum* the chytrid is roughly spherical with a discharge papilla projecting from the surface (Fig. 6.1), a good analogy being an inflated balloon with the neck of the balloon representing the discharge papilla. The mature fungal body is called a zoosporangium (Longcore et al., 1999). Some zoosporangia have several discharge papillae. The discharge papilla projects beyond the surface epithelium and has a plug at the mouth (Berger et al., 1998). Discharge papillae can be seen in histological sections, but they are not common. Zoospores that develop in the zoosporangium escape through the open discharge tube.

6.3.4.2 Wall

The wall of the zoosporangium is smooth on outer and inner surfaces and very uniform in thickness. Its staining characteristics vary from eosinophilic to slightly basophilic or refractile. Variable amounts of keratin may closely surround the organism.

6.3.4.3 Contents

The contents of the zoosporangia vary with the developmental stage of the chytrid (Figs. 6.1 & 6.2). On histological sections four stages can be identified. The earliest stage contains a central mass which is basophilic and roughly spherical or oval. The mass is fairly homogenous in staining characteristics (Fig. 6.1). Sporangia become multinucleate and then the cytoplasm divides to form zoospores. Zoospores are basophilic and appear in cross-section as round or oval bodies often with poorly defined margins (Fig. 6.1), usually numbering about 4 to 10 depending on the plane of section. Once the zoospores are released via the discharge papilla, the zoosporangium is empty and retains its spherical shape (Fig. 6.1). In some empty colonial stages, thin septa may be visible dividing the sporangium into internal compartments (Longcore et al., 1999). The empty sporangium may collapse into an irregular shape (Fig. 6.2). During this terminal stage the empty shell sometimes becomes colonised by bacteria, and these are seen in section as basophilic rods or cocci inside the "empty" sporangium (Fig. 6.2).

Bacteria can be confused with zoospores, but zoospores are larger than bacteria and less numerous. In frogs in terminal stages of chytridiomycosis, large numbers of bacteria may be seen between layers of sloughing keratin. Empty sporangia are the most common stage present, particularly in the sloughing surface layer. Empty sporangia may be missed unless looked for carefully. When examining sections, a useful guide to detection is the presence of clear spaces in the surface epithelium. If these are seen, examination should be made at higher magnification including oil immersion.

6.3.4.4 Size

In histological sections the diameter of zoosporangia varies with the plane of section with identifiable sections through the edge of a sporangium being 5 µm while maximum diameters are roughly 13 µm. Discharge tubes have a diameter of 2 µm and a variable length, usually between 2-4 µm, but up to 10 µm. The developing mass in early sporangia has a diameter of approximately 4-8 µm. Zoospores are about 2 µm in diameter. Size can be a useful guide in distinguishing zoosporangia from other bodies in the *stratum corneum* since zoosporangia are usually less than 15 µm in diameter.

6.3.4.5 Stains

Chytridiomycosis can be diagnosed using routine H&E staining. Special fungal stains, such as periodic acid-Schiff (PAS) or silver stains (Drury and Wallington, 1980) may be used, but appear to offer little additional benefit, except for confirming infection in cases where a few indistinct stages are present, and in differentiating *B. dendrobatidis* from other fungal species. Experiments are planned to evaluate if special stains will improve the sensitivity of diagnosis particularly by people less familiar with *B. dendrobatidis*.

6.3.4.6 Other dermal mycoses

Occasionally other fungi invade the epidermis of amphibians, for example cutaneous mucormycosis (Taylor et al., 1999c). Most have thread-like hyphae, but in cross-section the hyphae can often have a circular appearance, and if the number of planes of section are few, the inexperienced examiner may confuse hyphae with chytrid sporangia. If special fungal stains (PAS, silver stain) are used in these cases, the filamentous nature of the hyphae is more apparent than with H&E, and more oblique sections plus septa become obvious.

In the USA *Basidiobolus ranarum* has been reported to cause very similar superficial mycotic skin infections in dwarf African clawed toads (Groff et al., 1991) and in Canadian toads and Wyoming toads (Taylor et al., 1999a, b). However, there is controversy about whether these cases were due to *Basidiobolus*, or are misidentified cases of chytridiomycosis.

6.3.4.7 Artifacts

To consistently make a diagnosis of chytridiomycosis knowledge of the structures seen in normal skin is required. In addition there are a number of artifacts both in normal skin and in skin with other diseases that can be confused with chytrids. However, no other structure has exactly the same morphology, size, location and association with hyperkeratosis. Ducts from dermal glands often appear as spherical spaces between epidermal cells and may be confused with empty zoosporangia although they lack a distinct and complete wall, and are extracellular (Fig. 6.3). The basophilic immature stages of the chytrid in the subsurface layer can appear similar to epidermal cell nuclei, but are often surrounded by a clear halo. A PAS stain demonstrates the fungal wall around immature chytrids.

6.3.4.8 Tadpoles

The usual range of fungal stages may be present in tadpole mouthparts (Fig. 6.4). They can occur on all surfaces of the horny jaws, and on caudal surfaces of the labial fringes. Tadpoles may have light infections accompanied by minimal pathology, or heavier infections with hyperkeratosis and occasional bacterial invasion.

6.3.5 Histopathology of chytridiomycosis

In most cases the chytrid fungus is associated with focal hyperkeratosis and erosions in the area of *stratum corneum* adjacent to the organisms. Irregular thickening of the epidermis (hyperplasia) may be present as well as mild focal necrosis of epidermal cells. Vesicles may form between cells in the basal layer which coalesce and progress to ulcerations. There may be a slight increase in inflammatory cells in the dermis. In light infections in clinically normal amphibians in which a single focus of organisms may be found, the hyperkeratosis is confined to that focus (Fig. 6.5), while in fatal infections hyperkeratosis is often widespread as are organisms. In some fatal cases there may be

extensive sloughing of the hyperkeratotic layer leaving an epidermis with few organisms (Fig. 6.6). However, in these cases chytrids can be detected in low numbers in the slightly keratinised surface layer, or chytrids may be seen in large numbers if the sloughed skin is examined. Sporangia are not present in areas of extensive ulceration.

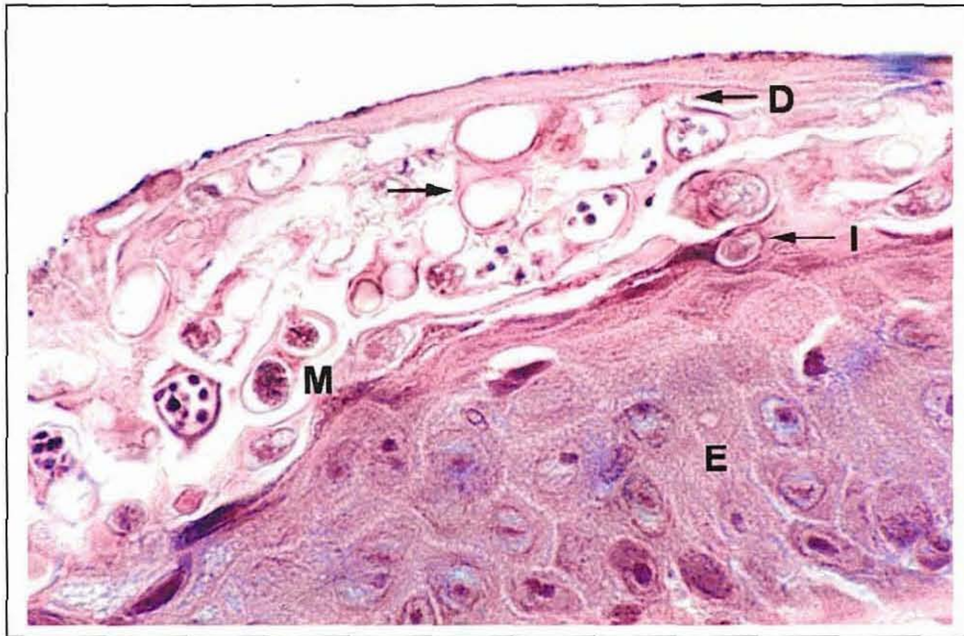

Figure 6.1 Section of skin from a heavily infected adult of *Litoria caerulea*. Note homogenous immature stage (I), larger multinucleate stages (M), zoosporangium with discharge tube (D) containing zoospores, and empty zoosporangium after zoospores have discharged (arrow). E = epidermis.

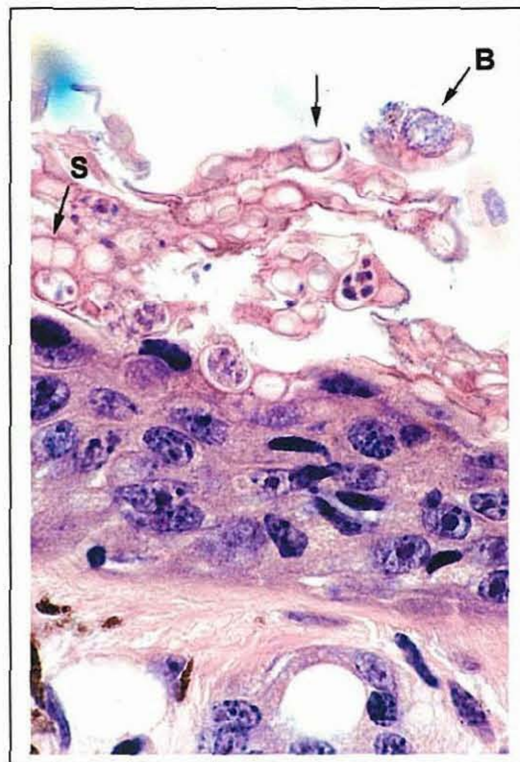

Figure 6.2 Section of skin from a juvenile of *Mixophyes fasciolatus* with mostly empty sporangia present. Note empty collapsing sporangium (arrow) and one containing bacteria (B). A sporangium is divided by an internal septum (S).

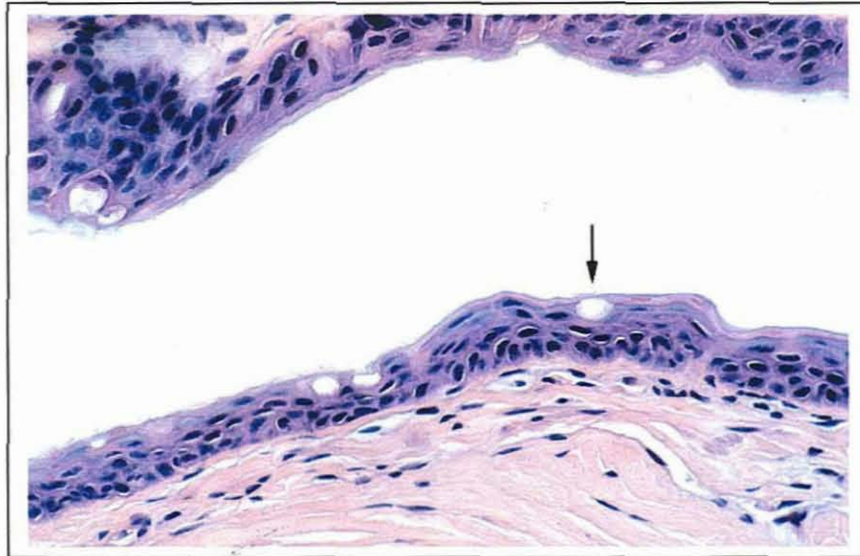

Figure 6.3 Normal toe skin from a *Litoria chloris* with vesicular structures in the epidermis (arrow). These are likely to be ducts from dermal glands but appear similar to empty sporangia. Note that the clear spherical space lacks a cell wall and the adjacent epidermal cells are not hyperkeratotic.

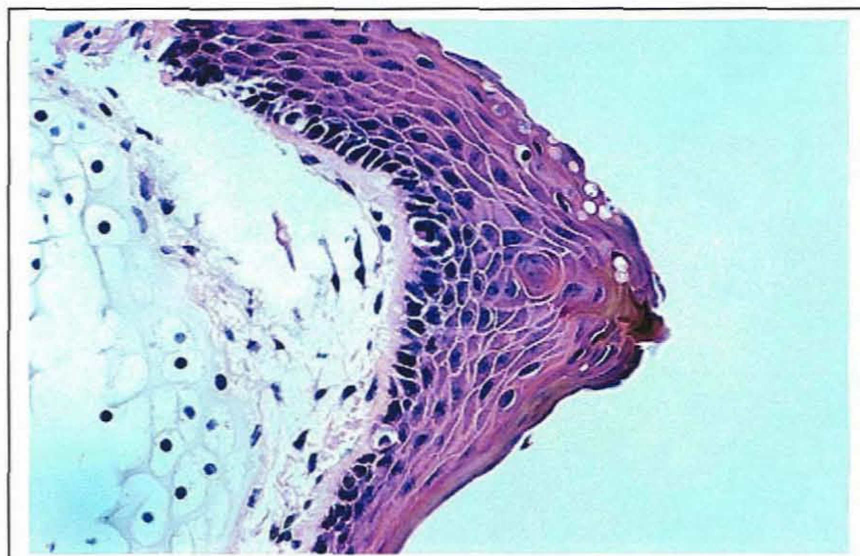

Figure 6.4 Section through the mouthparts of a tadpole of *Mixophyes fasciolatus* with various stages of *B. dendrobatidis* present in the superficial keratinised epidermis.

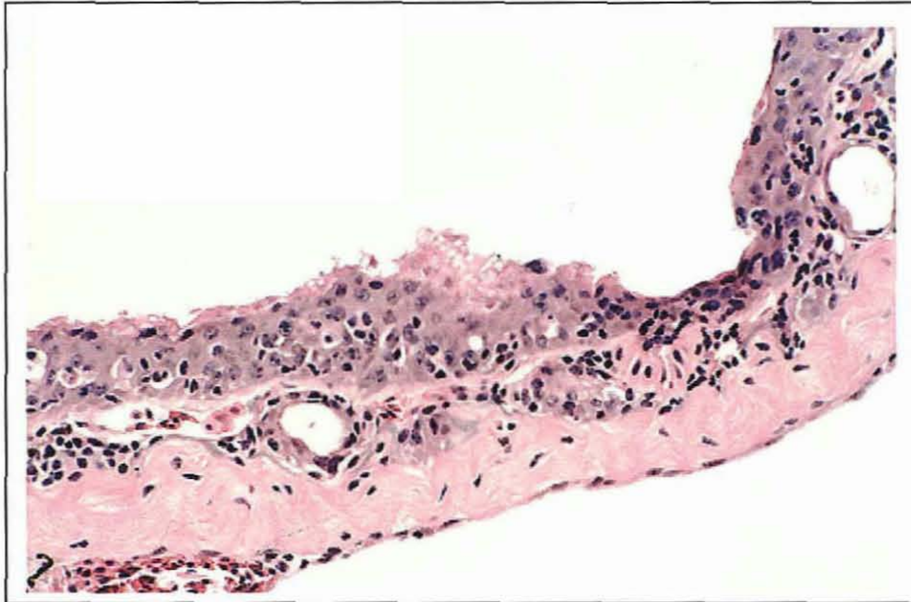

Figure 6.5 Section of skin from a lightly infected *Mixophyes fasciolatus* with focal hyperkeratosis. Sporangia of *B. dendrobatidis* appear as small, clear circular spaces in the surface of the thickened epidermis.

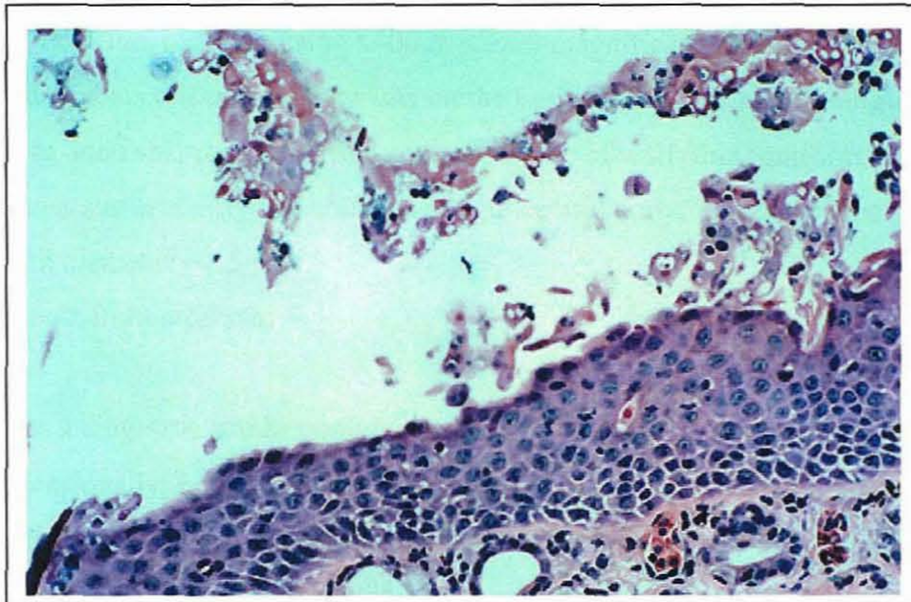

Figure 6. 6 Section of skin from a metamorph of *Mixophyes fasciolatus* with the heavily infected *stratum corneum* sloughing, leaving few organisms in the epidermis in the left half of the image. Sporangia appear as round spaces or dark circles in the shedding skin.

6.3.6 Technique of examination

The epidermis can be scanned at x200 magnification to detect suspicious organisms or areas of skin. These suspicious areas should be examined at x400 magnification to confirm the diagnosis. If one is unsure of the identity of a suspicious structure examination under oil immersion (x1000) will usually improve the confidence of the decision. In heavy infections oil immersion is not required, but for light infections with few chytrids oil immersion may be required to make a definite diagnosis.

6.3.6.1 Making a diagnosis

We use the following steps to maximise the sensitivity of diagnosis of chytridiomycosis in histological sections.

1. Examine the *stratum corneum* or sloughed skin from the digits or ventral skin.
2. Scan the *stratum corneum* at x200 or x400 magnification.
3. Look in the *stratum corneum* for i) structures that have any of the morphological features described above and ii) areas of hyperkeratosis or areas where the epidermal surface is irregular.
4. If these are found, examine using x400 or x1000 magnification.
5. Make a diagnosis of *Batrachochytrium* on the basis of shape of zoosporangium (spherical, balloon shape, or collapsed sphere), nature of wall (thin, uniform, smooth outer and inner surfaces), type of contents (single central body, zoospores, septa, bacteria), and diameter (<15 µm).
6. Differentiate from artifacts.

In most cases a diagnosis can be confidently made following the schema under points 1-5 above. Occasionally, however, for examiners with an inadequate level of knowledge of other structures occurring in the skin, the degree of confidence of the diagnosis may not be high. A diagnosis can be made easily if mature zoosporangia are seen, but is more difficult if only a few early or late stages are present. To confirm infection or to identify hyphal fungi use fungal stains such as PAS or silver stains, examine additional histological sections, or refer the slide to an expert. In screening amphibians for chytridiomycosis we classify results into negative (no chytrids found), confirmed positive (chytrids found), and suspect positive (possible chytrids found, but confirmation needed).

6.3.7 Acknowledgements

Thanks to Gerry Marantelli for supplying frogs, and Megan Braun and Gail Russell for excellent histology.

6.3.8 References

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6.4 Diagnosis of chytridiomycosis by examination of skin scrapings

Examination of skin scrapings by light microscopy is a quick and simple method of diagnosis. Shedding skin is lifted or scraped off the frog, spread out flat on a slide with a drop of water, a cover-slip is placed on top and the preparation is examined under a compound light microscope. Ideally, an even monolayer of keratinised epidermal cells is obtained. Magnification of 100x is used initially to scan a section, then 400x is used to confirm the presence of sporangia. The refractile walls of the sporangia are more distinctive if the condenser is racked down.

The round to oval intracellular sporangia (5 -13 μm) occur in clumps. Old empty sporangia are the most prevalent stage in shedding skin, although sporangia containing zoospores are commonly found (Fig. 6.7). As discharge tubes usually point perpendicularly to the skin surface, they can be difficult to discern and appear as small circles. The observation of internal septa within sporangia increases confidence in the diagnosis (Fig. 6.8). Epidermal cell nuclei are a similar size to sporangia but can be differentiated by their irregular, indistinct membranes and flat, granular, grey appearance.

It is important to store frogs separately to avoid cross-contamination of shedding skin that can result in a false-positive result.

Tadpole mouth-parts can be examined by cutting off pieces of the pigmented horny jaws or lips and squashing under a cover-slip (Fig. 6.9). Large numbers of tadpoles were not examined so data comparing histology and scrapings are not presented.

Diagnosis of chytridiomycosis by staining skin scrapings or smears has also been described. Lactophenol cotton blue and KOH is an effective stain in wet preparations (Rolando Mazzoni, pers comm 2000). DipQuick (Jorgensen, USA) was used to stain dried smears (Nichols et al., 2001). These stains may improve accuracy of diagnosis, and may be particularly useful for inexperienced examiners.

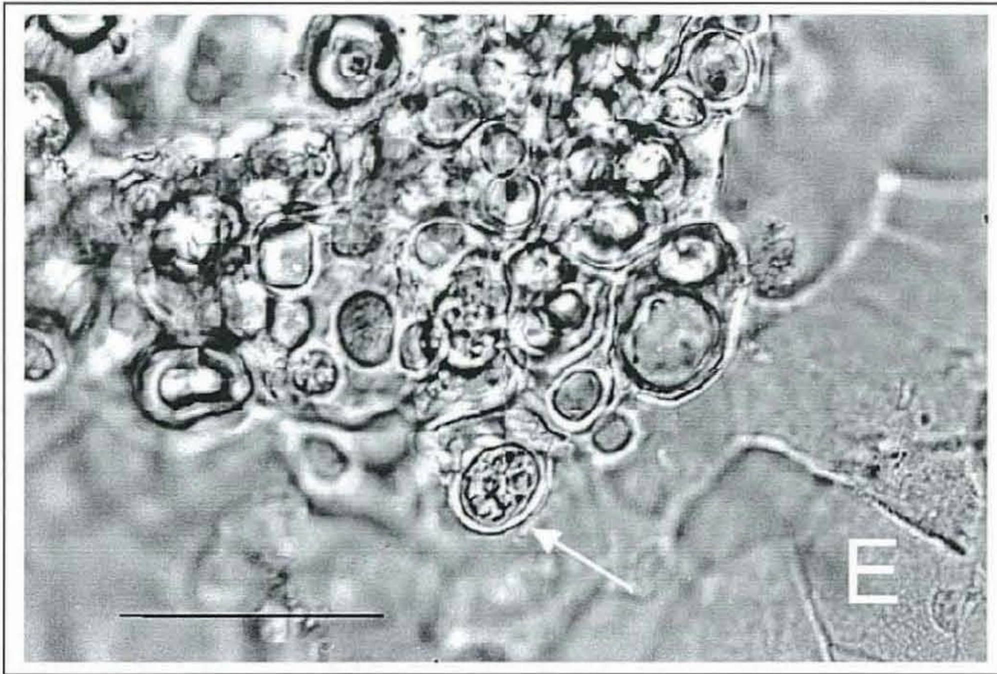

Figure 6.7 Unstained wet mount of shedding skin from an infected adult of *Litoria caerulea*. Note refractile round and oval sporangia. Most are empty but one contains developing zoospores (arrow). E = epidermal cell. Bar = 30 μm.

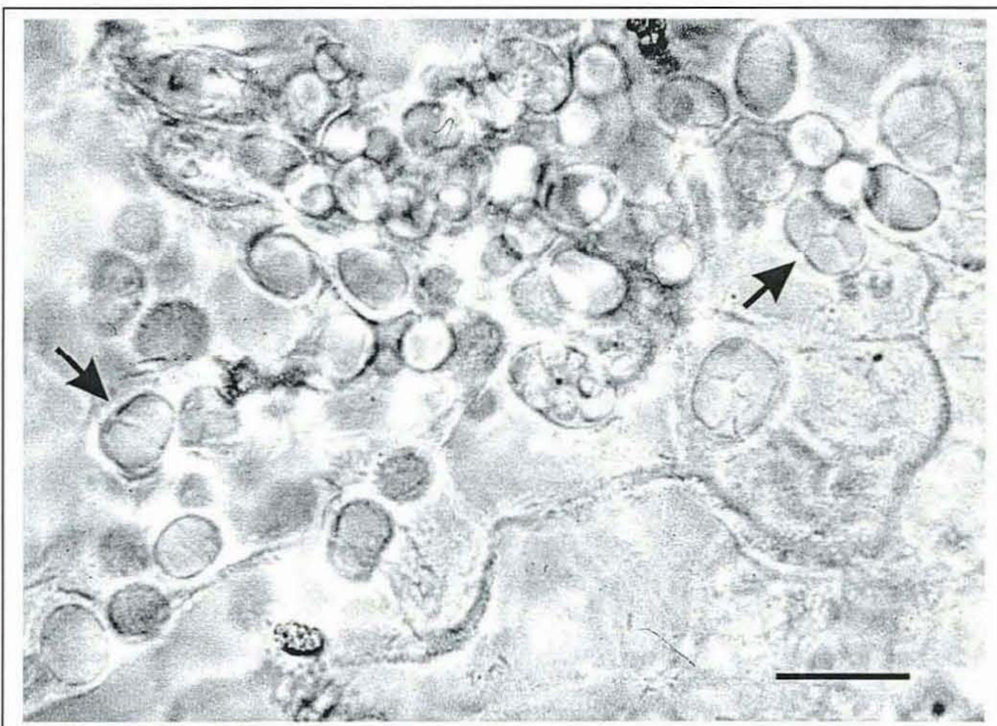

Figure 6.8 Shedding skin from an infected *Mixophyes fasciolatus*. There are a few colonial sporangia with internal septa dividing the thallus in to two or four compartments (arrows). Bar = 15 μm.

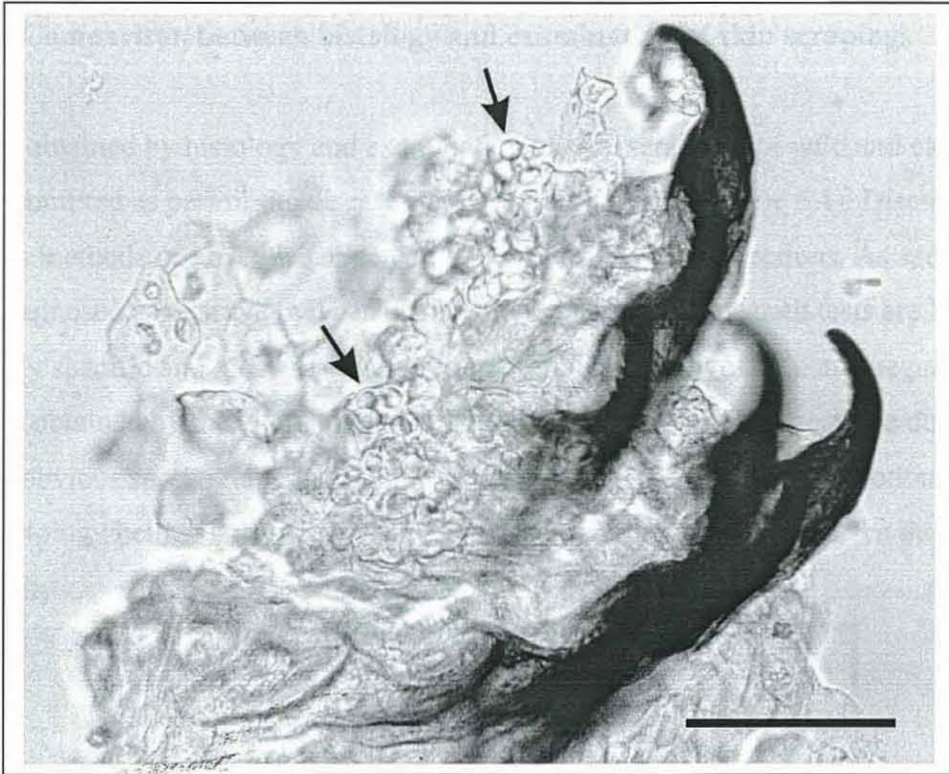

Figure 6.9 Unstained squash preparation of pigmented keratinised lips from an infected tadpole of *Mixophyes fasciolatus*. The arrows indicate clusters of sporangia of *B. dendrobatidis*. Bar = 80 µm.

6.5 Comparison between histology and examination of skin scrapings

Results obtained by histology and examination of skin scrapings of wild and captive frogs submitted as part of the disease survey were compared (Table 6.1). Discrepancies between methods occurred when detecting light or incidental infections. As samples were diagnosed as positive by visualising convincing structures, both tests are likely to be highly specific and most positives are therefore true positives. When a negative result is obtained by the alternative method, it is likely to be a false negative due to the lack of obvious sporangia in the sample. Overall results were similar for both methods, with histology being slightly more sensitive. Use of both methods increases the sensitivity.

		Histology			**Total**
		Pos	**Neg**	**Suspicious**	
Skin Scraping	**Pos**	72	6	1	79
	Neg	8	78	2	88
	Suspicious	3	1	1	5
Total		83	85	4	172

Table 6.1 Comparison of diagnosis of chytridiomycosis by histology and skin scrapings in sick frogs. These results are from examination of 172 sick frogs that were submitted to AAHL for diagnosis and examined by both skin scrapings and histology. This table includes diagnoses made early in the project and so results are relevant for investigators with a range of skill levels.

The advantages of skin scrapings are that preparation of the slide is much quicker and cheaper than preparing a histological section, and it allows a non-invasive antemortem diagnosis. A disadvantage is that interpretation can be more difficult for an inexperienced worker. Suitable samples can usually be obtained from sick or freshly dead frogs, even if they have diseases other than chytridiomycosis. However, it can be difficult to collect sheets of epidermal cells from healthy frogs unless they happen to be undergoing a normal moult. Homogenous, mushy samples obtained from autolysed frogs, or when viable epidermis is scraped, are difficult to interpret. Although a larger surface area of skin can be checked by examination of skin scrapings compared to a 5 μm section, sporangia may be more difficult to identify.

6.6 Diagnosis by immunoperoxidase staining

This manuscript describes the production of polyclonal antibodies against *B. dendrobatidis* in rabbits and sheep, characterisation of the cross-reactivity to other fungal species, and production of an immunoperoxidase test. Preliminary comparison of the immunoperoxidase stain with H&E staining shows it is more sensitive in detecting light infections.

Berger, L., Hyatt, A. D., Olsen, V., Hengstberger, S., Boyle, D., Marantelli, G., Humphreys, K., Longcore, J. E. 2001. Production of polyclonal antibodies to *Batrachochytrium dendrobatidis* and their use in an immunoperoxidase test for chytridiomycosis in amphibians. Diseases of Aquatic Organisms (in press).

6.6.1 Abstract

Polyclonal antibodies were produced for diagnosing chytridiomycosis in amphibians. Two sheep and four rabbits were inoculated with homogenised whole culture of *Batrachochytrium dendrobatidis* in Freund's complete adjuvant or triple adjuvant. Antisera from all animals reacted strongly with all stages of *B. dendrobatidis* and stained the walls, cytoplasm, rhizoids and zoospores in an indirect immunoperoxidase test. Significant cross-reactivity occurred only with some fungi in the Chytridiomycota, and there are no known members of this phylum besides *B. dendrobatidis* that infect frogs. The immunoperoxidase stain is a useful screening test when combined with recognition of the morphology and infection site of *B. dendrobatidis*.

6.6.2 Introduction

Chytridiomycosis is a fatal disease of amphibians caused by the fungus *Batrachochytrium dendrobatidis*, the only member of the Chytridiomycota that causes disease in vertebrates (Berger et al., 1999). This fungus has a broad amphibian host range and occurs worldwide. A total of 94 amphibian species from 15 families have been found infected with *B. dendrobatidis,* from Australia, South America, Central America, North America, Europe, New Zealand and Africa (Speare et al., 2001). Chytridiomycosis is the most common disease of Australian frogs (Berger et al., 1999).

Although amphibian population declines are often due to habitat modification and multi-factorial causes (Alford and Richards, 1999), the suspected introduction of chytridiomycosis to wild amphibians is the most likely cause of many declines in protected areas (Berger et al., 1999).

Chytridiomycosis is a highly infectious disease that appears to have spread nationally and internationally, possibly by movement of infected animals. Diagnosis of infected amphibians will be an important aspect of quarantine regulations aimed at preventing the introduction of disease to wild or captive populations. Current diagnostic tests rely on knowledge of the morphology of the fungus for identification by histology or examination of wet mounts of skin scrapings. Most sick frogs with chytridiomycosis have heavy infections with *B. dendrobatidis* that are easily recognised by standard histopathological techniques (Pessier et al., 1999; Berger et al., 2000). However histological diagnosis is insensitive when dealing with light infections in healthy animals, autolysed samples, or when performed by inexperienced workers. Diagnostic methods with improved sensitivity and ease of testing are needed. The production of polyclonal antibodies and the introduction of an immunoperoxidase stain are the first steps in the development of more sophisticated tests for the detection of antigen.

6.6.3 Materials and methods

6.6.3.1 Antigen for immunisation

A culture of *Batrachochytrium dendrobatidis* (isolate A98 1810/3) was obtained from a sick, wild adult Australian lacelid (*Nyctimystes dayi*). The culture was maintained on tryptone, gelatin hydrolysate agar (TGhL) (Longcore et al., 1999) for 5 mo before use. Sporangia were harvested by lightly scraping a 10 day old culture. 30 mg of culture was mixed with 1 ml distilled water and left for 24 h at room temperature, then manually homogenised in a sterile petri dish and frozen at -80°C. After defrosting, the mixture was diluted with phosphate buffered saline (calcium and magnesium free) (PBSA) to a final concentration of 3.25 mg ml^{-1}. The protein concentration was determined using a Pyr Unicam PU8800 UV/VIS spectrophotometer at 280 nm, by interpolation of its absorbance from a standard curve calculated from known concentrations of bovine serum albumin (0.25, 0.5, 1.0 and 5.0 mg ml^{-1}). Freund's complete adjuvant and triple

adjuvant (Quil A, DEAE-dextran, Montanide 888 oil) (Than et al., 2001) were prepared using a Sorvall Omnimixer to emulsify. The final protein concentration of antigen in both preparations was 0.5 mg ml^{-1} antigen.

6.6.3.2 Immunisation

Two rabbits (666 & 667) and one sheep (322) were inoculated with triple adjuvant intradermally with boosters at 7 and 11 wk post inoculation (pi). Two rabbits (668 & 669) and one sheep (386) were inoculated with Freund's complete adjuvant subcutaneously and boosted with Freund's incomplete adjuvant at 7 and 11 wk pi. At each inoculation, the rabbits received 0.5 mg fungus in 1 ml adjuvant and the sheep received 1 mg fungus in 2 ml adjuvant.

At 13 wk pi, serum from all animals showed strong staining to *B. dendrobatidis* in the immunoperoxidase test (refer below), and at 15 wk pi animals were bled out under anaesthesia (AAHL animal ethics approval no. 97-797). Blood was collected into SST gel and clot activator vacutainers. It was stored at 4°C for up to 2 d before centrifuging at 3000 rpm (Beckman J-6 M centrifuge) for 3 min. Serum was stored in 10 ml sterile Starstedt tubes at -80°C.

6.6.3.3 Fungal isolates for cross reactivity testing

Specimens of *B. dendrobatidis* from Australia, Ecuador, New Zealand and Germany were tested with the antisera. Ten other chytridiomycetes and 18 fungi from other phyla causing animal infections (including fungi from frogs, reptiles and fish) were obtained from various collections (Table 6.2). Most fungi were in agar cultures, but some identifiable fungi within animal tissues were also used. Two protozoans in amphibian tissue were also tested - a myxozoan and a coccidian. Samples of agar or tissue were fixed in 10 % neutral buffered formalin, processed into paraffin blocks and sectioned for immunoperoxidase staining.

6.6.3.4 Immunoperoxidase (IPX) test

For indirect immunoperoxidase staining, paraffin sections were dewaxed and incubated for 20 min 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 (Shandon, England) and incubated with 200 µl of the anti-chytrid antisera at various

dilutions in 0.1 % skim milk powder/PBSA for 1 h 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 min at 37°C. Slides were rinsed with PBSA then incubated with 3 % H_2O_2 in distilled water for 20 min at room temperature (22°C) to block endogenous peroxidase activity. After rinsing in PBSA, slides were incubated with streptavidin peroxidase conjugated (DAKO LSAB kit) for 20 min at 37°C, rinsed again then removed from the Sequenza cassettes. The antigenic complex was visualized using an AEC (3-amino-9-ethyl carbasole)(Sigma, St. Louis, USA) chromogen system. The substrate and chromogen (freshly made AEC solution [2 mg AEC powder in 200 µl dimethyl formamide] added to 10 ml 0.05 M acetate buffer with 5 µl 30 % hydrogen peroxide) were added and incubated at room temperature for 5 min. After washing, slides were counterstained in Lillie's modified haemalum, blued in Scott's tap water, rinsed in tap water and mounted in an aqueous mounting medium.

Negative controls consisted of test slides incubated with normal sera, pre-bleed sera or 1 % skim milk instead of primary antibody. Sections of *B. dendrobatidis* culture (A98 1810/3) and sections of infected skin from a green tree frog (*Litoria caerulea*) (A99 1385/1) were used as positive control slides.

The IPX test was used to indicate the titre of antibodies from each animal (rabbits and sheep) and to characterise the cross-reactivity of antibodies with other fungi. These fungi are listed in Table 6.2.

6.6.3.5 Evaluation of the IPX test for diagnosis

Preliminary evaluation of the IPX as a diagnostic assay was conducted on 55 lightly infected and 15 control toe-clip samples from an experimental infection using juveniles of *Litoria caerulea*. The toe-clips were collected 19 d after frogs were exposed to 50,000 zoospores. All infected frogs eventually died with chytridiomycosis between 24 and 67 d post exposure. Sections of toe-clips were stained using antiserum from rabbit 667 at 1:1000. The results from the IPX test were compared with results from haematoxylin and eosin (H&E) staining. Sections of a naturally infected wild tadpole of *Mixophyes fasciolatus* were tested with antisera from all animals.

6.6.3.6 Fluorescence staining

For direct fluorescence staining, cultured *B. dendrobatidis* grown on glass slides were incubated with rabbit 667 antiserum at 1:100 in 0.1 % BSA/PBSA for 30 min at 37°C. After washing in PBSA, slides were incubated with 1:100 anti rabbit IgG conjugated with FITC (Silenus, Boronia, Australia) for 30 min at 37°C. They were then washed and mounted in glycerol. Slides were examined with a Reichert–Jung (Leica) Polyvar microscope with a tungsten lamp and a filter for fluorescence.

6.6.3.7 Electron microscopy

Broth cultures of *B. dendrobatidis* were pelleted in 1.5 % low gelling temperature agarose (Type VII) (Sigma, St. Louis, USA) in 0.1 M cacodylate buffer (pH 7.2, 300 mosmol kg^{-1}) and fixed in 0.25 % (v/v) buffered glutaraldehyde for 40 min. The samples were then processed as described by Hyatt (1991) and embedded in LRW. Ultra-thin sections were cut with a Reichart-Jung (Leica) Ultracut E and immuno-gold labelled (Hyatt, 1991) with rabbit (666) anti *B. dendrobatidis* (1:1000). Sections were then double stained with uranyl acetate and lead citrate prior to examination with a Philips CM 120 at 100 kV.

6.6.4 Results

Antisera from all animals reacted strongly with isolates of *Batrachochytrium dendrobatidis* when used at dilutions between 1:100 and 1:1600 in the indirect IPX test on sections of culture and of infected skin. All stages of *B. dendrobatidis* were stained, and the walls, cytoplasm, septa, rhizoids and internal zoospores were highlighted (Figs. 6.10, 6.11, 6.12). There was little, or no, background staining of amphibian skin.

When titrated at doubling dilutions from 1:100, serum reactivity began falling at 1: 1600. Antisera from rabbit 669 and sheep 386 showed no staining at 1:12,800, while antisera from the other animals still had weak staining at 1:25,600 (Table 6.3). The sheep and one of the two rabbits inoculated with Freund's adjuvant had lower titres of antibody than the three animals given triple adjuvant.

Sera collected from rabbits prior to vaccination showed some distinct, although minimal, staining of cultured *B. dendrobatidis* when used at 1:100 but not at 1:500. Sera

collected from sheep prior to vaccination showed some staining at 1:100 and 1:500 but not at 1:1000. It was not determined if this staining was non-specific or due to pre-existing antibodies in the animals.

There was cross-reactivity between antisera and a large number of fungi (Table 6.4). Significant staining is represented by >2+ in the table. The strongest staining occurred with fungi in the Chytridiomycota, but reactions also occurred with fungi in other phyla. Staining of other fungal species was sometimes limited to one or two distinct structures e.g. zoospores, rhizoids, cytoplasm, or cell walls, demonstrating that these fungi had different antigens in common with *B. dendrobatidis*. When used at 1:100, antisera cross-reacted with between 6/10 and 9/10 other chytridiomycetes. Most of this cross-reactivity disappeared when the antisera were used at 1:1000, although a *Karlingiomyces* sp. (no. 93) and an undescribed isolate known as "multiple axes" (no. 142) were still stained by rabbit antisera. Less cross-reactivity occurred with fungi from other phyla; antisera from rabbits and sheep reacted with between 3/18 and 8/18 fungi when used at 1:100, but staining was negligible at 1:1000. The two protozoans were not stained. Fungi that cross-reacted at 1:1000 were also incubated with antisera diluted out to 1:2000, but they were still stained (data not shown).

Evaluation of the stain on toe-clips from experimentally infected frogs showed the IPX test was more sensitive than H&E staining for diagnosis of chytridiomycosis. With the IPX test, 34/55 (61.8 %) frogs tested positive, compared with 29/55 (52.7 %) positives and 3 suspicious positives with H&E staining. Fifteen controls were negative by both methods. On re-examination of three negative and two suspicious H&E sections that were positive by immunostaining, a few sporangia could be recognised in the sections - these were mostly solid, immature stages within viable epidermal cells and were difficult to differentiate from cell nuclei. The stain was also effective in highlighting infection in the mouth of a tadpole (Fig. 6.12).

Fluorescence staining of cultured fungi resulted in strong staining of the surface of sporangia, and rhizoids were clearly outlined. Transmission electron microscopy of ultra-thin sections showed sporangia and zoospores to be gold labelled (Fig. 6.13). Labelling was strongly associated with the inner aspect of the sporangial wall, the limiting membrane of the zoospores, flagella and the contents of the zoospore vacuoles.

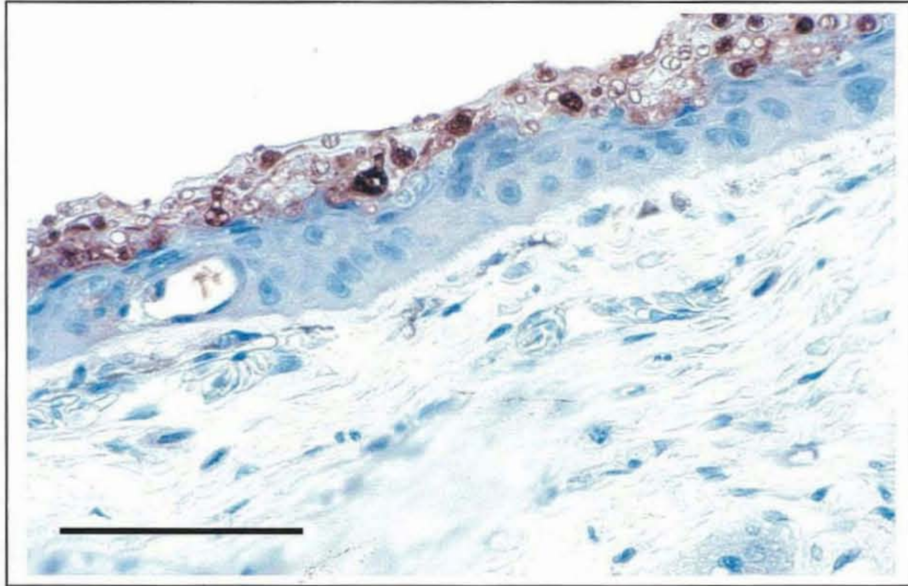

Figure 6.10 Immunoperoxidase stain on skin of a *Litoria caerulea* with a heavy infection of *B. dendrobatidis*. There is strong staining of fungal walls, cytoplasm, zoospores and septa. Antiserum from rabbit 667 was used at 1:1000. Scale bar = 80 μm.

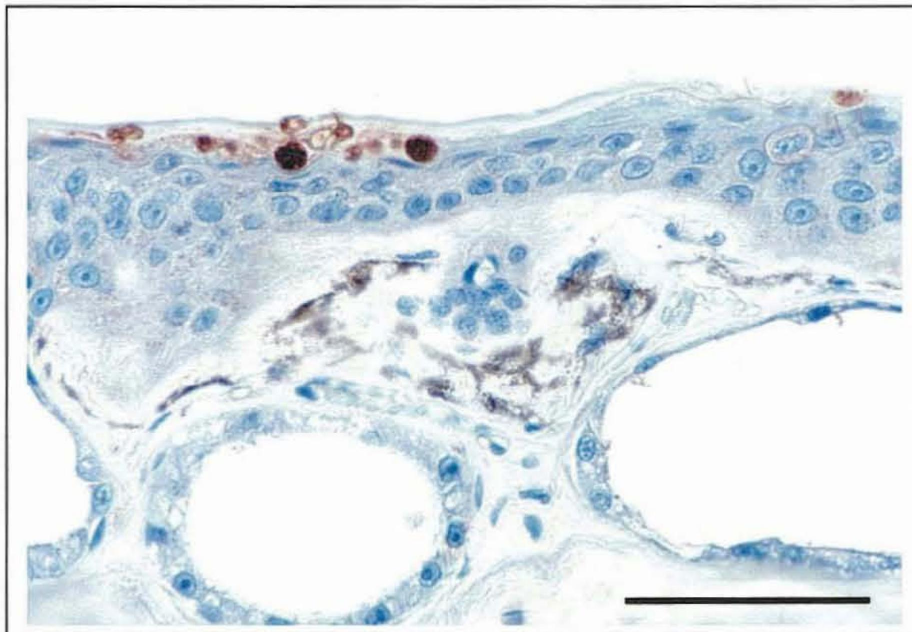

Figure 6.11 Immunoperoxidase stain on skin of a *Litoria caerulea* with a light infection of *B. dendrobatidis*, demonstrating the sensitivity of the test in highlighting a few sporangia. Scale bar = 80 μm.

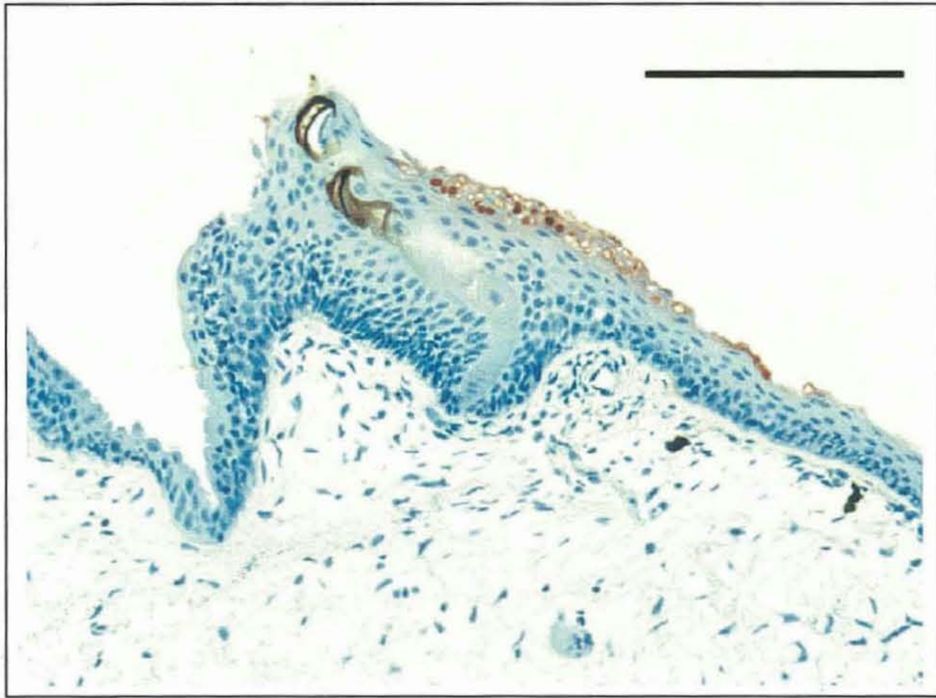

Figure 6.12 Immunoperoxidase stain on the mouth-parts of a tadpole of *Mixophyes fasciolatus* infected with *B. dendrobatidis*. Scale bar = 200 µm.

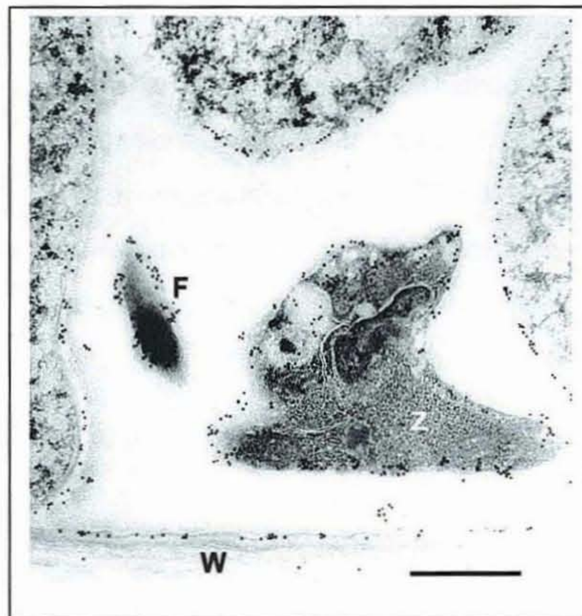

Figure 6.13 Electron micrograph of a gold-labelled cultured zoosporangium, using polyclonal antiserum from rabbit 666. Note dense gold particles adhering to the inner edge of the wall (W), the membrane of the zoospore (Z), and the surface of the flagellum (F). Scale bar = 500 nm.

6.6.5 Discussion

The antisera that were produced in this study will be valuable in improving diagnostic screening assays for the detection of *Batrachochytrium dendrobatidis* in amphibians. Although there was some cross-reactivity with other fungi, the antisera did not cross-react with other fungi known to infect amphibians. When the rabbit antisera were diluted out to 1:1000, apart from two chytridiomycetes, only *B. dendrobatidis* remained strongly stained. As structures of other fungi were still stained at the higher dilution the antibodies cannot be considered as species specific. The only other spherical fungus reported to infect amphibian skin in Australia is *Mucor amphibiorum* (Speare et al., 1997) and this fungus was not stained. The two fungi that cross-reacted strongly are chytridiomycetes, none of which are known to infect amphibians. One of these was the undescribed chytrid labelled "multiple axes" (JEL 42) which groups most closely with *B. dendrobatidis* in some 18S rDNA phylogenetic analyses (James et al., 2000). The other strongly stained species, *Karlingiomyces* sp. (JEL 93), is phylogenetically distant in analyses of 18S rDNA sequence data.

Polyclonal antibodies are usually cross-reactive among fungal species, and even monoclonal antibodies (MAbs) are often not specific (Fenelon et al., 1999). Carbohydrates and glycoconjugates on cell walls may be highly antigenic and have epitopes common to a range of species (Gabor et al., 1993). Electron microscopy of gold-labelled sections demonstrated that our polyclonal antibodies were directed mainly to the sporangial wall and zoospore membrane. Preparations containing only internal structures have been found to generate MAbs that are more species specific (Gabor et al., 1993).

The polyclonal antibodies we produced are a basis for further work and the IPX test may be useful for detecting or confirming infection with *B. dendrobatidis* in frog skin. Histological methods may be less sensitive than other methods (e.g. ELISAs) as only small skin samples, such as toe-clips, can be collected ante mortem. Furthermore, sections contain just a thin strip of skin and may miss any sporangia. The IPX test can be used instead of, or as an adjunct to, H&E staining when increased sensitivity of testing is required, such as for importation of amphibians. It is useful for screening toe-clip samples from healthy frogs where only a few sporangia may be present, and these

can be easily seen even at low magnification. The stain is also useful for testing necrotic or autolysed samples from sick frogs that may contain few distinctive sporangia. In addition, the ease of interpretation of the assay permits scientists other than specialists (e.g. pathologists and mycologists) to diagnose infection. The benefits of increased sensitivity and quicker examination of slides stained by the IPX test must be weighed against the greater complexity of the staining method, which takes about four hours.

It is recommended that the rabbit antisera are used at 1:1000 for immunostaining, and that interpretation of results includes examination of stained structures for the characteristic appearance of *B. dendrobatidis* (Berger et al., 1998; Pessier et al., 1999).

The data reported in this paper are part of a research effort into improving the diagnostic capability for the detection of chytridiomycosis (Hyatt et al., 2000). The immunostaining protocol is the first reported assay for the detection of *B. dendrobatidis* antigens. The polyclonal antibodies are currently being used in the development of an indirect ELISA. Monoclonal antibodies to *B. dendrobatidis* are being evaluated for use in more specific tests. At present it is not known which type of antibody or test will prove to be the most useful for detection of *B. dendrobatidis* in amphibians, but the immunoperoxidase test described here using polyclonal antibodies can now be used for improved diagnosis.

6.6.6 Acknowledgements

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Table 6.2 List of fungal species used, their strain identification numbers, the order and phylum they belong to, and their source.

Species	Strain identification *	Order	Phylum	Source
Batrachochytrium dendrobatidis	A 98 1810/3	Chytridiales	Chytridiomycota	Australian lacelid (*Nyctimystes dayi*)
Chytriomyces angularis	JEL 45	Chytridiales	Chytridiomycota	Pollen bait
Diplochytridium lagenarium	JEL 72	Chytridiales	Chytridiomycota	*Oedogonium* sp. (algae)
Karlingiomyces sp.	JEL 93	Chytridiales	Chytridiomycota	Snakeskin bait
Powellomyces sp.	JEL 95	Spizellomycetales	Chytridiomycota	Pollen bait
Rhizophydium sp.	JEL 136	Chytridiales	Chytridiomycota	Pollen bait
Multiple axes	JEL 142	Chytridiales	Chytridiomycota	Onion skin bait
Rhizophydium sp.	JEL 151	Chytridiales	Chytridiomycota	*Lyngbya* sp. (algae)
Gonapodya sp.	JEL 183	Monoblepharidales	Chytridiomycota	*Lemna* sp.
Asterophlyctis sarcoptoides	JEL 186	Chytridiales	Chytridiomycota	Shrimp chitin bait
Allomyces macrogynus	JEL 204	Blastocladiales	Chytridiomycota	Snakeskin bait
Fusarium culmorum	T 801/99	Hypocreales	Ascomycota	Blotched blue-tongued lizard (*Tiliqua nigrolutea*)
Phaeoacremonium parasiticum	T 819/99	Moniliales	Ascomycota	Bearded dragon (*Pogona barbata*)
Fusarium oxysporum (1)	T	Hypocreales	Ascomycota	Blotched blue-tongued lizard (*Tiliqua nigrolutea*)
Fusarium oxysporum (2)	T 801/99	Hypocreales	Ascomycota	Blotched blue-tongued lizard (*Tiliqua nigrolutea*)
Trichophyton terrestre	T 1067/00	Onygenales	Ascomycota	Blue-tongued lizard (*Tiliqua scincoides*)
Paecilomyces lilacinus	T 1618/1	Eurotiales	Ascomycota	Yellow anaconda (*Eunectes notaeus*)
Penicillium sp.	T 1440.1	Eurotiales	Ascomycota	Green and gold bell frog (*Litoria aurea*)
Candida sake	T 1103.1	Saccharomycetales	Ascomycota	Green and gold bell frog (*Litoria aurea*)
Fusarium sp.	T 1670.1	Hypocreales	Ascomycota	Gouldian finch (*Erythrura gouldiae*)
Gliocladium sp.	T 1212	Hypocreales	Ascomycota	Murray cod (*Maccullochella peelii*)
Verticillium sp	T 1212	Moniliales	Ascomycota	Murray cod (*Maccullochella peelii*)
Chrysosporium sp.	T 1333	Onygenales	Ascomycota	Hosmer's skink (*Egernia hosmeri*)
Acremonium sp.	T 1697.1	Hypocreales	Ascomycota	Diamond python (*Morelia spilota*)

Scopulariopsis brevicaulis	T 1258	Microascales	Ascomycota	Black-headed python (*Aspidites melanocephalus*)
Trichoderma viride	T 1344	Hypocreales	Ascomycota	Freshwater crocodile (*Crocodylus johnstoni*)
Monochaetia sp.	T 1226	Melancoliales	Ascomycota	Little penguin (*Eudyptula minor*)
Mucor amphibiorum (tissue)	A 96 1429/3	Mucorales	Zygomycota	Cane toad (*Bufo marinus*)
Aphanomyces sp. (tissue)	A 95 517	Saprolegniales	Oomycota	Cane toad (*Bufo marinus*)
B. dendrobatidis (tissue)	N 30916-00B	Chytridiales	Chytridiomycota	Southern bell frog (*Litoria raniformis*)
B. dendrobatidis (tissue)	E QCAZ 3691	Chytridiales	Chytridiomycota	*Atelopus* sp.
B. dendrobatidis (tissue)	G 99 1743/5	Chytridiales	Chytridiomycota	Yellow–lined poison dart frog (*Phyllobates lugubris*)
Myxidium immersum (tissue)	A 96 570/1	Bivalvulida	Myxozoa	Stony creek frog (*Litoria lesueuri*)
Goussia-like coccidian (tissue)	A 01 95/4	Eimeriidae	Apicomplexa	Great barred frog (*Mixophyes fasciolatus*)

*JEL = from the chytrid collection of Joyce Longcore, University of Maine, America
T = from Kaye Humphreys and Karrie Rose, Taronga Zoo, Australia
A = from Lee Berger, AAHL, Australia
N = from Bruce Waldman and Richard Norman, Massey University, New Zealand
E = from Andrés Merino-Viteri, Museo de Zoologia, Quito-Ecuador
G = from Frank Mutschmann, Tierarztpraxis, Berlin, Germany

Table 6.3 Intensity of staining in the immunoperoxidase test using various concentrations of antisera against *Batrachochytrium dendrobatidis.* Animals 666, 667 and 322 were inoculated with triple adjuvant, animals 668, 669 and 386 were inoculated with Freund's adjuvant. At higher dilutions, cell walls faded before zoospores.

Dilution	Rabbit 666	Rabbit 667	Rabbit 668	Rabbit 669	Sheep 322	Sheep 386
1:400	+++++	+++++	+++++	+++++	+++++	+++++
1:800	+++++	+++++	+++++	++++	+++++	++++
1:1600	++++	++++	++++	+++	+++++	++++
1:3200	+++	+++	++++	++	++++	++
1:6400	+++	+++	+++	++	+++	+
1:12800	+	++	++	-	++	-
1:25600	+	+	+	-	+	-

Table 6.4 Reactions of antisera with fungi in the immunoperoxidase test. Key: 1+ = ill-defined pale staining, 2+ = minimal staining of some elements, 3+ = moderate staining of some or all elements, 4+ = strong staining of some elements, 5+ = strong staining all elements. ND = not done.

Species	Rabbit 666			Rabbit 667			Rabbit 668			Rabbit 669			Sheep 322			Sheep 386		
	1:100	1:500	1:1K	1:100	1:500	1:1K	1:100	1:500	1:1K	1:100	1:500	1:1K	1:100	1:500	1:1K	1:100	1:500	1:1K
B. dendrobatidis	+++++	+++++	+++++	+++++	+++++	+++++	++++	+++++	++++	+++++	++++	++++	+++++	+++++	++++	++++	++++	+++++
Chytriomyces angularis	+++	++	-	+	-	ND	+++	+++	-	++++	-	ND	+++	++	-	+++	+	-
Diplochytridium lagenanum	++++	+++	++	++++	+	-	+++	+	+	++++	+++	++	+++	++	-	++++	+++	+
Karlingiomyces sp.	++++	++++	+++	++++	++++	+++	+++++	+++	+++	++++	+++	+++	++++	++	++	++++	++	++
Powellomyces sp.	+	+	-	++	-	-	+++	-	-	++	-	-	++	+	+	++	+++	+++
Rhizophydium sp.	+++	-	-	++	-	-	+++	+	+	++	-	-	++	++	++	++	+	-
Multiple axes	+++++	++++	+++	+++++	++++	++++	++++	++++	++++	+++++	++++	+++	+++++	+++++	++++	+++	++++	+++
Rhizophydium sp.	+++	++	++	+++	-	-	++++	+	+	+++	-	-	++++	+++	++	+++	+++	++
Gonapodya sp.	+++++	++++	++	++++	+++	++	+++++	++++	++	++++	++	++	++++	++++	++++	++++	+++	+
Asterophlyctis sarcoptoides	+	+	-	++	+	-	++++	+++	+	+++	-	-	+++	-	++	+++	++	+
Allomyces macrogynus	++	-	ND	+++	-	ND	++	++	+	++	-	ND	++	+	ND	+++	++	ND
Subtotal of staining >++	7	4	2	6	3	2	9	5	2	7	3	2	7	3	2	8	5	2
Fusarium culmorum	-	ND	ND	-	ND	ND	-	ND	ND	-	ND	ND	+	-	ND	+	-	ND
Phaeoacremonium parasiticum	-	ND	ND	-	ND	ND	-	ND	ND	-	ND	ND	-	ND	ND	+	-	ND
Fusarium oxysporum (1)	++	++	+	+	+	ND	+	-	ND	+	+	ND	+	+	ND	++	++	+
Fusarium oxysporum (2)	-	ND	ND	-	ND	ND	-	ND	ND	-	ND	ND	+	-	ND	+	-	ND
Trichophyton terrestre	+++	+	+	+++	-	ND	+++	++	-	+	-	ND	++	-	ND	++++	-	ND
Paecilomyces lilacinus	+++	+	-	++++	++	+	++	-	ND	+++	-	ND	++	-	ND	+++	++	-
Candida sake	+	+	-	++	-	ND	++	+	ND	++	+	-	-	-	ND	++	+++	+
Penicillium sp.	+++	-	ND	++	-	ND	+++	-	ND	+	-	ND	++	-	ND	++	-	ND
Fusarium sp.	+	-	ND	-	-	ND	-	ND	ND	+++	-	ND	++	-	ND	+++	-	ND

Gliocladium sp.	+	-	ND	+	-	ND	++	-	ND	-	ND	ND	++	+	ND	++++	++	ND
Verticillium sp	+++	+	-	+++	++	+	+++	++	+	+++	++	+	+++	++	+	++++	+	+
Chrysosporium sp.	++	+	+	++	++	-	+++	++	+	++	+	-	+++	+++	+++	+	+	-
Acremonium sp.	+	-	ND	++	-	ND	+	-	ND	-	ND	ND	-	ND	ND	++	-	ND
Scopulariopsis sp.	+++	+	-	++	-	ND	++	-	ND	++	+	-	-	ND	ND	+++	-	ND
Trichoderma sp.	++++	++	-	++++	-	ND	++	-	ND	++++	++	-	-	ND	ND	++++	+	+
Monochaetia sp.	++	-	ND	+	-	ND	++	-	ND	++	-	ND	-	ND	ND	++++	+	-
Aphanomyces sp.	-	ND	ND	-	ND	ND	-	ND	ND	-	ND	ND	-	ND	ND	-	ND	ND
Mucor amphibiorum	+	-	-	+	-	-	+	-	-	++	-	-	+++	-	-	+	-	-
Goussia sp.	-	ND	ND	-	ND	ND	-	ND	ND	-	ND	ND	+	ND	ND	-	ND	ND
Myxidium immersum	-	ND	ND	-	ND	ND	-	ND	ND	-	ND	ND	-	ND	ND	-	ND	ND
Subtotal of staining >++	6	0	0	4	0	0	4	0	0	4	0	0	3	1	1	8	1	0
Total staining >++	13	4	2	10	3	2	13	5	2	11	3	2	10	4	3	16	6	2

6.7 Conclusion on diagnostic techniques

All three methods of diagnosis using light microscopy are useful, although they have various advantages and disadvantages (Table 6.5).

Table 6.5 Comparison of the characteristics of each test.

Type of test	Skin scrapings	Histology	Immunostaining
Complexity and cost of preparation	*	**	***
Ease of interpretation	*	**	***
Sensitivity	**	**	***

The choice of test depends on a few criteria - whether the frog is ill or healthy, sensitivity required, time and facilities available, and the experience of the tester.

Ill frogs with chytridiomycosis usually have heavy infections that are easily seen by skin scrapings and histology, and sensitivity is not usually a concern. The rapidity of diagnosis by examination of skin scrapings is an advantage. Histology may be necessary to confirm light infections and to check internal organs for concurrent diseases. Occasionally immunoperoxidase staining is useful for sick frogs with ulcerations or autolysis when few distinctive sporangia are present and other tests have both resulted in unconfirmed diagnoses. Skin scrapings are also useful for ante mortem testing as sick frogs often have accumulations of shedding skin on the body and collecting this skin is less invasive than taking a toe-clip for histology. For testing healthy frogs, it can be difficult to obtain shedding skin, so toe-clips are collected for histology.

The immunoperoxidase test results in a slightly increased chance of detecting a positive slide compared with H&E staining, and is particularly useful for detecting light infections. A few immature sporangia in a healthy epidermis may be overlooked with H&E staining but are obvious with the immunoperoxidase stain. The immunoperoxidase stain is recommended for situations where it is important not to miss an infected animal, for example when testing frogs before translocation.

Although histological examination of toe-clips is currently the most useful test for live, healthy animals, it was found to be insensitive in detecting light infections of *B. dendrobatidis*. Using the immunoperoxidase stain, infections in 61.8% of experimentally infected frogs were detected at 19 days post exposure, while 52.7% were detected using H&E staining (Section 6.6).

For an inexperienced person involved in testing frogs, it is easier to recognise sporangia by routine histology or by immunoperoxidase staining.

Although the immunoperoxidase test is a longer and more complicated method, its effectiveness in highlighting sporangia enables slides to be screened much more rapidly.

Tests that do not rely on experience with microscopy will be available soon (Hyatt et al., 2000). An indirect ELISA is being developed using the polyclonal antibodies (described in section 6.6) and new monoclonal antibodies. The ELISA is useful for testing large numbers of frogs in any laboratory. The use of PCR is being investigated at AAHL for more sensitive diagnosis. We also hope to develop an environmental test that will detect zoospores in water bodies.

CHAPTER 7

Investigation of the pathogenesis of chytridiomycosis using experimental transmission

7.1 Introduction

The first experimental transmission of *B. dendrobatidis* was conducted before the fungus had been described or cultured, and was accomplished by using fresh skin scrapings from an infected frog (Berger et al., 1998; this chapter). As all six infected *M. fasciolatus* died, this demonstrated that chytridiomycosis was a fatal disease and indicated that more detailed investigations were warranted. Since the development of methods for culturing *B. dendrobatidis* (Longcore et al., 1999), infection using pure culture has also been shown to cause high mortality rates. Longcore et al. (1999) and Nichols et al. (2001) transmitted chytridiomycosis to dendrobatid frogs, which died between 13 and 31 days post-exposure, and we have used different doses of zoospores to infect two Australian species - *M. fasciolatus* and *L. caerulea* (Berger et al., 1999a; this chapter).

As outbreaks of chytridiomycosis occur more commonly in winter (Berger et al., 1999a; Chap 8) and frogs at higher altitudes have undergone the most severe declines (McDonald and Alford, 1999) it appeared likely that the disease was enhanced by lower temperatures. *In vitro* studies of an isolate of *B. dendrobatidis* showed that 23°C produced the most rapid growth which was reduced at higher temperatures with no substantial growth occurring at 29°C, and death occurring at 30°C and above (Longcore et al., 1999; Chap 9). The fungus grew well at 15°C and grew slowly at 6°C (Longcore et al., 1999; Longcore, pers comm 2000). Immunity in poikilotherms is often reduced at lower temperatures, so frogs may be more susceptible to infectious disease (Maniero and Carey, 1997). This has been documented in fish succumbing to winter saprolegniasis after cold spells (Bly et al., 1993). To determine how pathogenicity is ultimately affected by the temperature effects on *Batrachochytrium* and on the amphibian host, we infected frogs at 17°C, 23°C and 27°C.

Experiments reported in this chapter were conducted with Gerry Marantelli and staff at the ARC, to investigate the pathogenicity of *B. dendrobatidis* and how this varies with dose, temperature, and fungal isolate. Additional information on the temporal aspects of the disease, such as incubation times and progression of clinical signs, were also obtained.

7.2 Methods

Although we attempted to maintain consistency between experiments, significant variations were unavoidable. Besides the obvious change from using skin scrapings to using cultured fungus for infections, we also used different isolates of different passage number so that the most recent, and potentially less attenuated, strain was always used. The different frog species behaved differently and required different enclosure designs and methods of exposure. Three experiments were conducted in rooms in the large animal facility (LAF) at AAHL, and four were conducted at the Amphibian Research Centre (ARC). Enclosures and substrates used at AAHL were obtained from the ARC.

For the experiments at ARC, I prepared the zoospores, infected the frogs and did the diagnostic work, while the husbandry, observations, euthanasia and preservation of specimens were done by Gerry Marantelli, Raelene Hobbs and other staff at the ARC.

7.2.1 Animals

All frogs (but not the toads) used in experiments were captive-bred juveniles from the ARC that can be considered to be specific pathogen free. Juveniles were used instead of adults, as large numbers of similar young animals (sibling groups) with a known history were available. These were kept under strict quarantine, had no unusual mortality, and any unexplained deaths were tested for *B. dendrobatidis*.

7.2.1.1 *Mixophyes fasciolatus*

Juveniles

M. fasciolatus (Myobatrachidae) were originally chosen as a model species (Fig. 7.1). *M. fasciolatus* are a fossorial species and occur in rainforest and wet sclerophyll forest in southeast Queensland and eastern NSW. They are closely related to the declining

species *M. fleayi*. They are large frogs and adults are about 80 mm long, but the metamorphs and juveniles used in these experiments were between 30 and 46 mm. In captivity they usually burrow under gravel or leaves during the day (Fig. 7.2).
They were known to be highly susceptible to chytridiomycosis after an outbreak in 1996-1997 decimated a captive spawning group (Berger et al., 1998). In captivity at the ARC they are easily produced in large numbers, and were bred from wild caught adults that were originally collected from lowland areas at Mount Glorious in southeast Queensland.

The *M. fasciolatus* were housed in separate 40x28x16 cm plastic tubs (Decor 670 storage bins) with mesh lids. About 1.5 litres of 7 mm diameter gravel in one half provided a dry area and plastic aquarium leaves provided shelter (Fig. 7.3). Enclosures were initially disinfected with 0.2% hypochlorite for 10 minutes. Each tub contained 800 ml water. Aged tap water or softened water was used at AAHL, whereas tap water direct from the reticulated system was used at the ARC. Frogs were fed 5-7 mm cultured crickets two to three times a week *ad lib*. Crickets were fed dry dog food and were dusted with vitamins and minerals (Repcal Calcium with vitamin D3, plus Herptivite Multivitamins [Rep-Cal Research Laboratories, Los Gatos, California] combined in equal parts). The tubs had a row of drainage holes at one end, and tubs were cleaned by flushing through water and removing faeces. The number of water changes varied with the experiment (see below).

Tadpoles

Tadpoles of *M. fasciolatus* were housed in individual 1.4 litre clear plastic tubs containing tap water. The water was not changed for two days after exposure to zoospores, then an automatic water flow system produced a flow of 2 litres/hour for 4 hours a day. Tadpoles were fed frozen endive *ad lib*. As they reach up to about 30 mm in body length, they are an easy tadpole species to work with, and appropriate sections of mouthparts are consistently obtained for histological detection of *B. dendrobatidis*.

Figure 7.1 Healthy metamorph of *Mixophyes fasciolatus*. The common name is "great barred frog" due to the bars on the hindlimbs.

Figure 7.2 Captive metamorphs of *Mixophyes fasciolatus* usually hide during the day by burrowing in gravel or under leaves. They may be completely submerged in the gravel.

Figure 7.3 A tub used for housing *Mixophyes fasciolatus*, with gravel and plastic leaves at one end.

7.2.1.2 *B. marinus*

B. marinus (Bufonidae) were introduced to Australia in 1935 and are still expanding their range (Freeland, 1985). They were known to be susceptible to chytridiomycosis as a high mortality rate occurred during an outbreak in captive metamorphs at AAHL (Berger et al., 1998). Fourteen out of 63 juveniles in the same room also died with chytridiomycosis at this time but none of the adults kept nearby became infected (H. Parkes, unpub 1996).

Metamorphs used in the experiment were 1 to 4 weeks post metamorphosis and were about 12 mm long. They had been bred from wild caught adults by Michael Tyler at the Department of Zoology, Adelaide University, and sent to AAHL as tadpoles.

Toads were housed in 10 litre mouse trays with 6 mm thick perspex sheets with 12 holes drilled in them as lids. A strip of adhesive foam seal (Raven products) was placed around the top edges of the tray to block any gaps. Black polypipe of 10 cm diameter was cut in half longitudinally and used as shelter. A flat plastic dish containing 125 ml softened tap water was placed in each tub. The toads were fed everyday with hatchling crickets and vestigial-winged (i.e. flightless) drosophila, supplemented with calcium and vitamins. Everyday, faecal matter was removed and the water was topped up. Every second day, toads were removed, the tubs were rinsed thoroughly and half the water in the dish was replaced.

7.2.1.3 *Lim. peronii*

Lim. peronii (Myobatrachidae) are a common frog on the east coast and inhabit permanent ponds or slow moving streams. They grow to about 65 mm long (Cogger, 1992). This species has not declined and only one wild frog has been found dying from chytridiomycosis (Chap 8). Juveniles were used in the experiments that were between 19 and 25 mm. They were housed and fed the same as the *M. fasciolatus*.

7.2.1.4 *L. caerulea*

L. caerulea (Hylidae) occur across the northern and eastern half of Australia (Cogger, 1992). They occur in a large variety of coastal and inland habitats and are the most common frog collected from the wild dying from chytridiomycosis (Chap 8). They

grow to about 100 mm. In captivity they usually sit high on the enclosure walls during the day.

Juvenile frogs (~20 mm) were used that had been bred from "banana box frogs" from Queensland. They were housed individually in clear plastic 1.4 litre tubs (Hagen mini PalPens) with 7 mm diameter gravel at one end. Frogs were fed crickets twice a week. Automatic drippers flushed through 2 litres of water daily which ran out into gutters. Tubs were placed side-by-side on shelves separated by plastic sheeting. This arrangement with the automated water flow was highly effective in preventing cross contamination between containers, as evidenced by the lack of infection in the 15 control frogs in experiment 6 that were randomly placed among infected frogs (see below).

7.2.2 Collection and preparation of *B. dendrobatidis*

For the first two experiments, fresh skin scrapings were collected from a *M. fasciolatus* metamorph (96 1431/52) that died during the outbreak at the ARC described originally (Berger et al., 1998; Chap 5). Skin scrapings were stored in PBSA with penicillin and streptomycin for two days at 4°C. The number of sporangia in pieces of shedding skin was estimated by counting with a compound microscope at 400X magnification. Different stages of development were not differentiated, although many sporangia had already released their zoospores. One group of controls received 1 ml PBSA that had stored the skin sloughs and had been filtered through a 0.45 μm filter, and the second group of controls was untreated.

In subsequent experiments, cultured *B. dendrobatidis* was used for infections. Isolates were obtained from sick frogs by culturing on TGhL agar (Longcore et al., 1999; Chap 3). Three strains were used in these experiments - strain 98 1469/10 cultured from a captive bred metamorph of *L. dumerilii* from the ARC in December 1998, strain 99 1385/12 cultured from a wild adult of *L. caerulea* from Rockhampton in August 1999, and strain 00 545 cultured from a wild-caught captive metamorph of *L. lesueuri* from the ARC in March 2000. Cultures were maintained in TGhL broth at 4°C and were passaged every 2-3 months.

Zoospores (the infective stage) were collected from 4-5 day old agar cultures, prepared from actively growing 1-2 week old broth cultures. At this stage in culture development, colonies are surrounded by masses of actively swarming, recently released zoospores. Zoospores were harvested by flooding plates with distilled water for 5 minutes, then removing water by pipette at a spot distant to any colonies of sporangia. The concentration of zoospores was counted in a haemocytometer. They were counted unstained and live, as their movement aids in recognition. Zoospores collected from one or two plates were typically diluted in about 30 ml distilled water to achieve an appropriate dilution for infections. The zoospore suspension was stored in plastic tissue culture flasks and kept below 20°C until frogs were infected. For the experiments conducted at the ARC, the flasks were kept in an esky containing a well-insulated ice block. We attempted to minimise the time between collection of zoospores and exposure of the frogs, which occurred up to 6 hours later. The motility of zoospores was confirmed by microscopic examination immediately before infections were conducted. For treatment of control frogs, a sterile agar plate was rinsed with distilled water that was kept in a flask.

The survival of zoospores in distilled water at 4°C and 23°C was investigated before the first experiment with cultured fungus. Most zoospores kept in distilled water in plastic petri dishes were still active after 12 hours but few were motile after 24 hours, although more were motile at 4°C than at 23°C. This meant that although it was preferable to expose frogs to freshly collected zoospores, a few hours were available to transport zoospores to the ARC. Based on these results we chose to expose frogs to zoospores for 24 hours.

7.2.3 General experimental design

The general methods of infection, observation, euthanasia and sample analysis are described here. Details of experimental procedures that differed in each experiment are described in the next section where each experiment is described separately.

Frogs were randomly assigned to size-matched treatment or control groups. They were acclimatised to the experimental enclosures for various times, depending on the experiment.

In experiments 1 and 2, skin scrapings were added to the water in the tubs. As the lifecycle and infective stage were unknown, an indefinite period of exposure was selected. For subsequent experiments with cultured zoospores, frogs were exposed for 24 hours by placing them in individual plastic containers with holes punched in the lids. *M. fasciolatus* and *Lim. peronii* were exposed in 10 ml distilled water in 90 x 85 mm translucent round-bottom take-away containers, and *L. caerulea* were exposed in 5 ml distilled water in 45 mm x 50 mm yellow top specimen containers (Labserv). Containers were kept in the dark to reduce stress and then frogs were returned to their usual individual enclosures. Control frogs were treated the same but with water washed off a clean agar plate. Each tub was kept completely separate - disposable latex gloves were changed between every tub and separate equipment was used for each tub. For experiments at AAHL, infected and uninfected groups were kept at opposite ends of the room. At the ARC, control and infected frogs were placed randomly on shelving.

Animals were housed in individual tubs except for the *B. marinus* (experiment 2) and the *Lim. peronii* (experiment 5).

All animals were checked daily for signs of disease, such as changes in behaviour and in appearance of skin. Pieces of shedding skin were collected for testing when seen on frogs or if present in the enclosure.

Frogs were euthanased when clinical signs of disease became obvious, to reduce suffering. Lethargy and a delayed righting reflex were considered to indicate that death was certain. Euthanasia was performed by bathing frogs for a few minutes in about 1 cm deep 0.2% MS 222 (tricaine methanesulfonate) (Ruth Consolidated Industries, Annandale, Australia). As there is usually a short period of time (< 2 days) from onset of obvious clinical signs until death, euthanasia is not likely to affect the results significantly. In the results, frogs are described as having "died" whether they were euthanased or died naturally.

For experiments conducted at AAHL, animals were post-mortemed as described in Chapter 3. Briefly, frogs were weighed, and their snout-vent-length measured. Any lesions in skin and internal organs were described. Shedding skin and samples from organs that were collected using aseptic techniques were frozen, and the rest of the carcass was fixed in 10% buffered neutral formalin. Some skin samples were also preserved in 2.5% glutaraldehyde. For experiments conducted at the ARC, frogs were fixed whole in 10% formalin then transported to AAHL for post mortems.

Skin scrapings from all frogs were examined with a compound microscope, as described in Chapter 6. Samples of skin and internal organs were examined histologically. The immunoperoxidase stain (Chap 6) was used when H&E staining was not conclusive.

In experiments 6 and 7, the times till death in infected groups were compared for significant differences using the Student's non-paired two sample *t*-test two-tailed or an ANOVA single factor test using Microsoft Excel. In experiment 7, mortality rates in control and infected groups were compared using a Chi-square test by calculator (Sokal and Rohlf, 1997). The coefficient of rank (Spearman's) correlation, two-tailed, was used to determine the correlation between size and time till death using SPSS® for Windows 6.1 (SPSS Inc., Chicago, Illinois, USA). Differences were regarded as significant when $P \leq 0.05$

7.3 Specific aims, methods and results of each experiment

7.3.1 Experiment 1: Initial transmission to *M. fasciolatus*

7.3.1.1 Aims

The aims of this initial experiment were to find a method to transmit chytridiomycosis and to determine its pathogenicity.

7.3.1.2 Methods

This experiment was conducted at AAHL using *M. fasciolatus* juveniles up to three months old. They weighed between 2.8 and 11.2 gm (mean 5.0 gm). Ten from the cohort were toe-clipped two weeks before the experiment and found to be negative for

chytrids by histology. Frogs were housed individually and tubs were flushed weekly with aged tap water.

Pieces of shedding skin from a naturally infected *M. fasciolatus*, each containing about 3000 sporangia, were added to the water of six *M. fasciolatus*. The two control groups each contained four frogs. As the time of infection was dependent on receiving a naturally infected frog to obtain fresh infective material, a period of acclimatisation was not possible and frogs were infected three days after arrival at AAHL.

7.3.1.3 Results

Clinical signs occurred 10-16 days after exposure. Early behavioural changes were that frogs sat out in the open and were inappetant. Shedding skin accumulated over the body and the skin became dark, similar to frogs with natural infections (see Fig. 5.2). Frogs became weak and they lost their righting reflex. All six frogs exposed to infected skin scrapings became terminally ill, usually two days after skin sloughs were first collected. One died (day 10) and the rest were euthanased at days 14, 15, 16, 18, and 18. *B. dendrobatidis* was seen in all samples of shedding skin collected from the six infected frogs, and typical histopathology was present in the skin. Three frogs had light infections with sporangia. Frogs in both control groups remained healthy and were free of infection when euthanased after 22 days and examined histologically. All frogs had small to moderate fat bodies at euthanasia.

7.3.2 Experiment 2: Attempted transmission to *B. marinus*

7.3.2.1 Aims

The aims of this experiment were to determine the pathogenicity of *B. dendrobatidis* to metamorphs of *B. marinus*, and to assess their use as a model species for studying chytridiomycosis.

7.3.2.2 Methods

This experiment was conducted at AAHL concurrently with the first experiment described above. Forty-five or 46 *B. marinus* (9.5-13.5 mm) were used in each of the three groups, which consisted of three tubs containing 15-16 toads. They were exposed to infective material by adding shedding skin to the water dish. Each tub in one group

was dosed with about 3000 sporangia (from the same *M. fasciolatus* used in Experiment 1), the second group of three tubs received filtered PBSA that had stored the sloughing skin, and the third group was left untreated.

7.3.2.3 Results

Some deaths occurred in all groups throughout the 49 days they were monitored post exposure. These deaths appeared to be due to husbandry problems or natural attrition in the young metamorphs, similar to that seen in toads kept for the cane toad biological control project (H. Parkes, pers comm 1997). The skin of toads became dark and they stopped eating. Eleven out of 46 (23.9%) died in the group exposed to skin scrapings, 12/46 (26.1%) died in first control group and 9/45 (20.0%) died in the second control group. Two healthy toads from each tub were euthanased on day 19, four healthy toads from each tub were euthanased on day 25, and the remainder were euthanased after 49 days. Nineteen toads from the group exposed to infected skin scrapings were examined histologically. These were nine toads found freshly dead or moribund, and 10 that were euthanased during the experiment when healthy (4 from day 19, and 6 from day 25). None were infected with *B. dendrobatidis* and the cause of the death was not determined.

7.3.3 Experiment 3: Infection of *M. fasciolatus* using cultured zoospores at different doses

7.3.3.1 Aims

After *B. dendrobatidis* was cultured, an experiment was conducted to confirm that *B. dendrobatidis* is pathogenic when used in pure culture, to test a new method to infect frogs with known doses of zoospores, and to gain information on the minimum dose required to cause death.

7.3.3.2 Methods

This experiment was conducted at the ARC using 33-40 mm *M. fasciolatus* which had metamorphosed 2-3 months earlier. An isolate of *B. dendrobatidis* was used that had been cultured one month previously from a captive bred, sick metamorph of *L. dumerilii* (98 1469/10) from the ARC. Nine frogs were exposed to the fungus by placing them in 10 ml distilled water in plastic takeaway tubs to which zoospores were

added. Approximately 10 zoospores were added to the water of each of 3 frogs, 100 zoospores were added to the water of each of 3 frogs and 1000 zoospores were added to the water of each of 3 frogs, while 3 frogs were kept as controls. After 24 hours, they were transferred to individual tubs that were kept at 20-22°C. The water was flushed twice weekly.

7.3.3.3 Results

The frogs exposed to 1000 zoospores died at 23, 25 and 38 days post exposure, and the frogs exposed to 100 zoospores died at 35, 36 and 47 days post exposure. Chytridiomycosis with typical pathology was confirmed by examination of skin scrapings and by histology. Frogs given 10 zoospores and control frogs did not succumb to chytridiomycosis.

7.3.4 Experiment 4: Infection of tadpoles of *M. fasciolatus*

7.3.4.1 Aims

Before commencing the experiment to test antifungal treatments on tadpoles (McInnes, 1999), we needed to develop a method for obtaining high infection rates in tadpoles. This experiment was also used to test the infectivity of zoospores to tadpoles.

7.3.4.2 Methods

This experiment was conducted concurrently with Experiment 3, using the same zoospore preparation. *M. fasciolatus* tadpoles were used that had been spawned 9 months previously and had a mean snout-vent length of 21.4 +/- 4.5 mm (range 11-27 mm). Apart from one tadpole, none had hindlegs. They were housed individually in 1.4 litre tanks. Infection was attempted in four groups of two tadpoles by adding zoospores to their water. Two tadpoles were given 10 zoospores each, two were given 100 zoospores each, and two were given 1000 zoospores each. For the fourth group of two tadpoles, a 6 day old agar plate was divided into 5 mm squares of agar containing approximately 1800 sporangia each, which were placed inside nylon mesh bags and suspended in the water of each tank so that zoospores could escape. Two tadpoles were kept as untreated controls.

7.3.4.3 Results

After 25 days the tadpoles were euthanased with MS222 and their mouthparts examined histologically. None of the tadpoles were infected except the two in the fifth group that were exposed to a high dose by placing agar inside a mesh bag.

7.3.5 Experiment 5: Attempt to infect *Lim. peronii*

7.3.5.1 Aims

We attempted to infect metamorphs of *Lim. peronii* in order to trial antifungal treatments.

7.3.5.2 Methods

Infection was attempted at the ARC with a group of captive bred metamorphs of *Lim. peronii*. A high dose (25,000 zoospores) was used of an isolate of *B. dendrobatidis* from a wild *L. caerulea*, 4 months after it was isolated. Thirty-five frogs were exposed to zoospores in distilled water in individual plastic take-away containers for 24 hours, and seven were kept as controls. They were then returned to their usual enclosures of six animals per tub.

7.3.5.3 Results

None of the frogs became sick during three months of observations. At 49 days, a frog from each of the seven tubs was euthanased and examined by histology and examination of skin scrapings, but infections were not detected. Frogs were not euthanased at the end of the experiment but were retained for other purposes.

7.3.6 Experiment 6: Comparison of pathogenicity of three isolates on *L. caerulea*

7.3.6.1 Aims

Three isolates of *B. dendrobatidis* were used at the same dose rate to infect juveniles of *L. caerulea*, to determine if there was variation in the pathogenicity of the isolates and to select an isolate for use in experiment 7.

7.3.6.2 Methods

This experiment was conducted at the ARC concurrently with the treatment trial described in Chapter 9, for which the infected frogs included here were used as positive controls and the uninfected ones as negative controls.

The isolates were cultured from a captive *L. lesueuri* (00 545), a wild *L. caerulea* (99 1385/12), and a captive *L. dumerilii* (98 1469/10). Zoospores were collected from cultures on agar plates prepared in the same manner for all three isolates. Three to six week old (17-26 mm) captive bred metamorphs of *L. caerulea* were used. Room temperature varied between 16 and 20°C. Frogs were exposed to zoospores within 6 hours of collection by placing in yellow-top sample jars with 5 ml distilled water. Fourteen or 15 frogs were exposed to 50,000 zoospores from each of the isolates and 15 were kept as controls. The high dose was used as a high infection rate was required for the treatment trial and as *L. caerulea* spend most of the time sitting on the container wall, their total contact time with the inoculated water was unknown.

Toe-clips were removed from all frogs at 19 days and processed for histology. The experiment was terminated at day 85 with euthanasia of uninfected controls. Chytridiomycosis was diagnosed by examination of skin scrapings from all frogs, and histology on five frogs from each group.

7.3.6.3 Results

All frogs in infected groups died, although frogs exposed to zoospores from the most recently isolated culture (from the *L. lesueuri*) died significantly sooner ($P < 0.05$) than frogs exposed to the other two isolates (Fig. 7.4). The mean time till death of frogs infected with the 1.5 month old *L. lesueuri* strain was 19.4 +/- 4.17 days (range 9-28 days) (n = 15), with the 9 month old *L. caerulea* strain was 37.9 +/- 9.3 days (range 30-67 days) (n = 14) and with the 17 month old *L. dumerilii* strain was 32.7 +/- 6.8 days (range 24-52 days) (n = 15). Three of 15 uninfected control frogs also died - at days 69, 76 and 83.

The first signs of disease were that frogs were more likely to be found on the floor of the enclosure rather than sitting high on the walls, and they appeared less active. Their skin became blotchy and accumulations of shedding skin were visible over the body.

They rapidly became inappetant and lethargic and sat in abnormal postures with legs adducted.

Chytridiomycosis was confirmed in all frogs in infected groups by examination of skin scrapings, apart from three in the *L. lesueuri* group that were not examined as they died without premonitory signs and were only found when autolysed. Five frogs from each group were also examined by histology and infection was confirmed associated with typical histopathological lesions. Sporangia of *B. dendrobatidis* were not found in the 3 control frogs that died, or in the 12 that remained healthy and were euthanased at day 85.

Results from histology on toe-clips collected from all frogs 19 days after exposure were that infection was detected in 8/8 (100%) frogs exposed to the *L. lesueuri* strain (that were still alive at day 19), in 8/15 (53.3%) exposed to the *L. dumerilii* strain and in 9/14 (64.3%) exposed to the *L. caerulea* strain. Suspicious positives were seen in one frog from each of the latter two groups.

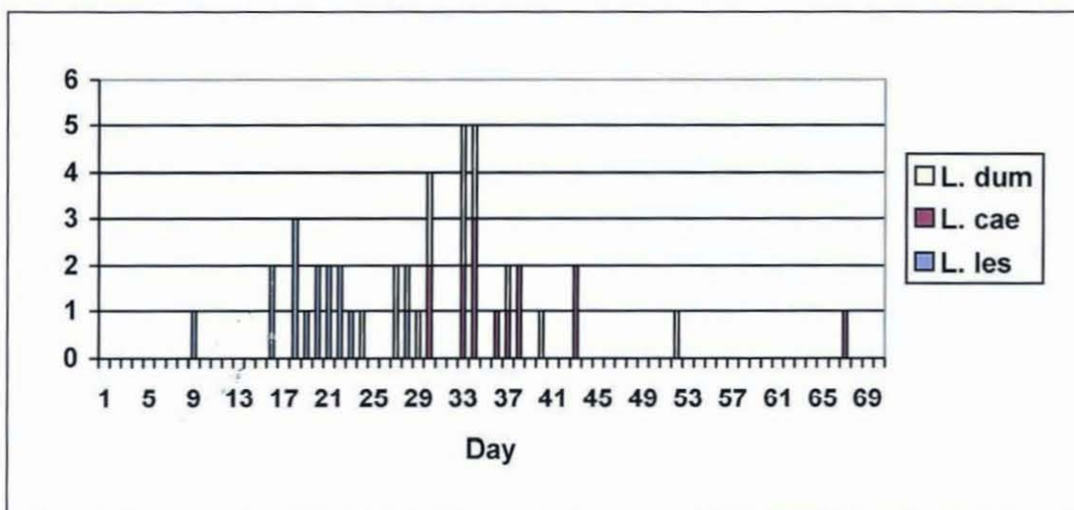

Figure 7.4 The number of frogs exposed to three isolates of *B. dendrobatidis* that died over time.

7.3.7 Experiment 7: Effects of temperature on the pathogenesis in *M. fasciolatus*

7.3.7.1 Aims

To investigate the effect of temperature on the pathogenesis of chytridiomycosis, frogs were infected in three rooms held at 17°C, 23°C and 27°C. The reasons for choosing

these temperatures were that 23°C was the optimum for growth of an isolate of *B. dendrobatidis* and 27°C was close to the upper limit that supported growth (Longcore et al., 1999), and constant temperatures below 17°C were considered unsuitable for housing *M. fasciolatus* (G. Marantelli, pers comm). These temperatures occur commonly in the natural distribution of this species.

7.3.7.2 Methods

The temperatures in three rooms in the LAF at AAHL were adjusted until the desired temperatures were achieved. Temperatures were initially logged over a 3 day period and were found to vary less than 1°C during this time. During the experiment, air, water and minimum/maximum temperatures were recorded daily. The average (mode) temperature in the cold room was 17.0°C (range 16.0 - 17.8°C), in the medium room was 23.0°C (range 22.4-24°C) and in the warm room was 27.0°C (range 26.5-28.0°C). Water temperatures were 2- 4°C lower than air temperatures and in each room were about 15°C, 19.5°C and 23°C respectively. Relative humidity was measured three times a week with a hand held electronic humidity probe. Humidity was dependent on the humidity of outside air and was similar in each of the three rooms, varying from 18% to 60%. Lights were set at a 12 hour light/dark cycle.

Frogs had been kept at 17-20°C at the ARC for the preceding months. On arrival at AAHL, frogs were randomly assigned to three size matched groups of 14 frogs which were further divided into 8 test and 6 control animals. This was achieved by dividing frogs into three groups based on their size, then sequentially allocating them to the different rooms. Frogs weighed from 3.0 - 11.7 gm (mean - 6.14) and had snout-vent lengths between 31 and 46 mm (mean 38.0 mm). There were no significant differences in weight or length between groups (ANOVA, $P > 0.05$). Initially, one group was placed in the 17°C room and two in the 23°C room. After four days, one of the groups at 23°C was moved to 27°C. Frogs were acclimatised for a total of 18 days. Frogs were fed crickets *ad lib*, but as frogs in warmer rooms had greater appetites, food intake, as well as temperature, was a variable. The water was topped up as necessary and two litres of softened water were flushed through each tub twice weekly.

Frogs were exposed to 1000 zoospores in plastic take-away tubs for 24 hours. The isolate used had been cultured from a captive *L. lesueuri* (00 545) three months previously. Infections were performed about an hour after collection of zoospores, and frogs in all rooms were infected over a 30 minute period in the following order - 27°C, 23°C and 17°C. Frogs were checked daily and the position of the frogs was recorded - whether they were buried under leaves and gravel or sitting in the open, and whether they were sitting with their bellies above or below the water line.

Frogs were tested opportunistically throughout the experiment by collecting shedding skin from the frog or from the water that was examined as a wet mount.

Controls in each room were euthanased when the last infected frog in that room died.

7.3.7.3 Results

Mortality

Twenty of the 24 frogs in the infected groups became ill: 13 were euthanased and 7 were found dead. Most frogs in the 17°C and 23°C rooms died by 38 days (Fig. 7.5). In the 17°C room, the mean time till death of the eight frogs that died with chytridiomycosis was 40.0 +/- 10.8 days (range 25-59). In the 23°C room the mean time till death of seven frogs that died with chytridiomycosis was 40.0 +/- 16.1 days (range 29-76 days). There was no significant difference between these two rooms ($P<0.05$). One frog in the infected group in the 23°C room died at day 18 but sporangia were not detected. The cause of death was not determined, and as this frog did not die from chytridiomycosis it is not included in the analysis of results.

Although four frogs in the 27°C room died with chytridiomycosis between 18 and 27 days, the other four remained healthy until the experiment was terminated at 98 days. Infection was confirmed in three of these during the experiment by examination of shed skin. Positive skin samples were obtained from each individual frog: at 76 days, at 28 and 63 days, and at 18, 28, 40 and 46 days. The two samples collected at 28 days had the heaviest infections, while very few sporangia were found in samples collected at 63 and 76 days. These frogs were euthanased when the experiment ended at day 98 and infection was no longer detectable by histology, examination of skin scrapings or

immunoperoxidase staining, although in one frog the thickness of the epidermis appeared slightly irregular.

When frogs in infected groups were compared using a Chi-square test, the mortality rate at 27°C was significantly lower than the mortality rate in frogs kept at 23°C or below ($P < 0.05$).

Histology was done on all dead frogs and typical pathology was seen with severe hyperkeratosis, irregular hyperplasia and necrosis of basal epidermal cells associated with heavy infections of *B. dendrobatidis*. Focal ulceration occurred in the skin of one frog that died at day 27 at 27°C, and extensive ulceration occurred in two frogs at 23°C that died at days 36 and 76. The four infected frogs that died at 27°C had lighter infections than those that died in the other two rooms.

None of the controls were infected with *B. dendrobatidis*. Unfortunately five of the 18 (27.8%) control frogs died during this experiment. One control frog died in the 27°C room on day 36 due to ingestion of gravel. The cause of death of the other frogs was not determined - one frog died in the 17°C room at day 20 and 3 died in the 23°C room each at days 7, 19 and 30. These frogs were all thin and had dark skin, and two had severely enlarged, opaque gall bladders. Few abnormalities were found on histology - one frog had red granular deposits in renal tubules, one frog had red granular hepatocytes, and one frog had a vacuolating dermatitis. When combined together, the mortality rate in control frogs (5/18) was significantly less than the mortality rate in the infected groups (20/24) ($P < 0.05$). (Significance was tested using a Chi-square test, as the properties in all three groups were identical so a three-way table analysis was not required).

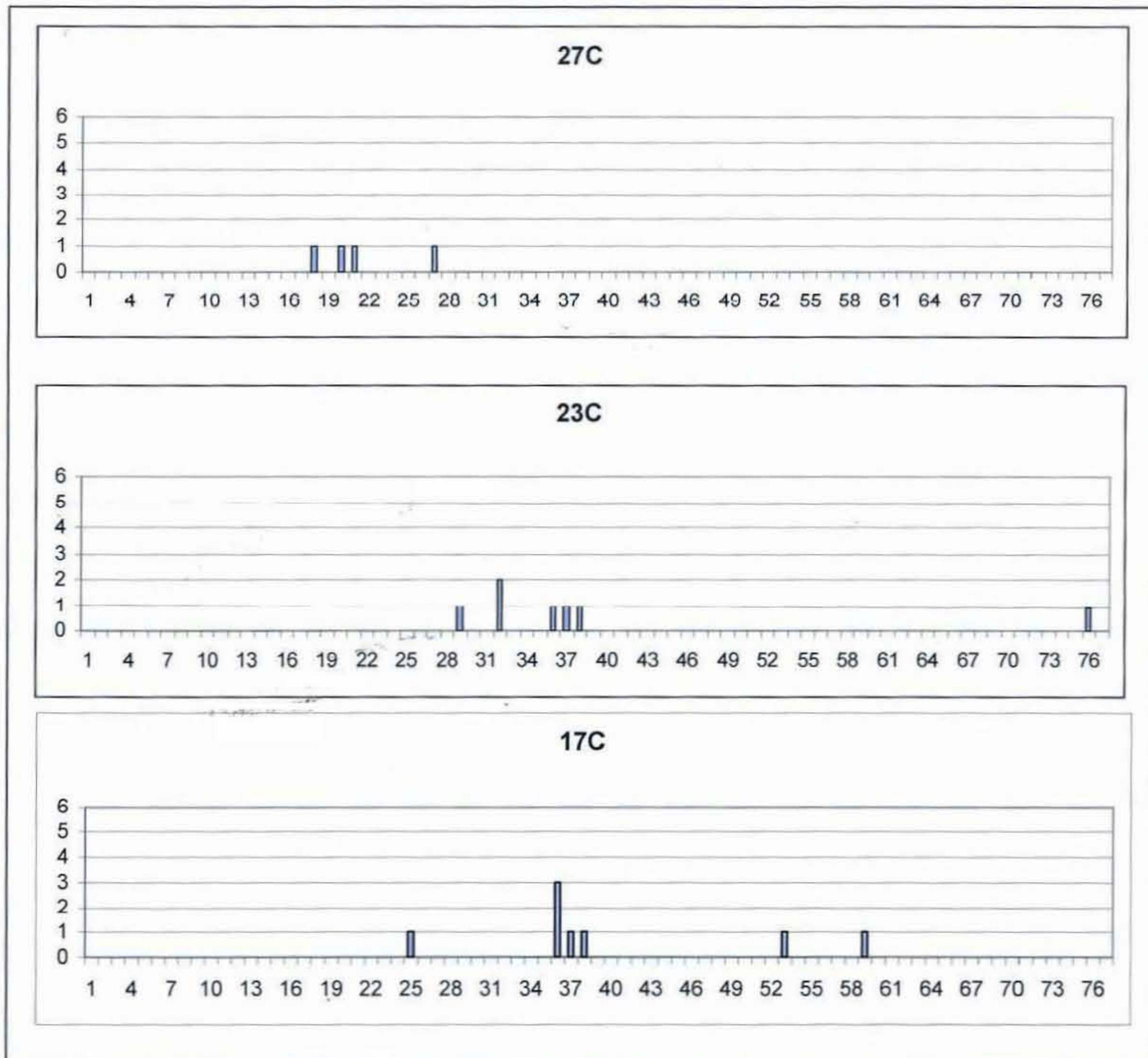

Figure 7.5 The number of frogs at each temperature that died with chytridiomycosis over time.

Clinical signs

Most frogs stopped burrowing in the gravel or hiding under leaves during the day about three days before they died (Fig. 7.6). Lethargy developed from about two days before death. Of the nineteen frogs that died with chytridiomycosis, thirteen spent their last days sitting dry while six were in contact with water.

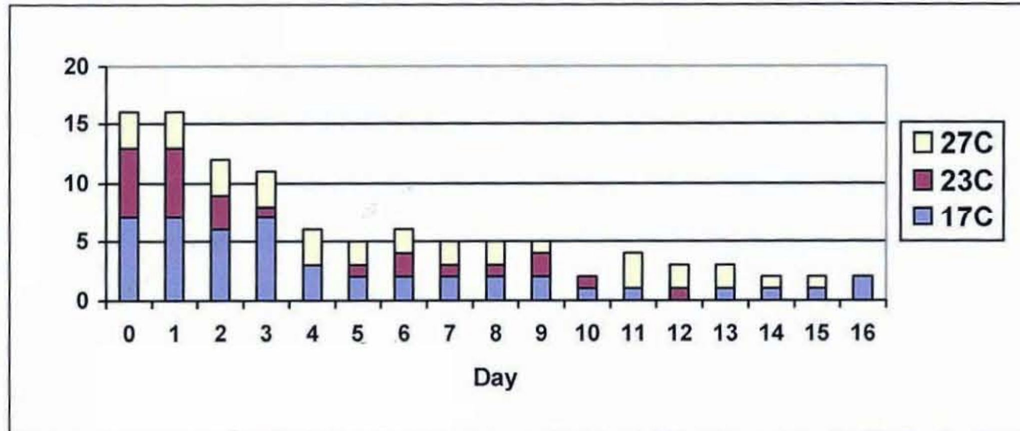

Figure 7.6 The numbers of frogs that were sitting uncovered during the day, of the 19 frogs that died with chytridiomycosis, where each day is the number of days before death (day 0).

Effects of body size and gender

In the 17°C and 23°C rooms, the weight and length of frogs did not correlate with time till death ($r = 0.14$, $P > 0.05$). However in the 27°C room, the three largest frogs died with chytridiomycosis while the three smallest ones survived.

Susceptibility did not appear to be associated with gender.

Diagnosis by examination of shedding skin

Shedding skin was collected opportunistically and examined. For the 19 frogs that died with chytridiomycosis, sporangia were detected in shedding skin a mean of 7.5 +/- 6.5 days before death (range 0-25 days). Negative results from examination of shedding skin were obtained from four of these frogs, with the latest time of obtaining a negative result being nine days before death. However, once infection was detected in a frog, sporangia were seen in all samples collected thereafter.

Other effects of temperature

Frogs in the warm room were more active and had greater appetites. They each ate about six crickets twice a week compared to four crickets twice a week in the cooler rooms. As water changes were the same in each room, this difference in feeding caused the water to be dirtier in the warm room due to greater production of faeces.

It was observed that most frogs at 17°C sat in contact with the water, whereas most frogs at 27°C sat dry above the water line. The position of frogs at 23°C was more variable.

Five out of five male frogs kept at 27°C were considered to have active testes when examined histologically as they had many mature sperm in the lumen. In contrast 4/8 male frogs kept at 23°C had active testes and 2/7 kept at 17°C had active testes.

7.4 Discussion

In summary, these experiments demonstrated that *B. dendrobatidis* is highly pathogenic to *M. fasciolatus* and *L. caerulea* (Table 7.1). *B. dendrobatidis* is highly infectious and even low experimental doses can result in fatal disease in susceptible species. Tadpoles can be infected experimentally. We were not able to infect juveniles of *B. marinus* and *Lim. peronii*. An isolate remained infectious and pathogenic after 17 months of being maintained in culture, although time to death may have been increased. Mortality rate was reduced at higher temperatures. Incubation times varied from 9 till 76 days, with most frogs (73.3%) dying between 18 and 48 days post exposure. Of the frogs that died with chytridiomycosis, only 5 (6.7%) survived longer than 48 days. The time till death varied with fungal dose and fungal strain.

Recent experiments at the ARC have demonstrated that frog species and environmental conditions also affect pathogenesis (Doug Woodhams, Katie Ardipraja, Gerry Marantelli, Ross Alford, unpub 2001). These experiments used the same experimental design developed here, showing it is a reproducible model for infection.

Clinical signs and histopathology seen in experimental infections using *M. fasciolatus* and *L. caerulea* were similar to those seen in natural cases of disease (Chap 5), and to those produced in experimentally infected dendrobatid frogs (Nichols et al., 2001), with lethargy occurring a few days before death.

The initial experiment using infected skin scrapings provided the first strong evidence that *B. dendrobatidis* was pathogenic to frogs. The high doses given produced a short 10-18 day incubation period. An attempt to infect *B. marinus* metamorphs using the same infective material did not result in transmission of infection or disease. As cane toad metamorphs are known to be susceptible to chytridiomycosis (Berger et al., 1998), perhaps the differences in the experimental set up did not support transmission or saprobic growth of the fungus. The enclosures were different - toads had small water dishes on a clean plastic floor instead of sloping gravel with water, and the toads spent less time on the ground and were most commonly found perched high along the rim of the tubs. Conditions for saprobic growth may not have occurred in the water dish. Less likely explanations are that the fungus strain (collected from a *M. fasciolatus*) was not adapted to growth on toads, or that the dose of fungi was too low. Similarly, we cannot be certain of the reasons that disease was not produced in the *Lim. peronii* in Experiment 5. As this was the only species exposed at this time, it was not known if the zoospore preparation was not viable after transport and handling etc., or if this species is remarkably resistant. A recent experiment has shown that a closely related species, *L. tasmaniensis* is more resistant compared with *L. caerulea, M. fasciolatus* and *L. chloris* (K. Ardipraja and G. Marantelli, unpub 2001).

With the experiment using different doses of cultured zoospores (Experiment 3) we fulfilled Koch's postulates and demonstrated that doses down to 100 zoospores are capable of causing fatal disease. As many frogs do not live in close contact with others, and in nature transmission occurs even in frogs inhabiting fast flowing streams, a waterborne disease would need to be highly infectious to survive. A longer incubation period was observed with low doses, perhaps reflecting the increased number of generation times needed to produce a fatal infection.

In experiment 4, we achieved the first experimental transmission of *B. dendrobatidis* to tadpoles. Infection only occurred with the high dose. Doses of 100 and 1000 zoospores,

which resulted in severe infections in metamorphs at this time, did not result in infection in tadpoles, probably as the large volume of water (1.4 litres) in which they were exposed resulted in zoospores being at too low a concentration.

All isolates of *B. dendrobatidis* used to infect *L. caerulea* in experiment 6 resulted in 100% mortality, showing that cultures were still infective even after 17 months of growth *in vitro* (approximately 12 passages). However, there were significant differences in the incubation period. The most virulent strain that resulted in rapid deaths was the most recently isolated (isolated 1.5 months earlier), although there was no significant difference between the groups exposed to 9 and 17 month old isolates. This could be due to attenuation of the earlier isolated strains or to inherent differences between strains, which were isolated from different frog species and locations. Variation in the production of biochemical metabolites commonly occurs during laboratory cultivation of fungi (Turner, 1971). To reduce changes in cultures, growth of stocks should be kept to a minimum. Passages should be recorded as for viruses.

The transmission experiment performed at different temperatures (experiment 7) produced interesting results. Although three frogs in the hottest room (27°C) were the first to die, 4/8 small frogs did not succumb to chytridiomycosis. Infection was confirmed in three of these surviving frogs by examination of shedding skin, however by the end of the experiment the infection had been lost. Temporary carrier status was produced for the first time in experimentally infected frogs, and positive samples were obtained up to 35 days apart. Time till death was not significantly different in frogs in the 17°C and 23°C rooms, all of which died.

Although four uninfected control frogs died for unknown reasons, frogs in the infected groups that died had typical lesions of severe chytridiomycosis and this was believed to be the cause of their deaths, apart from one frog at 23°C that may have died from the same undetermined cause as the controls. Although these deaths indicated a problem with the frogs or their husbandry in this experiment, the crucial result is the survival of half the infected frogs at 27°C, and this is very meaningful when compared with the high mortality rates observed in previous experiments at 24°C or below, as well as in the other temperature controlled rooms.

This temperature effect supports the theory that frogs have disappeared at high altitudes and that mortalities from chytridiomycosis occur more in winter due to colder temperatures (Laurance et al., 1996; Chap 8). Growth of the fungus and immunity of amphibians are both affected by temperature. The combinations of these effects may explain the increased virulence of chytridiomycosis at lower temperatures.

In vitro studies of *B. dendrobatidis* showed that optimum growth occurs at 23°C and growth is reduced at higher temperatures, with fungal death occurring at 30°C (Longcore, 2000). Temperature affects the ecology of many aquatic fungi, and chytridiomycetes have been observed to bloom with seasonal temperature changes (Sparrow, 1968). It is possible that any toxin production by *B. dendrobatidis* may also be temperature dependent. Although the 27°C air temperature in the warmest room would have inhibited fungal growth, water temperature did not exceed 24.5°C and so any saprobic growth could have continued. The temperatures of frogs are usually close to ambient (Brattstrom, 1961).

Studies of the effect of temperature on poikilotherm immunity have shown that some aspects are reduced with lower temperatures (Maniero and Carey, 1997). Haematologic values vary seasonally and white blood cells counts are lower in autumn and winter in *R. pipiens* (Anver and Pond, 1984). Other factors that can influence haematologic values include altitude, weather, and geographic origin (Anver and Pond, 1984). Plasma electrolyte and glucose levels also change seasonally (Anver and Pond, 1984). Experimental exposure of *R. tigerina* tadpoles and wounded frogs to *Aeromonas* spp. caused mortality at 20°C but not at 30°C, even though 30°C is used for culturing the bacteria (Somsiri et al., 1997). Bacterial septicaemia occurred in captive *R. catesbiana* when temperatures were lowered (Carr et al., 1976). An outbreak of *Pseudocapillaroides xenopodis* occurred in captive *Xenopus* after a drop in temperature from 22°C to 15°C for 10 days, and was thought to be a result of decreased immunity (Tinsley, 1995). However, an investigation of cold on immune function involving measurements of mitogenic responses, differential blood cell count and complement assays in *B. marinus* demonstrated that the toads were immunostimulated by colder temperatures (Carey et al., 1996). Phagocytosis in cyprinid fish (*Tinca tinca*) appears to be more resistant to low temperatures than lymphocytes (Collazos et al., 1994).

Winter saprolegniasis in channel catfish (*Ictalurus punctatus*) occurs after a rapid drop in water temperature of about 10°C which causes immunosuppression, and maintenance of cold temperatures which favour growth of *Saprolegnia* sp. (Bly et al., 1993). Immunosuppression did not occur if fish were gradually acclimatised to the lower temperatures. Experiments demonstrated that both factors - high zoospore levels and immunosuppression - were necessary for disease to result. Immunosuppression was characterised by low *in vitro* mitogen responses and a lack of inflammatory response to foci of infection (Bly et al., 1993). After environmental temperatures change, ectotherms must adjust their cellular composition to continue to function. Studies in catfish have shown that adaptations in T and B lymphocytes take about 5 weeks when temperatures drop from 22°C to 11°C (Bly et al., 1992, 1993). Secretion of mucous onto the skin may also be impaired after a decrease in temperature, allowing zoospores time to encyst and germinate before the mucous layer is sloughed (Bly et al., 1996). Changes in water quality including oxygen levels, ammonia and pH did not affect disease development (Bly et al., 1992).

Maybe if frogs in the warm room had had longer than 14 days to acclimatise to 27°C the survival rate would have been higher than 50%. Thermal acclimatisation in frogs can occur at the same or different rate when anurans are moved from hot to cold temperatures and vice versa (Brattstrom, 1968).

Frogs kept at the higher temperature were observed to eat more and were more active, demonstrating they had an increased metabolic rate. Aspects of the innate immune system, such as antimicrobial skin secretions, may be more productive, and this could have caused the loss of infection. Turnover of epidermal cells is affected by temperature and an increased rate of shedding could be a factor in removing sporangia. Another explanation for the lack of clinical signs in the infected frogs at 27°C could be that they were more able to metabolise any toxins produced by the fungus.

A combination of effects on the growth and metabolism of *B. dendrobatidis* and on the immunity of frogs appears most likely to explain the increased pathogenicity at lower temperatures.

As *B. dendrobatidis* is highly pathogenic to *L. caerulea*, in experimental transmissions low temperature was not a requirement of the disease, but high temperatures reduced its effects. In areas where *B. dendrobatidis* has become endemic in the wild, seasonal temperature changes and altitude appear to be important in expression of disease.

Future work on temperature effects should attempt to determine whether the effects of temperature on chytridiomycosis are mediated more by host or pathogen. A combination is likely, as in channel catfish. Although cellular and humoral immunity have been shown to be affected by temperature (Collazos et al., 1994), these types of immunity may not be important in resistance to chytridiomycosis. Studies on the host could be done after the aspects of immunity that are effective against *B. dendrobatidis* are understood. Studies on the pathogen should involve measuring fungal growth at different temperatures and whether a drop in temperature causes a fungal bloom. In experiment 7 the rooms were kept at constant temperatures, which was appropriately simple for an initial experiment, but the effects of more realistic fluctuating temperatures could be studied.

Now that methods are available for freezing cultures of *B. dendrobatidis* (Boyle et al., 2002), comparison between strains can be attempted without the confounding factor of attenuation of isolates during periods of culture. Effects of attenuation can be examined by comparing the same isolate after different numbers of passages.

While the differences in susceptibility between species are currently being examined (Katie Ardipraja and Gerry Marantelli, pers comm 2001), other aspects to investigate are differences in susceptibility between adult and juvenile frogs, and differences within the same species of frogs from various geographic areas that may have had different lengths of time to develop resistance.

Table 7.1 Summary of seven transmission experiments where amphibians were exposed to *B. dendrobatidis*. Care must be taken with comparison between experiments, due to many variables.

Experiment No. Location	Amphibian species Life stage	Dose (Strain) #	Temperature	Mortality due to chytridiomycosis	Mean time till death from chytridiomycosis (Range) (Days)
1 AAHL	*M. fasciolatus* Juveniles	3000 sporangia (in skin scraping) (*M. fasciolatus* 96 1431/52)	24°C	6/6	15.2 (10-18)
2 AAHL	*B. marinus* Metamorphs	3000 sporangia per 15 toads (in skin scraping) (*M. fasciolatus* 96 1431/52)	24°C	0/46	-
3 ARC	*M. fasciolatus* Juveniles	10 zoospores 100 zoospores 1000 zoospores (*L. dumerilii* 98 1469/10)	20-22°C	0/3 3/3 3/3	- 39.3 (35-47) 28.7 (23-38)
4 ARC	*M. fasciolatus* Tadpoles	10 zoospores 100 zoospores 1000 zoospores 1800 sporangia (*L. dumerilii* 98 1469/10)	20-22°C	0/2* 0/2 0/2 2/2	-
5 ARC	*Lim. peronii* Juveniles	25,000 zoospores (*L. caerulea* 99 1385/12)	20-22°C	0/35	-
6 ARC	*L. caerulea* Juveniles	50,000 zoospores (*L. lesueuri* 00 545) 50,000 zoospores (*L. caerulea* 99 1385/12) 50,000 zoospores (*L. dumerilii* 98 1469/10)	16-20°C	15/15 14/14 15/15	19.4 (9-28) 37.9 (30-67) 32.7 (24-52)
7 AAHL	*M. fasciolatus* Juveniles	1000 zoospores 1000 zoospores 1000 zoospores (*L. lesueuri* 00 545)	17°C 23°C 27°C	8/8 7/7 4/8	40.0 (25-59) 40.0 (29-76) 21.5 (18-27)

Experiments 1 & 2, and experiments 3 & 4, were conducted simultaneously using the same infective material. * Proportion of tadpoles with sporangia.