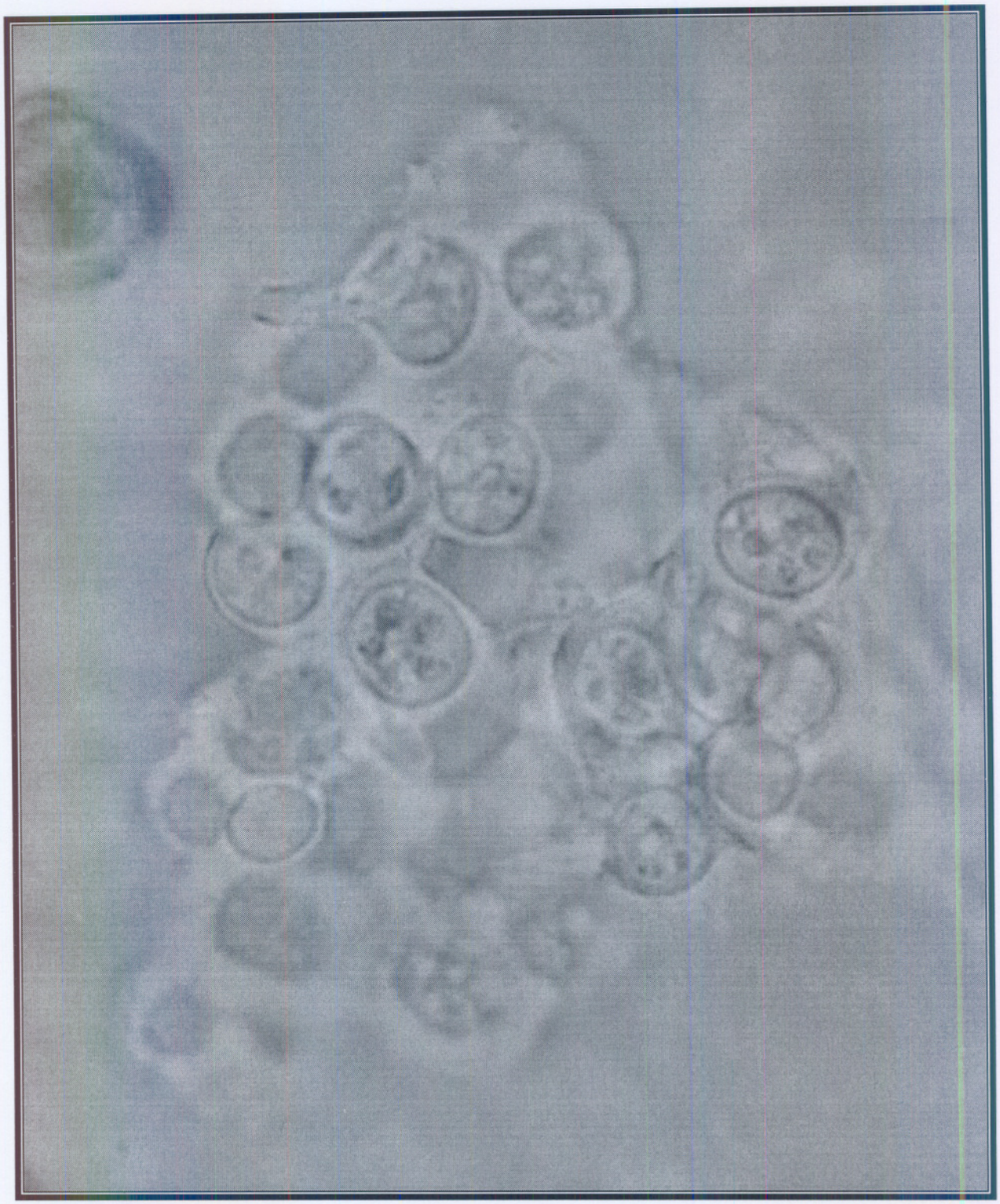
CHYTRIDIOMYCOSIS, AN EMERGING INFECTIOUS DISEASE OF AMPHIBIANS IN SOUTH AFRICA

C. WELDON

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Promoter: Prof. L.H. du Preez
Co-promoter: Prof. R. Speare

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Unstained skin scrape of the Cape river frog showing sporangia with zoospores of *Batrachochytrium dendrobatidis*.

ABSTRACT

Chytridiomycosis, an emerging infectious disease of amphibians in South Africa

The sudden appearance of chytridiomycosis, as the cause of amphibian deaths and population declines in several continents suggests that its etiological agent, the amphibian chytrid fungus *Batrachochytrium dendrobatidis,* was introduced into the affected regions. However, the origin of this virulent pathogen is unknown. Efforts were directed to determine the occurrence of chytridiomycosis in Africa, whether the disease had been introduced into South Africa in recent years and how wild frog populations were affected by infection. A chytridiomycosis survey of 2,300 archived and live specimens involving members of the Pipidae family in sub-Saharan Africa, as well as a number of unrelated frog species in South Africa was conducted by histological diagnosis of skin samples. The epidemiological evidence indicated that chytridiomycosis has been a stable endemic infection in southern Africa for 23 years before any positive specimens were found outside Africa. The occurrence of chytridiomycosis in South Africa can be described as widespread both in terms of geographical distribution and host species and generally infection is not associated with adverse effects at the individual or population level. It was proposed that the amphibian chytrid originated in Africa and that the international trade in the African clawed toad *Xenopus laevis* that commenced in the mid 1930s was the means of dissemination. A risk assessment of the *X. laevis* trade demonstrated that chytridiomycosis could spread through this pathway and culminated in the development of a management protocol to reduce the risks of spreading disease through this animate commodity. Initial comparative genetic analysis of *B. dendrobatidis* strains isolated from South African frogs with a global set of 35 strains, suggests that analysis of a more geographically diverse set of southern African strains is needed before this line of argument can support or reject the "out of Africa" hypothesis.

Keywords: Chytridiomycota, *Batrachochytrium dendrobatidis*, chytridiomycosis, disease, origin, epidemiology, prevalence, Africa, amphibian declines, *Xenopus laevis*

OPSOMMING

Chytridiomikose, 'n nuwe aansteeklike siekte van amfibië in Suid-Afrika

Die skielike verskyning van chytridiomikose, die oorsaak van amfibiër sterftes en hulle afname op verskeie kontinente veronderstel dat die etiologiese agent, die amfibiese chytrid swam *Batrachochytrium dendrobatidis,* aan die geaffekteerde gebiede geïnduseer is. Die oorsprong van hierdie aggressiewe patogeen, is egter onbekend. Pogings is aangewend om die voorkoms van chytridiomikose in Afrika te probeer vasstel en meer spesifiek hoe onlangs die siekte in Suid-Afrika geïnduseer is en hoe wilde paddapopulasies deur die infeksies beïnvloed is. 'n Chytridiomikose opname van 2,300 gebergde sowel as lewende eksemplare van die Pipidae familie in sub-Sahara Afrika, asook 'n aantal Suid-Afrikaanse nie-verwante paddaspesies is deur middel van histologiese diagnose van velmonsters uitgevoer. Dit is epidemiologies bewys dat chytridiomikose 'n stabiele endemiese infeksie vir 23 jaar in Suid-Afrika was voordat enige positiewe eksemplare buite Afrika gevind is. Chytridiomikose in Suid-Afrika is geografies wyd verspreid en daar is geen aanduiding van gasheerspesifisitiet nie. Oor die algemeen word infeksie nie met nadelige uitwerking op individuele sowel as populasievlakke geassosieer nie. Daar word voorgestel dat die amfibiese chytrid sy oorsprong in Afrika het en dat internasionale handel van die gewone platanna, *Xenopus laevis*, gedurende die middel 1930's die verspreidingsmeganisme was. 'n Risikobepaling van die *X. laevis* handel, demonstreer dat chytridiomikose op dié manier kan versprei en het gelei tot die ontwikkeling van 'n bestuursprotokol om die verspreidingsrisiko deur hierdie handelsmetode te verminder. Aanvanklike vergelykende genetiese ontleding van stamme van *B. dendrobatidis*, geïsoleer van Suid-Afrikaanse paddas met 'n globale stel van 35 stamme, suggereer dat die ontleding van 'n meer geografies uiteenlopende stel van suider-Afrikaanse stamme benodig word voordat hierdie argument die 'uit Afrika' hipotese kan ondersteun of verwerp.

Sleutelterme: Chytridiomycota, *Batrachochytrium dendrobatidis*, chytridiomikose, siekte, oorsprong, epidemiologie, prevalensie, Afrika, amfibiër afnames, *Xenopus laevis*

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The fear of the LORD is the beginning of knowledge, but fools despise wisdom and instruction.
Proverbs 1:7 ([1]KJV 1999)

[1] King James Version 1999, American Bible Society, New York

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Chapter 1

INTRODUCTION AND LITERATURE REVIEW

> So consider the frog. The frog has sensed a change, a displacement in the order of life as we know it. We may be responsible, at least in part, for causing the change. And we may be the next to feel it. This story is larger than we can imagine. The facts humble us. The evening silence is spreading.
> The frog is telling us something. Will we listen?
>
> William Souder (2000)

1.1 Declining Amphibian Populations

According to the World Wide Fund for Nature (WWF) the biodiversity index for freshwater habitats based on vertebrate data, including amphibians experienced a 50% decline between 1970 and 1999 (Loh 2000). It would seem therefore that freshwater habitats are under severe threat, a process that affects an array of biodiversity especially frogs and salamanders. Amphibians are often referred to as indicators of environmental stress, providing early signs of the deteriorating health of other species (Blaustein 1994, Sparling et al. 2000) and therefore raise concern when populations start disappearing. Furthermore, amphibians form an integral part of many aquatic and terrestrial food webs. Adults and most larvae are predators of mainly invertebrate prey, while serving as a food source to a variety of larger predators including snakes, wading birds and fish. They are particularly vulnerable to environmental change, because they are in contact with both water and land for at least part of their development, they have moist, permeable skin and their unshelled eggs are directly exposed to soil, water and sunlight (Blaustein and Kiesecker 2002). An assessment of the global amphibian declines conducted by the World Conservation Union Global Amphibian Assessment (IUCN GAA) demonstrated that 32.5% (1,856 species) of amphibians are currently being threatened (Stuart et al. 2004). In addition, 34 species are known to have become extinct since 1500, while a further 122 species are believed to be possibly extinct (species that can no longer be found that await exhaustive surveys to establish their disappearance). Of great concern is that the majority of these disappearances (113 species) have occurred since 1980.

The sudden disappearance of amphibian populations seems to have started during the 1970s in western United States, Puerto Rico and northeastern Australia (Czechura and Ingram 1990,

Drost and Fellers 1996, Burrowes et al. 2004). These declines have not been limited to habitats heavily affected by human activities, but have occurred in pristine habitats as well. By 1989 when the First World Congress of Herpetology took place in Canterbury, England, scientists realised that rapid amphibian declines were a global phenomenon that necessitated urgent investigations into the extent and causes of the declines. Various reasons for declines have been proposed:

- Global warming, caused by the greenhouse effect, has a serious effect on climate. Alterations in weather patterns can cause changes in rainfall that adversely affect amphibian reproduction. Depletion of stratospheric ozone causes an increase in UV-B radiation that greatly reduces hatching success of embryos and tadpole survival in some amphibians (Kiesecker et al. 2001, Blaustein et al. 2003).
- Modification of habitats can have long-lasting effects and is the most obvious factor contributing to amphibian declines (Alford and Richards 1999). For example, deforestation not only removes large trees but also changes the microclimate of the undergrowth.
- Chemical pollution has been shown to have devastating effects on amphibian populations, including developmental and behavioural abnormalities (Bridges and Semlitsch 2000, Sparling et al. 2000).
- Pollutants include not only residues released by industry, but also fertilizers, herbicides, fungicides and pesticides. Several of these pollutants disrupt endocrine functions and thereby the reproductive systems of amphibians and even induce immunosuppression (Colborn and Clement 1992, Stebbins and Cohen 1995).
- Introduced predators can have a serious effect on amphibian populations. Such predators can compete with native species for natural resources, as well as carry and transmit disease, hybridise with natives, and prey on amphibians (Kiesecker and Blaustein 1998, Lawler et al. 1999, Gillespie 2001, Kats and Ferrer 2003).
- The harvest of frogs from natural habitats for human consumption also has a huge effect. It was estimated that in 1993, no less than 4,700 tons of frogs' legs were exported to Europe from Indonesia alone (Patel 1993). This represents an astonishing 94-235 million animals (Veith et al. 2000). Various amphibians have possibly been driven to the brink of extinction through the pet trade. The more rare a species is, the higher the asking price, and the more severe the threat to those few remaining in the wild.
- Like other animals, amphibians fall prey to viruses and fungi, and to parasitic infections by protozoans and various helminths. In recent years it has become increasingly clear that infectious diseases are one of several causes of global amphibian declines

(Laurance et al. 1996, Carey 2000, Williams et al. 2002, Collins and Storfer 2003, Daszak et al. 2003). According to Daszak et al. (2000), emerging infectious diseases are diseases that are newly recognised, newly appeared in a population or rapidly increasing in incidence, virulence or geographical range. These pathogens may induce sub-lethal damage (e.g. developmental deformities, physiological and behavioural deviations) or may directly cause mortality.

Despite reports of amphibian disease going back to the first decade of this century, knowledge of amphibian pathology and medicine has not kept pace with that of other animal groups. According to Elkan (1960) the published literature on amphibian pathology is extremely sparse and the knowledge concerning the amphibian reaction to the factors commonly causing disease is even less incomplete. The existence of this situation, Crawshaw (1992) attributes to the fact that it has usually been easier and cheaper to obtain new animals than investigating or treating medical problems. The main causes of morbidity in amphibians are parasites, tumours, inflammatory conditions and fungal infection (Elkan 1960). The most commonly reported disease in frog culture is a form of bacterial septicaemia called red-leg disease and is usually caused by gram-negative bacilli belonging to the family Vibrionacidae, Pseudomadaciae or Enterobacteriacae (Crawshaw 1992, Pariyanonth and Daorerk 1995). The disease has been studied extensively and preventative measures as well as treatment have been suggested by many that encountered the condition (Koivastik 1950, Nace 1968, Ruddock and Ruffle 1972). Three of the most commonly implicated diseases of amphibians associated with population declines are chytridiomycosis, saprolegniosis and infections with ranavirusses (Cunningham et al. 1996, Berger et al. 1998, Kiesecker et al. 2001). Data available on the emergence of chytridiomycosis has the strongest association with amphibian population declines (Daszak et al. 2003).

A distinction can be made between the causes of amphibian declines on the basis of the timing of threatening processes and recent amphibian declines (Hero et al. 2004). For example, continuing historical threats include habitat modification and changing fire regimes that have potentially affected frog habitats for over 200 years, whereas introduced fish and amphibian species have been an eminent threat for more than 70 years. Factors such as global climate change and emerging infectious diseases are relatively new threats (post 1970s). It is well known that animals have evolved ways to deal with most natural stressors in their environment. However, anthropogenic stressors are often novel to organisms and introduced abruptly, precluding natural evolution of adaptive processes (Westerman et al. 2003). The geographic pattern that emerged from rapidly declining amphibians shows a concentration of overexploited species in East and Southeast Asia, a wider occurrence of species suffering

from reduced habitat especially in Southeast Asia, West Africa and the Caribbean, whereas declines due to enigmatic causes (declines in spite of suitable habitat for poorly understood reasons such as disease and climate change) are mainly confined to South America, Mesoamerica, Puerto Rico and Australia (Stuart et al. 2004). Linking specific factors or stressors to an actual decline or extinction event of populations is a difficult process. The reality is that the causes of relatively few amphibian population declines are clearly understood (Westerman et al. 2003). Amphibian declines can seldom be linked to a single factor. More often complex mechanisms that involve abiotic and biotic interactions are responsible for amphibian population declines (Blaustein and Kiesecker 2002). For example, during extremely dry years, possibly brought on by sea-surface warming in the Pacific (Kiesecker et al. 2001), aquatic breeding sites of the western toad, *Bufo boreas* become very shallow, which enhances the effects of harmful UV-B and encourages lethal invasion of the fungus *Saprolegnia ferax* (Pounds 2001). According to Krest et al. (2003), improved experimental designs, especially for integrated field and laboratory studies, are important when characterising cause-effect relationships that would support adaptive management programmes intended to benefit amphibians.

1.2 The Amphibian Chytrid Fungus

The short history during which the amphibian chytrid fungus is known to science has been met with rapid interest in the fields of pathology, epidemiology, molecular biology and diagnostic test development because increasingly more discoveries were made of associations of this pathogen with amphibian declines. As we will see, some of these disciplines have evolved through the process of succession of current knowledge to a better understanding of the organism or related technique. Others disciplines still remain within the realm of hypotheses and complexity.

1.2.1 Discovery and Notice

In the first reports of amphibian deaths related to chytridiomycosis, the suspect organism was not recognised as a chytrid fungus. Nichols et al. (1996) reported on histological findings of skin taken off frogs (Whites tree frogs, *Litoria caerulea* and the ornate horned frog, *Ceratophrys ornata*) that died in captivity from the National Zoological Park (NZP), Washington D.C. in 1988 and 1990 and off dead California arroyo toads (*Bufo microscaphus californicus*) in 1991 from the University of California - Santa Barbara. A skin disease, thought to have been caused by a protist-like fungus, was identified as the most likely cause of death. The amphibian chytrid was also detected in 1993 in several frog species that died at upland sites in tropical Queensland, Australia, but was thought to be a *Perkinsus* sp.-like protozoan at the

time (Berger et al. 1997). Mass mortality events with similar histological findings had occurred in the Fortuna Forest Reserve, western Panama in 1996 and together with the investigators from Australia the suspect organism was described as a chytrid fungus for the first time and the disease referred to as chytridiomycosis (Berger et al. 1998). DNA sequencing of rDNA showed that the fungus was a chytrid fungus (Chytridiomycota: Chytridiales), a finding that was supported by the ultrastructure of the spores of the organism, known as zoospores. Berger et al. (1998) also established that the amphibian chytrid caused disease and fatalities in anurans by exposing healthy frogs to skin from infected frogs. It was not possible to fulfil Koch's postulates, a standard clinical test designed to prove that a suspect organism is responsible for death in a host organism, because it requires that the suspect organism be isolated from typical cases and grown in pure cultures. Concurrent with this publication two more papers appeared that described chytridiomycosis in poison dart frogs (*Dendrobatidis* spp.), *L. caerulea* and *C. ornata* (Nichols et al. 1998, Pessier et al. 1999).

The species description of the amphibian chytrid fungus was presented in a separate paper by Longcore et al. (1999) and the amphibian pathogen was placed in a new genus *Batrachochytrium*, meaning chytrid fungus of frogs, and named *Batrachochytrium dendrobatidis* after the genus of the frog from which the type culture was isolated (*Dedrobatis azureus*; blue poison dart frog). The description is based on morphology in pure culture in which the development is outlined and a detailed examination of zoospore ultrastructure as revealed by transmission electron microscopy. In addition Longcore et al. (1999) were able to reisolate *B. dendrobatidis* from frogs that became diseased after being experimentally infected with an isolate in pure culture, thus proving Koch's postulates of a pathogenic organism.

In the months to follow multiple-authored papers appeared that condensed the known knowledge from published and unpublished data concerning the biology, biogeography as well as clinical and histological diagnosis (including host response) of chytridiomycosis (Berger et al. 1999a, Daszak et al. 1999). These papers provided an explanation to the international scientific community concerned with amphibian population declines that had long suspected that an emerging infectious disease could be responsible for many of the declines and even extinction events, and together with the contribution by Pessier et al. (1999) and later by Berger et al. (1999b), enabled them to make their own diagnosis. This sparked a flurry of case reports of chytridiomycosis associated amphibian declines and mass mortality events from all over the world (Bishop 2000, Bosch 2000, Mutschmann et al. 2000, Speare and Berger 2000). It also provoked the suspicion that chytridiomycosis may have been responsible for population declines witnessed in the past and resulted in historic surveys of archived material collected during such events, some of which detected chytridiomycosis (Carey et al. 1999, Morell 1999,

Speare and Berger 2000, Sredl and Caldwell 2000). Soon a list of the global distribution of chytridiomycosis compiled by amphibian chytrid fungus experts, Prof. Rick Speare and Dr Lee Berger was filled with entries of almost 100 species of amphibians (Anura and Caudata) from 11 countries representing every continent where amphibians occur except Asia (Speare and Berger 2000). It had become clear that *B. dendrobatidis* was able to infect any amphibian and therefore posed a threat to the well being of amphibians worldwide. This lead to chytridiomycosis being nominated for listing as a key threatening process under the Environment Protection and Biodiversity Conservation Act 1999 (Speare and Core Working Group 2001). Henceforth chytridiomycosis could never again be ignored in investigations that follow a multiple stressor approach in identifying the causes of amphibian decline events where no direct link with a stressor is obvious such as with habitat destruction.

1.2.2 Biology

It was clear from the initial diagnosis of chytridiomycosis that the fungus occurred in various forms in epidermal skin that included a uninucleate form containing homogenous basophilic cytoplasm, a form containing multiple nuclei and a cyst-like form containing multiple round basophilic spores (Berger et al. 1998, Nichols et al. 1998, Pessier et al. 1999), but it wasn't before the development in pure culture was studied by Longcore et al. (1999) that the lifecycle was described. *Batrachochytrium* grows in the keratinised layer of the epidermis, the *stratum corneum* of metamorphosed frogs, concentrated in the region of the hind legs and pelvic patch, and in the keratinised mouthparts of tadpoles. At first it was thought that the localised occurrence of *B. dendrobatidis* in keratinised tissue meant that it utilised the protein as a nutrient, however, a resent study did not find proof that keratin is degraded by the extracellular proteases produced by the fungus (Daszak et al. 1999, Piotrowski et al. 2004). Isolates of *B. dendrobatidis* are able to grow at 4-25°C and at pH 4-8 and experience mortality when temperatures rise above 30°C (Longcore et al. 1999, Berger 2001, Piotrowski et al. 2004). A lot of the base work concerning the physiological confounders and preferences of *B. dendrobatidis* was done by Berger (2001) that paved the way for subsequent research to follow (Johnson et al. 2003, Johnson and Speare 2003). The lifecycle (Figure 1-1) takes about 4-5 days to complete when grown in pure culture (Longcore et al. 1999). The infective stage of the amphibian chytrid is a motile zoospore that can either reinfect the same individual or transmit to other hosts when released into water. After locating a susceptible animal, zoospores penetrate the skin and develop into spherical mature zoosporangia with discharge tubes. Zoospores are produced inside the sporangia. The zoospores are released through the discharge papillae. Sometimes thin septa form within the sporangium that divide the contents into two or more compartments, each with its own discharge papilla. Such a zoosporangium is referred to as a colonial thallus. After the zoospores have been released, all that is left are

empty sporangia that often become colonised by bacteria. The mechanism of transmission could explain why the infection is more severe on the ventral parts of the body because these areas are in more frequent contact with a wet substrate (Pessier et al. 1999).

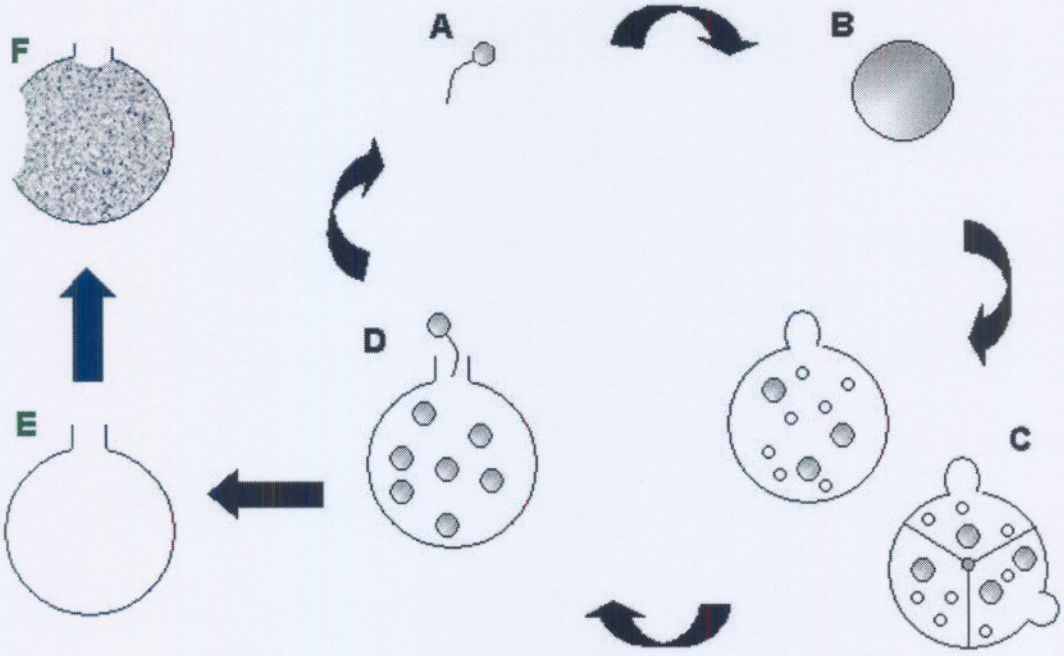


Figure 1-1 Diagrammatic representation of the lifecycle of *Batrachochytrium dendrobatidis*. The following developmental stages can be identified: A) zoospore, B) developing sporangium, C) mature sporangia; sporangium on right has internal septa, D) zoospore being released through discharge papilla. Empty sporangia (E) remain in the superficial epidermis and often become colonised by bacteria (F) before the skin is shed.

Amphibians that have become infected with chytridiomycosis and are susceptible to disease can be identified and the condition described in terms of clinical symptoms and gross and microscopic lesions. However, although the clinical signs and pathology of chytridiomycosis is similar in amphibians, the extent of clinical signs varies greatly with the species of amphibian host. Chytridiomycosis manifests as clinical symptoms related to the central nervous system including abnormal behaviour and body posture i.e. nocturnal frogs squatting unprotected during the day with limbs in an unusual position away from the body (Figure 1-2), lethargy and loss of righting reflex, and causes sloughing of the skin (Berger et al. 1999a, Daszak et al. 1999). However, in only a few species is sloughing clinically obvious. In amphibians susceptible to disease, chytridiomycosis has a clinical course of around 3 weeks resulting in death of the infected animal. Gross lesions are often subtle and usually restricted to the legs

and ventrum of post-metamorphic amphibians and may vary from mild skin thickening and discoloration to swollen limbs and slight reddening of the skin (Nichols et al. 2001, Bradley et al. 2002, Pasmans et al. 2004). Infected tadpoles often have discoloured rostrodonts (jaw sheath) or missing keratodonts (teeth rows) despite a healthy appearance (Lips 1999, Fellers et al. 2001, Lips et al. 2004). Epidermal tissue in the area of infection usually develops hyperkeratosis and hyperplasia (thickening of the *stratum corneum*) and could include intercellular edema and ulceration, but inflammatory cell response is almost absent (Berger et al. 1998, Nichols et al. 2001, Bradley et al. 2002, Lane et al. 2003a). It appears that amphibians that do not become diseased by the infection do not consistently show a thickening of the outer epidermal layer or else show a mild response (Daszak et al. 2004, Hanselmann et al. 2004, Weldon et al. 2004).

Two mechanisms by which chytridiomycosis brings about death have been proposed (Berger et al. 1998, Pessier et al. 1999). Firstly, it could be that a fungal toxin is absorbed systemically. Research efforts involving neuronal cell cultures to investigate the role of toxins produced by *B. dendrobatidis* have not been able to implicate a cytotoxin ([2]M. Johnson pers. com. 2003). Alternatively, the dense aggregation of thalli in the epidermis could impair fluid and electrolyte homeostasis, respiration, and the skin's role as a barrier to toxic and infectious agents. The impaired ability to exchange gases becomes particularly threatening in lungless urodelans such as *Bolitoglossa dofleini* that rely completely on respiration through the skin and mucosal surfaces (Pasmans et al. 2004). It has been hypothesised that during spells of dry weather *Eleutherodactylus* species from Puerto Rico are more likely to die from chytridiomycosis due to their inability to uptake water as a direct result of thickening of the *stratum corneum* (Burrowes et al. 2004).

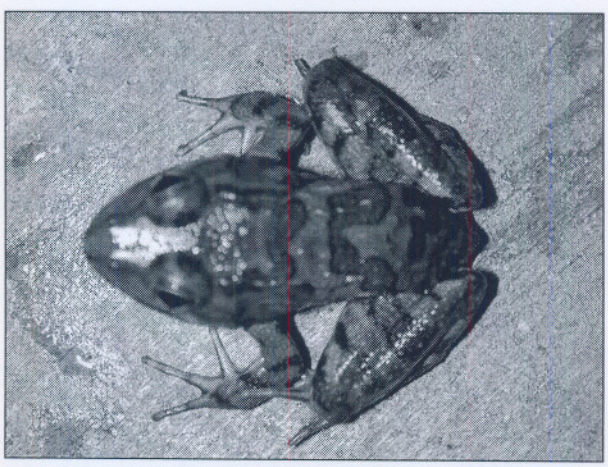


Figure 1-2 A Cape river frog infected with *B. dendrobatidis*. Note the limbs are angled away from the body, instead being tucked in underneath it.

[2] Dr Megan Johnson, James Cook University, Townsville, Queensland, Australia

1.2.3 Chytridiomycosis - an Emerging Infectious Disease

According to Stuart et al. (2004) amphibians of the Neotropical and Australasian-Oceanic realms are most prone to enigmatic declines (declining, even where suitable habitat remains for reasons that are not fully understood) of which disease and climate change are the most commonly cited causes. In Australia chytridiomycosis is notably associated with declines for 5 species and is a potential contributor for an additional 13 species and has been detected in 33 more species that are not declining (Hero et al. 2004). Chytridiomycosis is associated with most of the frog species known to have declined in parts of Latin America including Costa Rica, Ecuador, Panama and Puerto Rico (Lips 1999, Young et al. 2001, Lips et al. 2003, Ron et al. 2003, Burrowes et al. 2004). Chytridiomycosis is also associated with amphibian deaths and/or population declines in Africa (Hopkins and Channing 2003, Lane et al. 2003a), Canada (Ouellet et al. 2003), Europe (Mutschmann et al. 2000, Bosch et al. 2001), Mexico (Lips et al. 2004), New Zealand (Waldman et al. 2001, Bell et al. 2004) and 12 states in the USA (Carey et al. 1999, Fellers et al. 2001, Bradley et al. 2002, Green et al. 2002, Carey et al. 2003). The wide occurrence and range of amphibian taxa from which *B. dendrobatidis* has been detected, regardless of the degree of habitat conservation, suggests that any amphibian species can become infected. A distinction can, however, be made between species regarding susceptibility to disease. A difference in the severity of the disease among species has been attributed to ecological niche and life history strategy of the species (Daszak et al. 1999), as well as resistance to disease (Daszak et al. 2004). Susceptibility to chytridiomycosis may even vary within a species (Davidson et al. 2003). At the population level these differences may lead to death in only some individuals, mass deaths followed by population recovery or mass deaths resulting in population declines. Population declines are particularly associated with stream breeding amphibians at high elevations in Australia (Berger et al. 1998, Alford et al. 2001), Latin America (Lips 1999, Young et al. 2001, Lips et al. 2004) and Spain (Bosch et al. 2001). In addition to breeding in streams, Lips et al. (2003) found that the most susceptible species have restricted elevational ranges and have large body sizes. Diagnostic findings of amphibian declines in eastern Australia as well as trials on captive frogs have demonstrated that prevalence and mortality rate due to chytridiomycosis is highest at lower temperatures (Berger et al. 2004, Retallick et al. 2004). Bradley et al. (2002) observed that the breeding behaviour and over wintering strategy (congregation in high densities) of native Arizona frogs influenced disease transmission that led to die-offs during the cooler months. During the warmer months, temperatures may rise to the range within which the organism cannot effectively reproduce in adequate numbers to cause disease (Bradley et al. 2002). In the case of the common midwife toads (*Alytes obstetricans*) in central Spain, Bosch et al. (2001) suggests that an extended larval period and small clutch size contributed to this being the only species in the area that experienced mass mortality. Following a decline event in wild

populations and in captivity, *B. dendrobatidis* can become a stable infection in a previously susceptible species that has since recovered (Mazzoni et al. 2003, Retallick et al. 2004). It was suggested that such recoveries could be because of evolution of resistance to the pathogen or evolution of less pathogenic strains of the fungus.

A subject of debate is how *B. dendrobatidis* persists in the environment between outbreaks because no resting spore stage has been found for this organism. Explanations for several survival mechanisms have been offered. The amphibian chytrid can survive in different types of water (sterilised tap-, distilled- and lake water) between 3 and 7 weeks during which time sporangia may attach to plankton (Johnson and Speare 2003). However, the ability of zoospores to infect a host is constrained by a short swimming distance (20 mm) and limited time (24 h) before they encyst (Piotrowski et al. 2004). It can also survive in soil for 7 days and up to 21 days when nutrient is added (Porter 2004). Between seasons *B. dendrobatidis* survives in the keratinised mouthparts of tadpoles until the next outbreak (Berger et al. 1998). Healthy frogs of exotic and/or native species may also serve as a reservoir of infection (Daszak et al. 2004, Retallick et al. 2004, Weldon et al. 2004) that transmits the disease to susceptible species.

It has been hypothesised that infectious diseases are only the proximate cause of declines in wildlife and that the process is driven by anthropogenic environmental changes such as increased UV-B, chemical pollution and habitat change (Carey et al. 1999, Pounds et al. 1999, Daszak et al. 2000). The evidence suggests that chytridiomycosis is capable of directly causing mortality in amphibians. However, global climate change, drought, pH, land use change and alien species have been hypothesised as drivers of chytridiomycosis emergence (Daszak et al. 1999, Bosch et al. 2001, Daszak et al. 2003, Burrowes et al. 2004). It is important to distinguish between factors that have an effect regardless of chytridiomycosis presence (co-factors) and factors that worsen the effect in the presence of chytrid (synergistic factors) (Hero et al. 2004) when searching for the most probable cause of decline events. These synergistic factors can, by acting alone or together, stress amphibians sufficiently to compromise their immune system that in turn increases disease susceptibility (Pounds 2001, Blaustein et al. 2003, Carey and Alexander 2003, Carey et al. 2003).

The quick rate at which populations decline where chytridiomycosis is involved and the distinct geographic and temporal progression of the epidemic wave as witnessed in Australia (Laurance et al. 1996, Mahony 1996) is characteristic of an introduced virulent pathogen dispersing through a previously unexposed population (Daszak et al. 1999). Similar directional progress of the pathogen is occurring in amphibians from New Zealand and intercontinentally

from western North America southward through Mexico and into lower Central America (Lips 1998, 1999, Bell et al. 2004, Lips et al. 2004). The pattern of amphibian declines is supported by molecular evidence that suggests anthropogenic introduction is involved in the spread or emergence of chytridiomycosis (Daszak et al. 2003, Morehouse et al. 2003). If chytridiomycosis was recently introduced (within the past 2-4 decades) into these affected areas, then a common source exists from which the disease originally spread and might still be spreading. A compilation of epidemiological evidence derived from a histological survey of archived and live *Xenopus* spp. indicates southern Africa as the origin of the amphibian chytrid fungus (Weldon et al. 2004). According to the "out of Africa" hypothesis the sub-clinically infected African clawed toad, *Xenopus laevis*, has carried the disease around the world via the scientific trade. The emergence of chytridiomycosis in new regions has in part been the result of anthropogenic introduction. The national and international movement of amphibians, including tadpoles, in the pet trade has widely been implicated in the spread of chytridiomycosis (Bishop 2000, Mutschmann et al. 2000, Waldman et al. 2001, Pasmans et al. 2004). There is growing concern about the role the food industry plays in the emergence or spread of chytridiomycosis (Mazzoni et al. 2003, Hanselmann et al. 2004), a risk that is highlighted by the large numbers of amphibians involved. Other mechanisms of introduction include the importation for zoo collections (Pessier et al. 1999) and exotic species used in biocontrol such as cane toads in Australia (Berger et al. 1998). Amphibians that have some sort of resistance to chytridiomycosis and are part of an anthropogenic mechanism of introduction can be regarded as high-risk carrier species. *Xenopus laevis* fills this role in the scientific trade (Weldon et al. 2004). The bullfrog, *Rana catesbeiana* has been identified as a reservoir host in the food trade (Cunningham et al. 2003, Mazzoni et al. 2003, Daszak et al. 2004).

1.2.4 Applied Chytridiomycosis Research

The event that paved the way for research aimed at better understanding *B. dendrobatidis* (aspects of physiology, pathology, immunology etc.) was the successful isolation and culture of the fungus. Zoospores can be harvested from pure cultures for use in transmission experiments to achieve specific outcomes. A protocol for counting zoospores in inocula and techniques for inoculating handling and housing experimental animals for chytridiomycosis research was provided by Daszak et al. (2004). Experimental transmission involving *B. dendrobatidis* has been used to show that chytridiomycosis can be a primary pathogen (Nichols et al. 2001), indicate within species susceptibility variation (Davidson et al. 2003), demonstrate the role of reservoir hosts (Daszak et al. 2004), investigate the effect of temperature on mortality rate (Berger et al. 2004) develop diagnostic tests (Berger et al. 2002,

Boyle et al. 2004) and for the development of curative treatments (Marantelli et al. 2001, Nichols et al. 2001).

Molecular Analysis

The first amphibian chytrid fungus DNA that was sequenced enabled scientists to identify the then unknown pathogen of amphibians as a fungus belonging to the Chytridiomycota (Berger et al. 1998). The ssu-rDNA was amplified from DNA extracted from skin scrapings from two wild *L. caerulea* of Australia and aligned with that of 44 other eukaryotes. While more cases of chytridiomycosis were being reported, the next step was to determine if one or more species existed. In the first attempt to investigate the relationship of isolates the rDNA internal transcribed spacers (ITS gene) in strains from Australia, Central America and North America were used (Morgan 2000). Results with the ITS gene were variable and at conflict with epidemiological data. Subsequently, it was discovered that *B. dendrobatidis* had multiple copies of the ITS and that this gene was unsuitable for taxonomic work ([3]R. Speare pers. com. 2004). Early comparison of the 18S rDNA of chytrid strains from Australian and American frogs did not show significant differences (see Berger et al. 1999a). *Batrachochytrium dendrobatidis* strain JEL 197 from the chytridiomycete collection at the University of Maine was one of 54 chytrids included in a phylogenetic study of the class Chytridiomycota (James et al. 2000). The phylogenetic trees that were generated placed *B. dendrobatidis* in an isolated position. This outcome supported what was already known about the zoospore ultrastructure because *B. dendrobatidis* contains unique character states among the chytrids (Longcore et al.1999, James et al. 2000). The most authoritative work to date regarding the population genetics of the amphibian chytrid was the use of multilocus sequence typing by Morehouse et al. (2003) to examine genetic diversity among 35 amphibian chytrid strains from Africa, Australia, Central America and North America. A low genetic variation of five variable nucleotide positions was detected among 10 loci. This study provides evidence to support the hypothesis that chytridiomycosis is an introduced emerging disease and implicates anthropogenic introduction as a mechanism of spread.

Diagnostic Test Development

Accurate diagnosis of infected amphibians is important when determining the cause of death in wild and captive amphibians as well as for quarantine regulations concerned with the import and export of amphibians. There have been three generations of diagnostic techniques for chytridiomycosis since the initial discovery of the fungus. The first tests relied on the morphology of the fungus. These were followed by tests that are based on antigen detection.

[3] Prof. Rick Speare, James Cook University, Townsville, Queensland, Australia

The third generation of diagnostic tests make use of DNA technology. With every new generation the level of sensitivity and specificity increased and the test becomes less invasive. Third generation tests have the added benefit of delivering a result in rapid time. On the down side, advanced diagnostic tests are more expensive to use and less accessible due to the high level of expertise involved in the respective disciplines. For this reason and due to the length of time they have been in practice, first generation diagnostic tests have been used far more often than second and third generation tests combined.

1998: In the original paper on chytridiomycosis Berger et al. (1998) detected the fungus through examining haematoxylin and eosin (H&E) stained sections of toe clips prepared by standard histology techniques. Morphology, position in the skin and host tissue response of the *stratum corneum* are used to make a positive diagnosis. A clear and conscious account of the use of the histological technique is provided by Berger et al. (1999b) that allows investigators that are unfamiliar with histopathology to positively diagnose chytrid infection. Skin from the pelvic patch, toes and ventral thighs of specimens as well as tadpole mouthparts are usually prepared for examination.

Berger et al. (1998) also examined wet mounts of skin scrapings from fresh specimens using a light microscope. Longcore (2001) provided the first detailed step-by-step instructions for using sloughed skin to identify chytridiomycosis. The use of skin sloughs or scrapings requires training and more skill than H&E histology in identifying *B. dendrobatidis*, but saves a lot of time once the technique has been mastered. To increase differentiation between fungus and skin tissue, skin smears can be stained with a few drops of cotton blue and 10% aqueous potassium hydroxide (1:1 solution) (Mazzoni et al. 2003) or with Congo red (Biggs and Bergin 2003).

In addition, tissue samples were examined by Berger et al. (1998) with a scanning transmission microscope (SEM, for external ultrastructure morphology) and transmission electron microscope (TEM, for internal ultrastructure morphology and function). Although the use of electron microscopy can accurately identify the amphibian chytrid, its primary function remains the study and description of ultrastructure morphology.

Nichols et al. (1998) and subsequently Longcore et al. (1999) succeeded in isolating and growing cultures of *B. dendrobatidis* on TGhL (tryptone, gelatin hydrolysate, lactose) agar. Although *B. dendrobatidis* can be identified through culture, it is not used as a primary diagnostic technique and is usually the means to an auxiliary objective such as molecular and pathogenicity studies.

The co-occurrence of healthy tadpoles with damaged mouthparts in streams with dead frogs with chytridiomycosis prompted Lips (1999) to realise that the lesions are due to chytridiomycosis and therefore an indication of infection. Damaged or missing mouthparts are

only an indication of possible infection (Fellers et al. 2001, Lips et al. 2004) because an incomplete oral disc could be due to mechanical damage (Rachowicz 2002) and should therefore be confirmed with specific techniques.

2001: An enzyme-linked immunosorbant assay (ELISA) was developed that uses an antibody detection system that binds to the specific antigen of *B. dendrobatidis* (Hyatt et al. 2001). ELISA is distinctive in that organisms from a broth culture are used instead of histological sections.

2002: An immunoperoxidase test was developed after polyclonal antibodies to *B. dendrobatidis* were produced (Berger et al. 2002). Antiserum from rabbit reacts strongly with all developmental stages of *B. dendrobatidis* and stains the walls, cytoplasm, rhizoids and zoospores. The test stains *B. dendrobatidis* tissue dark brown to red that contrasts strongly with host tissue (blue). Although the immunostaining is a more elaborate process than H&E, it is superior in its sensitivity for diagnosing chytridiomycosis (Berger et al. 2002) and therefore a valuable test for confirming infection status in suspicious cases. The value of the test for inexperienced diagnosticians was increased by addition of a stain for keratin (Olsen et al. 2004).

2004: A sensitive and specific polymerase chain reaction (PCR)-based assay was recently developed by Annis et al. (2004). The assay makes use of small skin samples (1-3 mm^2) and is able to detect as few as 10 zoospores (less than the average number per sporangium) in a sample. Large samples can be processed in a short time; an advantage for chytridiomycosis surveys of wild populations.
Boyle et al. (2004) developed a real-time PCR Taqman assay that is not only able to identify *B. dendrobatidis* with a high degree of sensitivity in skin samples, but is also able to quantify the infection (can detect 1 zoospore). The ability to detect early or low levels of infection allows for the prevention of spread between enclosures and for possible treatment of infected animals (Boyle et al. 2004).

The next advancement in diagnostic test development could be DNA-based assays that are able to detect *B. dendrobatidis* in pond water and even fish and reptiles (Annis et al. 2004).

Sterilisation

Simple sterilisation regimes based on the physiological characteristics of *B. dendrobatidis* as well as the application of chemical disinfectants were derived through experimental trial on *in vitro* cultures by Johnson et al. (2003). These physical techniques can be used to sterilise

equipment, protective clothing and enclosures contaminated with *B. dendrobatidis* during fieldwork, in the laboratory and in captive husbandry. Techniques recommended on the basis of their sensitivity to the environment and practical application include treatment by heat, desiccation, 70% ethanol, 1% Virkon (Antec International), 2% sodium hyopchlorite, didecyl dimethyl ammonium chloride, 1% formaldehyde, 10% sodium chloride (salt) and 2% potassium permanganate.

Treatment

Successful treatment of chytridiomycosis can prevent deaths of not only the animals being treated, but prevent the disease from spreading to other species and regions. An effective and safe treatment could be used as part of official requirements for movement of amphibians within countries and between countries. In the case of frogs with commercial value (food-, scientific- and pet trade) early detection and treatment can prevent huge economic losses.

Documented accounts on investigations into the cure of chytridiomycosis through the application of chemotherapeutic agents have been few and never tested on a range of taxa. Marantelli et al. (2001) were able to reduce the prevalence of *B. dendrobatidis* in great barred frogs, *Mixophyes fasciolatus* tadpoles through benzalkonium chloride and fluconazole baths. The topical application of itraconizole successfully treated chytridiomycosis in adult yellow poison dart frogs, *Dendrobates tinctorius* (Nichols et al. 2001), however, the treatment has not been tested on tadpoles. Treatment of captive *Xenopus tropicalis* with formalin/malachite green baths eliminated infection and cured sick animals that survived the epizootic (Parker et al. 2002). An elevation of temperature to 37°C was effective against *B. dendrobatidis* in captive Australian red-eyed tree frogs, *Litoria chloris* (Woodhams et al. 2003). Benzalkonium chloride treatment, a recognised therapy for infections with non-hyphal fungi, was not able to reduce mortality rates in infected *Rana catesbeiana* from a frog farm in Uruguay (Mazzoni et al. 2003). No immunisations are currently available for chytridiomycosis or for other significant infectious diseases of amphibians.

Berger (2001) was able to demonstrate that antifungal products from the skin of amphibians (*L. caerulea*) could kill *B. dendrobatidis in vitro*. It has been suggested that the inherent peptide defence mechanism in amphibian skin may play a role in preventing and limiting infection by the amphibian chytrid and other pathogens associated with amphibian declines (Rollins-Smith et al. 2003). This follows after it was demonstrated that antimicrobial peptides, of which each species has a unique set, inhibit the growth of *B. dendrobatidis* and *Basidiombolus ranarum* (Rollins-Smith et al. 2002). The ability to disrupt the cell membrane and resistance to fungal proteases, may explain differences in peptide potency (Rollins-Smith et al. 2003).

1.3 Study Proposal

1.3.1 Research Problem

It is not known where *B. dendrobatidis* initially originated. The most likely hypothesis holds that *B. dendrobatidis* escaped from a host and locality in which it evolved, into host species and environments novel to the pathogen. Alternatively, it may be a widespread organism that has emerged as a pathogen to amphibians because of either an increase in virulence or an increase in host susceptibility caused by unknown factors (Berger et al. 1998). In Australia epidemiological evidence suggests that *B. dendrobatidis* was introduced into southeast Queensland in the late 1970s and spread north along the Queensland coast at 100 km per year (Laurance et al. 1996).

Three records of the amphibian chytrid have emerged from Africa since 1999. These include the tropical platanna *X. tropicalis* from West Africa (Reed et al. 2000), Anchieta's ridged frog *Ptychadena anchietae* from Kenya (Speare and Berger 2000) and the African clawed toad *X. laevis* from the Western Cape, South Africa (Speare and Berger 2000). Because massive amphibian declines have not been reported in Africa, there is a possibility that chytridiomycosis is endemic to this continent and has spread to other parts of the world by way of trade in amphibians such as *X. laevis* (Speare and Core Working Group 2001, Weldon et al. 2004).

Whether and how chytridiomycosis arrived in South Africa and what its effect is on wild populations is uncertain. We know that *Xenopus* from the Cape is a high-risk species for dissemination of chytridiomycosis through the scientific trade in amphibians. This puts amphibians both nationally and internationally at risk of acquiring chytridiomycosis through transmission via *X. laevis*. An international conference in Cairns, Australia recommended that risk assessments be carried out in all countries to determine the occurrence of chytridiomycosis, its geographical distribution and the species of amphibians infected (Speare 2001). Given that South Africa is a major supplier of *X. laevis* for scientific research in many countries, there is an urgent need to quantify the risks of infected frogs being exported and to protect the trade. Because the majority of the exported frogs are drawn from natural populations there is also a need to determine the status of chytridiomycosis in this region. In addition, the Office International des Epizooties (OIE), the equivalent in the animal field of the World Health Organization, has recently accepted amphibian chytridiomycosis on its wildlife list as a disease requiring quarantine restrictions in movement of amphibians between countries. Given that South Africa is a member state of OIE, it is timely to pre-empt any quarantine or movement restrictions that may be recommended.

1.3.2 Objectives

- Objective 1: Occurrence of *B. dendrobatidis* in Africa

  1a) Determine which species of *Xenopus* from Africa are infected with *B. dendrobatidis* by examining specimens from archived collections.

  1b) Determine also the extent of infection in other South African frogs from archived collections as well as from field surveys.

- Objective 2: Dissemination risk of *B. dendrobatidis* via the *X. laevis* trade

  2a) Determine the role of the *Xenopus* trade in the historical and current dissemination of chytridiomycosis locally and globally.

  2b) Develop strategies to reduce this dissemination.

- Objective 3: DNA analysis of South African strains

  3a) Isolate and cultivate *B. dendrobatidis* from South African frogs from a wide range of localities.

  3b) Determine the level of genetic variation in *B. dendrobatidis* strains from South Africa through multilocus sequence typing. Relationships with isolates from other parts of the world (e.g. Australia, Panama and USA) will be determined to investigate geographical patterns and the origin of the fungus.

- Objective 4: Electron microscopy on *B. dendrobatidis*

  Examine the surface and macro molecular characteristics of *B. dendrobatidis* with the use of conventional scanning electron microscopy and laser scanning confocal microscopy.

- Objective 5: Quantification of infection

  Develop a technique that will enable investigators to quantify an infection of chytridiomycosis in live amphibians. Naturally infected Cape river frogs, *Afrana fuscigula* will be used in the investigation.

- Objective 6: Investigation of the Kihansi spray toad decline

  6a) Investigate the circumstances that lead to the disappearance of the Kihansi spray toad, *Nectophrynoides aspergenis* from the Udzungwa Mountains, Tanzania.

  6b) Develop a pathogen hygiene protocol for Kihansi Gorge.

1.3.3 Phases of Research

The project can be divided into four phases; the outcome of each phase determines the course of the next (Figure 1-3).

Phase A centres around the formulation of research questions applicable to studying chytridiomycosis in Africa through a comprehensive study of available literature and by connecting with the international network of scientists involved in *B. dendrobatidis* research.

Phase B involves determining the host species and geographical distribution of *B. dendrobatidis* in Africa by conducting surveys of both archived and live frogs.

Phase C includes investigations into the epidemiology of chytridiomycosis by conducting a risk assessment of the *Xenopus* trade, investigating the role of chytridiomycosis in population declines, and by comparing South African strains of *B. dendrobatidis* (attained through isolation and cultivation) to global strains.

Phase D involves the drafting of strategies to control chytridiomycosis and reduce the risks of disseminating the disease in the trade sector, environmental science practices and wildlife management.

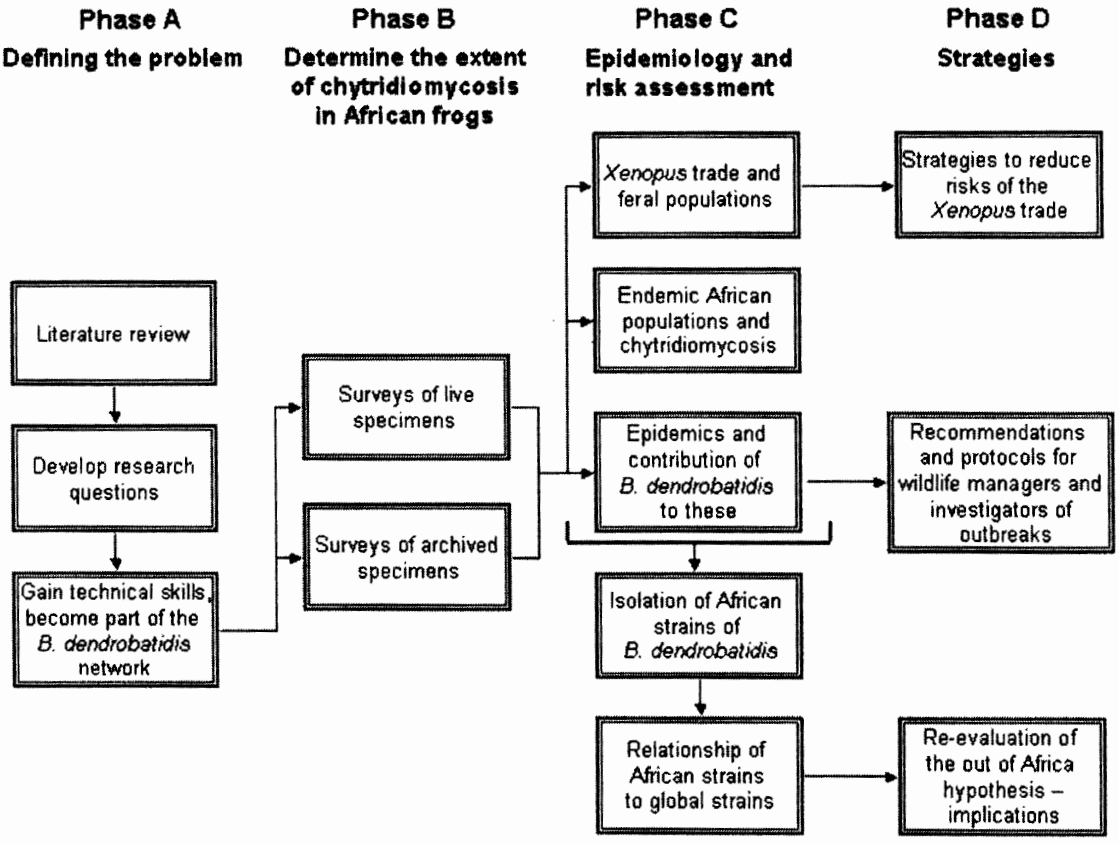


Figure 1-3 Flowchart of research design showing phased approach.

1.3.4 Thesis Chapters, Collaboration and Research Outputs

At the start of this project in 2002 no one else was actively involved in amphibian chytrid research in Africa and little was known about this amphibian pathogen among the herpetological community in Africa. Achieving the objectives set out in this thesis therefore required that the candidate become part of the international network of scientists involved in *B. dendrobatidis* research. The nature of collaboration either involved the acquisition of expert advice or technical skills needed for *B. dendrobatidis* research, or making use of specialised services provided by world experts in the relevant field of research.

Three publications in accredited journals (Lane et al. 2003a, Weldon et al. 2004, Harkewicz et al. 2005), a book chapter (Weldon and Du Preez 2004a) and a number of newsletters (Weldon 2002a, b, Weldon 2003, Lane et al. 2003b, Weldon and Du Preez 2004b) have stemmed from this research. Four more manuscripts are in various stages of preparation for publication. Since the onset of this project, the findings have received maximum exposure at scientific conventions. Various aspects of this project have been delivered as oral presentations at six national and two international symposia. Scientific posters have also been presented at two national and one internal symposium. Invitations have been received to present aspects of this project at two symposia (Declining Amphibia-Symposium, *Xenopus*-Symposium) at the 5th World Congress of Herpetology in June 2005. Our research on the threat that the amphibian chytrid fungus poses to the global amphibian biodiversity received exposure in a number of local and national newspapers.

The thesis is divided into ten chapters including an introduction and literature review, a methodology chapter, seven text chapters dealing with various aspects of chytridiomycosis research in Africa, and a final discussion chapter. Examples of data sheets and major publications by the candidate that are related to this project have been included as annexures following the last chapter. A complete list of references that have been used throughout this thesis has been placed at the back of the thesis. The format for references provided by the scientific journal *Biodiversity and Conservation* was used in this thesis.

Chapter 1 deals with the literature review of amphibian declines and specifically the amphibian chytrid fungus and contains the study proposal that serves as an introduction to the rest of the thesis.

<u>Collaboration</u>

- Prof. Rick Speare, James Cook University (JCU), Townsville, Queensland, Australia was involved with the project from the planning phase and consistently throughout its

duration in an expert advisory capacity on chytridiomycosis biology, pathology and epidemiology. Prof. Speare was appointed co-promoter for the project in January 2002.

Publications

- At the onset of the project the candidate wrote a memo to the newsletter of the African Herpetological Association of Africa as a notification of his intended research and as a call for the involvement of fellow herpetologists in the event of them finding dead or sick frogs during fieldwork (Weldon 2002a).
- The candidate co-authored a roundtable manuscript in *Journal of Herpetological Medicine and Surgery* that deals with aspects of the biology, pathology and epidemiology of *B. dendrobatidis* (Harkewicz et al. 2005).

Chapter 2 is a synthesis of general materials and methods that are applicable to most of the contents chapters that follow. The chapter includes sections on permits, frog collection methods, field hygiene protocol and chytridiomycosis diagnostic techniques.

Collaboration

During November/December 2002 the candidate spent five weeks in Australia with the purpose of developing and advancing skills that would aid in South Africa's competence to recognise and manage amphibian diseases, especially chytridiomycosis.

- Training in histological techniques was arranged by Prof. Speare and provided by Dr Diana Mendez, (JCU), Townsville, Queensland, Australia.
- On-hands experience was gained at the Animal Health Laboratory, Geelong, Victoria under the guidance of Dr Alex Hyatt regarding the operation of two diagnostic tests specific for chytridiomycosis: immunoperoxidase test (polyclonal antibodies are used in the diagnoses) and an ELISA test (enzyme linked immunosorbant assay that uses an antibody detection system that binds to the specific antigen of *B. dendrobatidis*).
- The candidate also participated in the annual Frog Search at Eungella National Park, Queensland organised by the Queensland Parks and Wildlife Service. The aim was to search and monitor some of central Queensland's most threatened montane frog species such as the tusked frog, *Adelotus brevis* that is now critically endangered after suffering serious population declines due to chytridiomycosis. During fieldwork a strict pathogen hygiene protocol was followed to prevent potential spread of diseases between streams and between frogs.

Chapter 3 is a study of the morphology and development of *B. dendrobatidis* with the use of scanning electron microscopy and laser scanning confocal microscopy. The data are supported with a comprehensive set of high-resolution digital images.

Collaboration

- Dr Lourens Tiedt from the Laboratory for Electron Microscopy, North-West University provided expert assistance with the examination of *B. dendrobatidis* using scanning electron microscopy.
- Me. Anne Grobbler from the School of Pharmacy, North-West University provided expert assistance with the examination of *B. dendrobatidis* using confocal microscopy.

Chapter 4 deals with the occurrence of chytridiomycosis in African frogs and includes species lists and distribution maps. In this chapter is presented evidence to support the hypothesis that *B. dendrobatidis* originated in Africa.

Collaboration

- In April 2002 Dr Lee Berger (JCU), Townsville, Queensland, Australia visited our facility at North-West University. Dr Berger provided training in the histological diagnosis of chytridiomycosis as well as diagnosis using skin sloughs prepared on wet mounts.
- The candidate collaborated with Dr Emily Lane, a veterinary surgeon from the University of Pretoria, Onderstepoort to diagnose chytridiomycosis in dead frogs that were submitted to the animal health facility at Onderstepoort.
- Prof. Speare helped to formulate the "out of Africa" hypothesis that was proposed for the origin of *B. dendrobatidis*.
- Dr Hyatt provided antibodies for the specific immunoperoxidase test (IpX) and helped to diagnose chytridiomycosis in archived specimens of *X. laevis* collected prior to 1945 using IpX.

Publications

- After detecting chytridiomycosis during a survey of frogs from the Western Cape, the initial results were posted in the newsletter of the Declining Amphibian Population Task Force (DAPTF) (Weldon 2002b). This served to expose our research activities from the North-West University to the international herpetological community concerned with the study of declining amphibians and as a call for collaboration with researchers involved in the rest of Africa.
- These preliminary results were also presented in the newsletter of the School of Environmental Sciences and Development of the North-West University to inform our colleagues of our progress (Weldon 2003).
- In a joint effort with scientists affiliated with the University of Pretoria, Onderstepoort to identify possible pathogens in a dead frog, the first report of chytridiomycosis in a free-ranging frog from South Africa was published (Lane et al. 2003a, Annexure A).

- A summary of this publication, including a call for collaboration, was distributed among veterinary surgeons in the newsletter of the Veterinary Association of South Africa (Lane et al. 2003b).
- Histological evidence supporting the hypothesis that *B. dendrobatidis* originated in Africa, was presented in a multi-authored publication in the December 2004 edition of *Emerging Infectious Diseases* (Weldon et al. 2004, Annexure B). This paper generated a lot of attention from the international herpetological community. It lead to a radio interview with an environmental correspondent (John Nielsen) with NPR, a Washington, D. C. -based radio station. A PDF-version of the paper has since been circulated on the list servers of two international herpetological associations (RANA – Network of Scientist for Threatened Neotropical Amphibians, and AZA - The American Zoo and Aquarium Association).
- A chapter contribution dealing with the role of infectious diseases on amphibian declines in South Africa was included in the *Atlas and Red Data book of the Frogs of South Africa, Lesotho and Swaziland* (Weldon and Du Preez 2004a).

Chapter 5 concerns the trade, both domestic and international in *X. laevis* and related conservation issues are discussed.

Collaboration

- Atherton De Villiers from the Western Cape Nature Conservation Board (WCNCB) provided information on the numbers and export destinations of *X. laevis* from South Africa.

Chapter 6 is a critical evaluation of the movement of chytridiomycosis through the *Xenopus* trade in the form of a risk assessment.

Collaboration

- Strategies to reduce risks of disseminating chytridiomycosis through the trade in *X. laevis* was submitted to the WCNCB and discussed with officials (Dr Ernst Baard, Atherton De Villiers) at the WCNCB head office in Jonkershoek during November 2003.
- Prof. Richard Tinsley, Bristol University UK and Dr John Measey, University of Antwerp, Belgium, provided specimens of feral *X. laevis* for determining the prevalence of chytridiomycosis.

Chapter 7 presents a new technique that was developed for the quantification of *B. dendrobatidis* infection on sloughed skin from live amphibians.

Chapter 8 deals with the molecular biology of the South African strains of *B. dendrobatidis* and discusses the implications of the analyses on the "out of Africa" hypothesis.

Collaboration

- In August 2004 Dr Joyce Longcore from the University of Maine, Orono, USA and Prof. Rick Speare, visited our laboratories at the North-West University during which time we received extensive training in the isolation and cultivation of *B. dendrobatidis* from infected frogs collected in Namaqualand, Northern Cape Province.
- Infected archived specimens were sent to Dr Hyatt for molecular analysis of *B. dendrobatidis* using the Taqman real-time PCR assay.
- Dr Tim James and Dr Rytas Vilgalys from Duke University, Durham USA performed multilocus sequence typing on the South African isolates.

Chapter 9 presents a case study of a chytridiomycosis related decline and presumed extinction of the Kihansi spray toad from Tanzania.

Collaboration

- The investigation into the decline of the Kihansi spray toad was performed under the auspices of the Lower Kihansi Environmental Management Project (LKEMP), Dar es Salaam, Tanzania. The project manager, Dr Wilfred Sarunday, provided logistical support and the technical advisor, Dr Jeremy Thompson, lead the expedition and provided assistance during fieldwork.

Publications

- The first report on chytridiomycosis associated population declines in Africa was posted in the newsletter of the DAPTF following the investigation (Weldon and Du Preez 2004b).

Chapter 10 is a synthesis of the most significant conclusions that were derived at in the contents chapters. Included in the chapter is a section on recommendations for future research.



Chapter 2

GENERAL MATERIALS AND METHODS



2.1 Ethical Clearance and Permits

Clearance by an ethical committee is mandatory for conducting research involving vertebrates at the North-West University. A proposal for obtaining ethical clearance for studying chytridiomycosis in anurans was therefore submitted to the ethical committee and ethical clearance for the project was subsequently granted (Reference no.: 02D02).

Before any wild frogs were collected permits were obtained from the relevant nature conservation authorities responsible for the regions in which fieldwork was intended (Table 2-1). A declaration form granting permission to collect frogs was completed for each site and signed by the respective land owner (Annexure C).

Table 2-1 Permits applied for during the project for the collection of wild frogs.

Permit No.	Issuing Body	Date Valid	Region	Specifications
354/2001	Western Cape Nature Conservation Board	2000-2002	Western Cape Province	*Xenopus laevis* (unlimited)
0209/04	Northern Cape Nature Conservation Service	2004	Namaqua region	*Xenopus laevis* (50) *Afrana fuscigula* (50)
0663/04	Northern Cape Nature Conservation Service	2004	Goegap Nature Reserve	*Afrana fuscigula* (10 live, as well as any dead animals)

2.2 Anuran Sampling Techniques

2.2.1 Visual Encounter Sampling

A visual encounter approach was used to collect mainly river frogs and toads that are associated with streams and ponds. Animals that were spotted on land hiding in vegetation or in the water clinging to the base of reeds, drifting logs etc., were individually collected by hand. The duration of search effort was not limited like when collecting qualitative data for monitoring amphibian assemblages, but was determined by the time it took to collect a sample of a certain size that would enable the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* to be

detected at a certain prevalence. The only prevalence data for chytridiomycosis in South African frogs at the start of the study was infection in 60% of a sample of African clawed toads, *Xenopus laevis* from the Western Cape that were collected in 1999 ([4]R. Speare pers. com. 2002). The assumption was made that for the range of infection within a sample to vary between 10 and 60%, a minimum of 50 frogs would have to be examined to detect a minimum number of 5 infected animals. Active sampling has the benefit of allowing the observer to assess the activity patterns of the frogs for abnormal behavior that may be present as a result of chytridiomycosis.

2.2.2 Dip-net Sampling

After a site was searched for frogs, a dip net (300 x 250 mm opening) with mesh size of 2 mm was used to sample aquatic larvae by sweeping among aquatic vegetation and in open water (Figure 2-1). Dip-netting is an invasive technique that disturbs the habitat and might chase frogs away and should therefore not immediately precede visual encounter sampling at the same site.



Figure 2-1 Dip-netting in a shallow roadside pond in the Kammiesberg near Kammieskroon.

2.2.3 Aquatic Traps

Aquatic bucket traps were constructed from 20 L plastic buckets fitted with a funnel near the bottom and air holes drilled at the top (Figure 2-2). These traps were exclusively used to collect post-metamorphic *X. laevis*, and they make use of the aquatic nature and feeding behaviour of the species in order to trap them. Strongly odoured bait (liver and marrow bones) were used to lure frogs into the traps because *Xenopus* strongly rely on their olfactory sense for locating food. The sampling method is based on the assumption that animals that enter the trap are unable to escape or escape is delayed because of the difficulty in locating the inward-directed funnel opening. Traps were placed and left overnight near the water's edge with one third protruding above the water level allowing the trapped animals to surface for air. Trapped animals were immediately transferred to a container half-filled with water from the specific locality after traps were lifted because *X. laevis* (especially young individuals) can suffocate from the froth produced by the beating of skin secretion when they are crowded. Data from

[4] Prof. Rick Speare, James Cook University, Townsville, Queensland Australia

field sampling was recorded onto data sheets (Annexure D) and transferred into spreadsheets generated in Microsoft Excell upon return from fieldwork.



CONOMODE=OFF
@PJL SET TRAY1TEMP=NORMAL
@PJL SET RESOLUTION=600
@PJL ENTER LANGUAGE

Figure 2-2 Setting an aquatic trap in a pond for the collection of *Xenopus laevis*.

2.2.4 Pathogen Control Protocol

Because the subject of fieldwork involved detecting an infectious disease of amphibians care was taken to prevent becoming a vector for transmitting chytridiomycosis between study sites. The Fieldwork Code of Practice developed by the Declining Amphibian Population Task Force provides a standard pathogen control protocol that should be followed when conducting amphibian related fieldwork (available from URL: http//ventura.fws.gov/es/protocols/dafta.pdf). By using this protocol as a guide, the following precautions and procedures were implemented during fieldwork.

When not in use footwear, nets and aquatic traps were cleaned and stored in a dry place.

A portable disinfection station was taken along during field visits. The station included adequate supplies of disinfectant solution (sodium hypochlorite) and tap water, two large square buckets (at least 30 L volume), a scrub brush and a pair of protective gloves.

Footwear and equipment was disinfected before entering a site. Disinfection involved scrubbing boots and traps and soaking nets in a 2% sodium hypochlorite solution for 2 min., followed by rinsing with tap water.

CHYTRIDIOMYCOSIS, AN EMERGING INFECTIOUS DISEASE OF AMPHIBIANS IN SOUTH AFRICA

C. WELDON

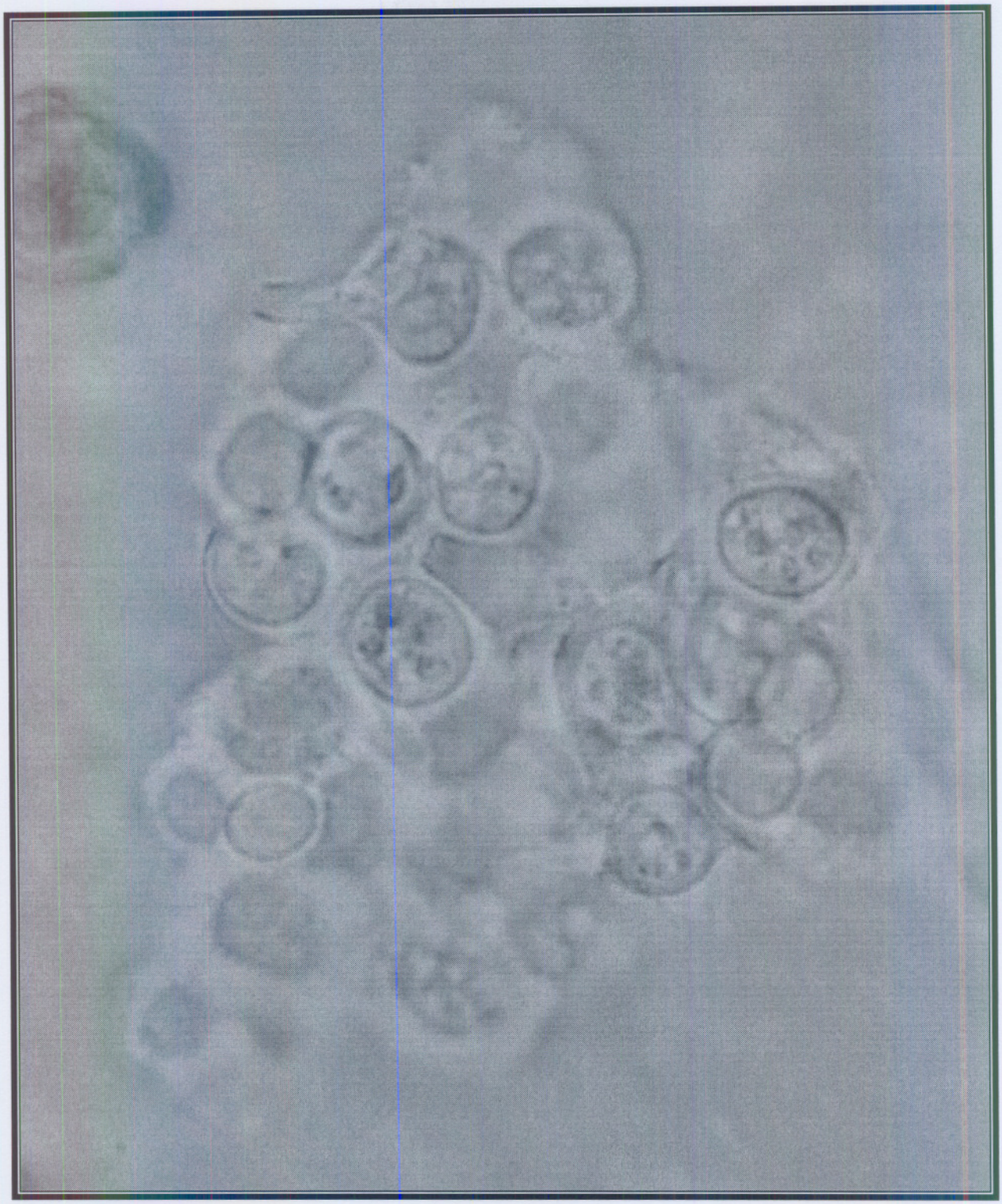
Thesis submitted for the degree Doctor of Philosophy in Zoology at the North-West University

Promoter: Prof. L.H. du Preez

Co-promoter: Prof. R. Speare

July 2005

Potchefstroom Campus



Unstained skin scrape of the Cape river frog showing sporangia with zoospores of *Batrachochytrium dendrobatidis*.

ABSTRACT

Chytridiomycosis, an emerging infectious disease of amphibians in South Africa

The sudden appearance of chytridiomycosis, as the cause of amphibian deaths and population declines in several continents suggests that its etiological agent, the amphibian chytrid fungus *Batrachochytrium dendrobatidis,* was introduced into the affected regions. However, the origin of this virulent pathogen is unknown. Efforts were directed to determine the occurrence of chytridiomycosis in Africa, whether the disease had been introduced into South Africa in recent years and how wild frog populations were affected by infection. A chytridiomycosis survey of 2,300 archived and live specimens involving members of the Pipidae family in sub-Saharan Africa, as well as a number of unrelated frog species in South Africa was conducted by histological diagnosis of skin samples. The epidemiological evidence indicated that chytridiomycosis has been a stable endemic infection in southern Africa for 23 years before any positive specimens were found outside Africa. The occurrence of chytridiomycosis in South Africa can be described as widespread both in terms of geographical distribution and host species and generally infection is not associated with adverse effects at the individual or population level. It was proposed that the amphibian chytrid originated in Africa and that the international trade in the African clawed toad *Xenopus laevis* that commenced in the mid 1930s was the means of dissemination. A risk assessment of the *X. laevis* trade demonstrated that chytridiomycosis could spread through this pathway and culminated in the development of a management protocol to reduce the risks of spreading disease through this animate commodity. Initial comparative genetic analysis of *B. dendrobatidis* strains isolated from South African frogs with a global set of 35 strains, suggests that analysis of a more geographically diverse set of southern African strains is needed before this line of argument can support or reject the "out of Africa" hypothesis.

Keywords: Chytridiomycota, *Batrachochytrium dendrobatidis*, chytridiomycosis, disease, origin, epidemiology, prevalence, Africa, amphibian declines, *Xenopus laevis*

OPSOMMING

Chytridiomikose, 'n nuwe aansteeklike siekte van amfibië in Suid-Afrika

Die skielike verskyning van chytridiomikose, die oorsaak van amfibiër sterftes en hulle afname op verskeie kontinente veronderstel dat die etiologiese agent, die amfibiese chytrid swam *Batrachochytrium dendrobatidis,* aan die geaffekteerde gebiede geïnduseer is. Die oorsprong van hierdie aggressiewe patogeen, is egter onbekend. Pogings is aangewend om die voorkoms van chytridiomikose in Afrika te probeer vasstel en meer spesifiek hoe onlangs die siekte in Suid-Afrika geïnduseer is en hoe wilde paddapopulasies deur die infeksies beïnvloed is. 'n Chytridiomikose opname van 2,300 gebergde sowel as lewende eksemplare van die Pipidae familie in sub-Sahara Afrika, asook 'n aantal Suid-Afrikaanse nie-verwante paddaspesies is deur middel van histologiese diagnose van velmonsters uitgevoer. Dit is epidemiologies bewys dat chytridiomikose 'n stabiele endemiese infeksie vir 23 jaar in Suid-Afrika was voordat enige positiewe eksemplare buite Afrika gevind is. Chytridiomikose in Suid-Afrika is geografies wyd verspreid en daar is geen aanduiding van gasheerspesifisitiet nie. Oor die algemeen word infeksie nie met nadelige uitwerking op individuele sowel as populasievlakke geassosieer nie. Daar word voorgestel dat die amfibiese chytrid sy oorsprong in Afrika het en dat internasionale handel van die gewone platanna, *Xenopus laevis*, gedurende die middel 1930's die verspreidingsmeganisme was. 'n Risikobepaling van die *X. laevis* handel, demonstreer dat chytridiomikose op dié manier kan versprei en het gelei tot die ontwikkeling van 'n bestuursprotokol om die verspreidingsrisiko deur hierdie handelsmetode te verminder. Aanvanklike vergelykende genetiese ontleding van stamme van *B. dendrobatidis*, geïsoleer van Suid-Afrikaanse paddas met 'n globale stel van 35 stamme, suggereer dat die ontleding van 'n meer geografies uiteenlopende stel van suider-Afrikaanse stamme benodig word voordat hierdie argument die 'uit Afrika' hipotese kan ondersteun of verwerp.

Sleutelterme: Chytridiomycota, *Batrachochytrium dendrobatidis*, chytridiomikose, siekte, oorsprong, epidemiologie, prevalensie, Afrika, amfibiër afnames, *Xenopus laevis*

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The fear of the LORD is the beginning of knowledge, but fools despise wisdom and instruction.
Proverbs 1:7 ([1]KJV 1999)

[1] King James Version 1999, American Bible Society, New York

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Chapter 1

INTRODUCTION AND LITERATURE REVIEW

> So consider the frog. The frog has sensed a change, a displacement in the order of life as we know it. We may be responsible, at least in part, for causing the change. And we may be the next to feel it. This story is larger than we can imagine. The facts humble us. The evening silence is spreading.
> The frog is telling us something. Will we listen?
>
> William Souder (2000)

1.1 Declining Amphibian Populations

According to the World Wide Fund for Nature (WWF) the biodiversity index for freshwater habitats based on vertebrate data, including amphibians experienced a 50% decline between 1970 and 1999 (Loh 2000). It would seem therefore that freshwater habitats are under severe threat, a process that affects an array of biodiversity especially frogs and salamanders. Amphibians are often referred to as indicators of environmental stress, providing early signs of the deteriorating health of other species (Blaustein 1994, Sparling et al. 2000) and therefore raise concern when populations start disappearing. Furthermore, amphibians form an integral part of many aquatic and terrestrial food webs. Adults and most larvae are predators of mainly invertebrate prey, while serving as a food source to a variety of larger predators including snakes, wading birds and fish. They are particularly vulnerable to environmental change, because they are in contact with both water and land for at least part of their development, they have moist, permeable skin and their unshelled eggs are directly exposed to soil, water and sunlight (Blaustein and Kiesecker 2002). An assessment of the global amphibian declines conducted by the World Conservation Union Global Amphibian Assessment (IUCN GAA) demonstrated that 32.5% (1,856 species) of amphibians are currently being threatened (Stuart et al. 2004). In addition, 34 species are known to have become extinct since 1500, while a further 122 species are believed to be possibly extinct (species that can no longer be found that await exhaustive surveys to establish their disappearance). Of great concern is that the majority of these disappearances (113 species) have occurred since 1980.

The sudden disappearance of amphibian populations seems to have started during the 1970s in western United States, Puerto Rico and northeastern Australia (Czechura and Ingram 1990,

Drost and Fellers 1996, Burrowes et al. 2004). These declines have not been limited to habitats heavily affected by human activities, but have occurred in pristine habitats as well. By 1989 when the First World Congress of Herpetology took place in Canterbury, England, scientists realised that rapid amphibian declines were a global phenomenon that necessitated urgent investigations into the extent and causes of the declines. Various reasons for declines have been proposed:

- Global warming, caused by the greenhouse effect, has a serious effect on climate. Alterations in weather patterns can cause changes in rainfall that adversely affect amphibian reproduction. Depletion of stratospheric ozone causes an increase in UV-B radiation that greatly reduces hatching success of embryos and tadpole survival in some amphibians (Kiesecker et al. 2001, Blaustein et al. 2003).
- Modification of habitats can have long-lasting effects and is the most obvious factor contributing to amphibian declines (Alford and Richards 1999). For example, deforestation not only removes large trees but also changes the microclimate of the undergrowth.
- Chemical pollution has been shown to have devastating effects on amphibian populations, including developmental and behavioural abnormalities (Bridges and Semlitsch 2000, Sparling et al. 2000).
- Pollutants include not only residues released by industry, but also fertilizers, herbicides, fungicides and pesticides. Several of these pollutants disrupt endocrine functions and thereby the reproductive systems of amphibians and even induce immunosuppression (Colborn and Clement 1992, Stebbins and Cohen 1995).
- Introduced predators can have a serious effect on amphibian populations. Such predators can compete with native species for natural resources, as well as carry and transmit disease, hybridise with natives, and prey on amphibians (Kiesecker and Blaustein 1998, Lawler et al. 1999, Gillespie 2001, Kats and Ferrer 2003).
- The harvest of frogs from natural habitats for human consumption also has a huge effect. It was estimated that in 1993, no less than 4,700 tons of frogs' legs were exported to Europe from Indonesia alone (Patel 1993). This represents an astonishing 94-235 million animals (Veith et al. 2000). Various amphibians have possibly been driven to the brink of extinction through the pet trade. The more rare a species is, the higher the asking price, and the more severe the threat to those few remaining in the wild.
- Like other animals, amphibians fall prey to viruses and fungi, and to parasitic infections by protozoans and various helminths. In recent years it has become increasingly clear that infectious diseases are one of several causes of global amphibian declines

(Laurance et al. 1996, Carey 2000, Williams et al. 2002, Collins and Storfer 2003, Daszak et al. 2003). According to Daszak et al. (2000), emerging infectious diseases are diseases that are newly recognised, newly appeared in a population or rapidly increasing in incidence, virulence or geographical range. These pathogens may induce sub-lethal damage (e.g. developmental deformities, physiological and behavioural deviations) or may directly cause mortality.

Despite reports of amphibian disease going back to the first decade of this century, knowledge of amphibian pathology and medicine has not kept pace with that of other animal groups. According to Elkan (1960) the published literature on amphibian pathology is extremely sparse and the knowledge concerning the amphibian reaction to the factors commonly causing disease is even less incomplete. The existence of this situation, Crawshaw (1992) attributes to the fact that it has usually been easier and cheaper to obtain new animals than investigating or treating medical problems. The main causes of morbidity in amphibians are parasites, tumours, inflammatory conditions and fungal infection (Elkan 1960). The most commonly reported disease in frog culture is a form of bacterial septicaemia called red-leg disease and is usually caused by gram-negative bacilli belonging to the family Vibrionacidae, Pseudomadaciae or Enterobacteriacae (Crawshaw 1992, Pariyanonth and Daorerk 1995). The disease has been studied extensively and preventative measures as well as treatment have been suggested by many that encountered the condition (Koivastik 1950, Nace 1968, Ruddock and Ruffle 1972). Three of the most commonly implicated diseases of amphibians associated with population declines are chytridiomycosis, saprolegniosis and infections with ranavirusses (Cunningham et al. 1996, Berger et al. 1998, Kiesecker et al. 2001). Data available on the emergence of chytridiomycosis has the strongest association with amphibian population declines (Daszak et al. 2003).

A distinction can be made between the causes of amphibian declines on the basis of the timing of threatening processes and recent amphibian declines (Hero et al. 2004). For example, continuing historical threats include habitat modification and changing fire regimes that have potentially affected frog habitats for over 200 years, whereas introduced fish and amphibian species have been an eminent threat for more than 70 years. Factors such as global climate change and emerging infectious diseases are relatively new threats (post 1970s). It is well known that animals have evolved ways to deal with most natural stressors in their environment. However, anthropogenic stressors are often novel to organisms and introduced abruptly, precluding natural evolution of adaptive processes (Westerman et al. 2003). The geographic pattern that emerged from rapidly declining amphibians shows a concentration of overexploited species in East and Southeast Asia, a wider occurrence of species suffering

from reduced habitat especially in Southeast Asia, West Africa and the Caribbean, whereas declines due to enigmatic causes (declines in spite of suitable habitat for poorly understood reasons such as disease and climate change) are mainly confined to South America, Mesoamerica, Puerto Rico and Australia (Stuart et al. 2004). Linking specific factors or stressors to an actual decline or extinction event of populations is a difficult process. The reality is that the causes of relatively few amphibian population declines are clearly understood (Westerman et al. 2003). Amphibian declines can seldom be linked to a single factor. More often complex mechanisms that involve abiotic and biotic interactions are responsible for amphibian population declines (Blaustein and Kiesecker 2002). For example, during extremely dry years, possibly brought on by sea-surface warming in the Pacific (Kiesecker et al. 2001), aquatic breeding sites of the western toad, *Bufo boreas* become very shallow, which enhances the effects of harmful UV-B and encourages lethal invasion of the fungus *Saprolegnia ferax* (Pounds 2001). According to Krest et al. (2003), improved experimental designs, especially for integrated field and laboratory studies, are important when characterising cause-effect relationships that would support adaptive management programmes intended to benefit amphibians.

1.2 The Amphibian Chytrid Fungus

The short history during which the amphibian chytrid fungus is known to science has been met with rapid interest in the fields of pathology, epidemiology, molecular biology and diagnostic test development because increasingly more discoveries were made of associations of this pathogen with amphibian declines. As we will see, some of these disciplines have evolved through the process of succession of current knowledge to a better understanding of the organism or related technique. Others disciplines still remain within the realm of hypotheses and complexity.

1.2.1 Discovery and Notice

In the first reports of amphibian deaths related to chytridiomycosis, the suspect organism was not recognised as a chytrid fungus. Nichols et al. (1996) reported on histological findings of skin taken off frogs (Whites tree frogs, *Litoria caerulea* and the ornate horned frog, *Ceratophrys ornata*) that died in captivity from the National Zoological Park (NZP), Washington D.C. in 1988 and 1990 and off dead California arroyo toads (*Bufo microscaphus californicus*) in 1991 from the University of California - Santa Barbara. A skin disease, thought to have been caused by a protist-like fungus, was identified as the most likely cause of death. The amphibian chytrid was also detected in 1993 in several frog species that died at upland sites in tropical Queensland, Australia, but was thought to be a *Perkinsus* sp.-like protozoan at the

time (Berger et al. 1997). Mass mortality events with similar histological findings had occurred in the Fortuna Forest Reserve, western Panama in 1996 and together with the investigators from Australia the suspect organism was described as a chytrid fungus for the first time and the disease referred to as chytridiomycosis (Berger et al. 1998). DNA sequencing of rDNA showed that the fungus was a chytrid fungus (Chytridiomycota: Chytridiales), a finding that was supported by the ultrastructure of the spores of the organism, known as zoospores. Berger et al. (1998) also established that the amphibian chytrid caused disease and fatalities in anurans by exposing healthy frogs to skin from infected frogs. It was not possible to fulfil Koch's postulates, a standard clinical test designed to prove that a suspect organism is responsible for death in a host organism, because it requires that the suspect organism be isolated from typical cases and grown in pure cultures. Concurrent with this publication two more papers appeared that described chytridiomycosis in poison dart frogs (*Dendrobatidis* spp.), *L. caerulea* and *C. ornata* (Nichols et al. 1998, Pessier et al. 1999).

The species description of the amphibian chytrid fungus was presented in a separate paper by Longcore et al. (1999) and the amphibian pathogen was placed in a new genus *Batrachochytrium*, meaning chytrid fungus of frogs, and named *Batrachochytrium dendrobatidis* after the genus of the frog from which the type culture was isolated (*Dedrobatis azureus*; blue poison dart frog). The description is based on morphology in pure culture in which the development is outlined and a detailed examination of zoospore ultrastructure as revealed by transmission electron microscopy. In addition Longcore et al. (1999) were able to reisolate *B. dendrobatidis* from frogs that became diseased after being experimentally infected with an isolate in pure culture, thus proving Koch's postulates of a pathogenic organism.

In the months to follow multiple-authored papers appeared that condensed the known knowledge from published and unpublished data concerning the biology, biogeography as well as clinical and histological diagnosis (including host response) of chytridiomycosis (Berger et al. 1999a, Daszak et al. 1999). These papers provided an explanation to the international scientific community concerned with amphibian population declines that had long suspected that an emerging infectious disease could be responsible for many of the declines and even extinction events, and together with the contribution by Pessier et al. (1999) and later by Berger et al. (1999b), enabled them to make their own diagnosis. This sparked a flurry of case reports of chytridiomycosis associated amphibian declines and mass mortality events from all over the world (Bishop 2000, Bosch 2000, Mutschmann et al. 2000, Speare and Berger 2000). It also provoked the suspicion that chytridiomycosis may have been responsible for population declines witnessed in the past and resulted in historic surveys of archived material collected during such events, some of which detected chytridiomycosis (Carey et al. 1999, Morell 1999,

Speare and Berger 2000, Sredl and Caldwell 2000). Soon a list of the global distribution of chytridiomycosis compiled by amphibian chytrid fungus experts, Prof. Rick Speare and Dr Lee Berger was filled with entries of almost 100 species of amphibians (Anura and Caudata) from 11 countries representing every continent where amphibians occur except Asia (Speare and Berger 2000). It had become clear that *B. dendrobatidis* was able to infect any amphibian and therefore posed a threat to the well being of amphibians worldwide. This lead to chytridiomycosis being nominated for listing as a key threatening process under the Environment Protection and Biodiversity Conservation Act 1999 (Speare and Core Working Group 2001). Henceforth chytridiomycosis could never again be ignored in investigations that follow a multiple stressor approach in identifying the causes of amphibian decline events where no direct link with a stressor is obvious such as with habitat destruction.

1.2.2 Biology

It was clear from the initial diagnosis of chytridiomycosis that the fungus occurred in various forms in epidermal skin that included a uninucleate form containing homogenous basophilic cytoplasm, a form containing multiple nuclei and a cyst-like form containing multiple round basophilic spores (Berger et al. 1998, Nichols et al. 1998, Pessier et al. 1999), but it wasn't before the development in pure culture was studied by Longcore et al. (1999) that the lifecycle was described. *Batrachochytrium* grows in the keratinised layer of the epidermis, the *stratum corneum* of metamorphosed frogs, concentrated in the region of the hind legs and pelvic patch, and in the keratinised mouthparts of tadpoles. At first it was thought that the localised occurrence of *B. dendrobatidis* in keratinised tissue meant that it utilised the protein as a nutrient, however, a resent study did not find proof that keratin is degraded by the extracellular proteases produced by the fungus (Daszak et al. 1999, Piotrowski et al. 2004). Isolates of *B. dendrobatidis* are able to grow at 4-25°C and at pH 4-8 and experience mortality when temperatures rise above 30°C (Longcore et al. 1999, Berger 2001, Piotrowski et al. 2004). A lot of the base work concerning the physiological confounders and preferences of *B. dendrobatidis* was done by Berger (2001) that paved the way for subsequent research to follow (Johnson et al. 2003, Johnson and Speare 2003). The lifecycle (Figure 1-1) takes about 4-5 days to complete when grown in pure culture (Longcore et al. 1999). The infective stage of the amphibian chytrid is a motile zoospore that can either reinfect the same individual or transmit to other hosts when released into water. After locating a susceptible animal, zoospores penetrate the skin and develop into spherical mature zoosporangia with discharge tubes. Zoospores are produced inside the sporangia. The zoospores are released through the discharge papillae. Sometimes thin septa form within the sporangium that divide the contents into two or more compartments, each with its own discharge papilla. Such a zoosporangium is referred to as a colonial thallus. After the zoospores have been released, all that is left are

empty sporangia that often become colonised by bacteria. The mechanism of transmission could explain why the infection is more severe on the ventral parts of the body because these areas are in more frequent contact with a wet substrate (Pessier et al. 1999).

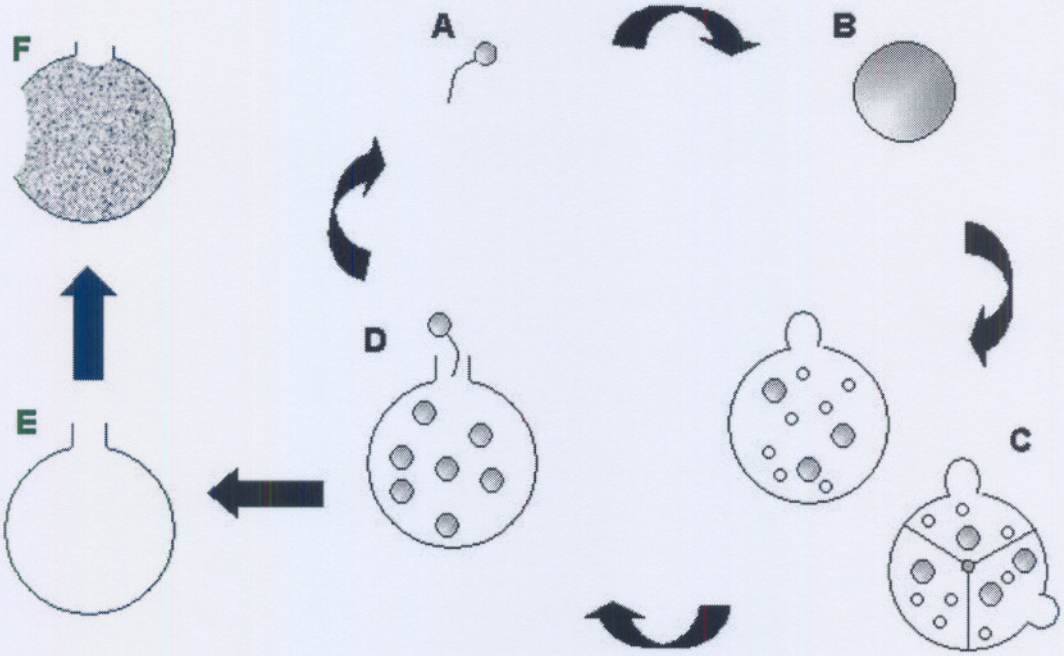


Figure 1-1 Diagrammatic representation of the lifecycle of *Batrachochytrium dendrobatidis*. The following developmental stages can be identified: A) zoospore, B) developing sporangium, C) mature sporangia; sporangium on right has internal septa, D) zoospore being released through discharge papilla. Empty sporangia (E) remain in the superficial epidermis and often become colonised by bacteria (F) before the skin is shed.

Amphibians that have become infected with chytridiomycosis and are susceptible to disease can be identified and the condition described in terms of clinical symptoms and gross and microscopic lesions. However, although the clinical signs and pathology of chytridiomycosis is similar in amphibians, the extent of clinical signs varies greatly with the species of amphibian host. Chytridiomycosis manifests as clinical symptoms related to the central nervous system including abnormal behaviour and body posture i.e. nocturnal frogs squatting unprotected during the day with limbs in an unusual position away from the body (Figure 1-2), lethargy and loss of righting reflex, and causes sloughing of the skin (Berger et al. 1999a, Daszak et al. 1999). However, in only a few species is sloughing clinically obvious. In amphibians susceptible to disease, chytridiomycosis has a clinical course of around 3 weeks resulting in death of the infected animal. Gross lesions are often subtle and usually restricted to the legs

and ventrum of post-metamorphic amphibians and may vary from mild skin thickening and discoloration to swollen limbs and slight reddening of the skin (Nichols et al. 2001, Bradley et al. 2002, Pasmans et al. 2004). Infected tadpoles often have discoloured rostrodonts (jaw sheath) or missing keratodonts (teeth rows) despite a healthy appearance (Lips 1999, Fellers et al. 2001, Lips et al. 2004). Epidermal tissue in the area of infection usually develops hyperkeratosis and hyperplasia (thickening of the *stratum corneum*) and could include intercellular edema and ulceration, but inflammatory cell response is almost absent (Berger et al. 1998, Nichols et al. 2001, Bradley et al. 2002, Lane et al. 2003a). It appears that amphibians that do not become diseased by the infection do not consistently show a thickening of the outer epidermal layer or else show a mild response (Daszak et al. 2004, Hanselmann et al. 2004, Weldon et al. 2004).

Two mechanisms by which chytridiomycosis brings about death have been proposed (Berger et al. 1998, Pessier et al. 1999). Firstly, it could be that a fungal toxin is absorbed systemically. Research efforts involving neuronal cell cultures to investigate the role of toxins produced by *B. dendrobatidis* have not been able to implicate a cytotoxin ([2]M. Johnson pers. com. 2003). Alternatively, the dense aggregation of thalli in the epidermis could impair fluid and electrolyte homeostasis, respiration, and the skin's role as a barrier to toxic and infectious agents. The impaired ability to exchange gases becomes particularly threatening in lungless urodelans such as *Bolitoglossa dofleini* that rely completely on respiration through the skin and mucosal surfaces (Pasmans et al. 2004). It has been hypothesised that during spells of dry weather *Eleutherodactylus* species from Puerto Rico are more likely to die from chytridiomycosis due to their inability to uptake water as a direct result of thickening of the *stratum corneum* (Burrowes et al. 2004).

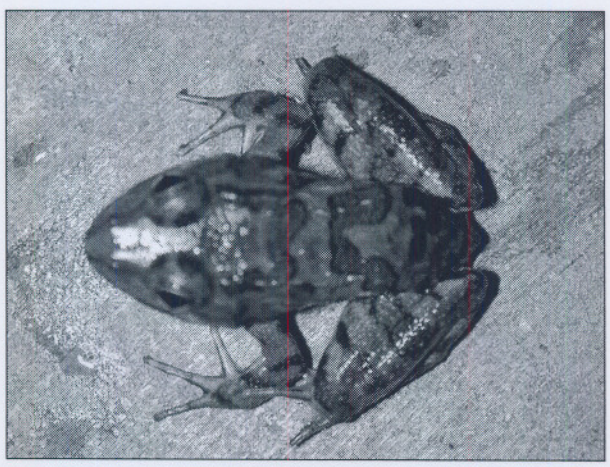


Figure 1-2 A Cape river frog infected with *B. dendrobatidis*. Note the limbs are angled away from the body, instead being tucked in underneath it.

[2] Dr Megan Johnson, James Cook University, Townsville, Queensland, Australia

1.2.3 Chytridiomycosis - an Emerging Infectious Disease

According to Stuart et al. (2004) amphibians of the Neotropical and Australasian-Oceanic realms are most prone to enigmatic declines (declining, even where suitable habitat remains for reasons that are not fully understood) of which disease and climate change are the most commonly cited causes. In Australia chytridiomycosis is notably associated with declines for 5 species and is a potential contributor for an additional 13 species and has been detected in 33 more species that are not declining (Hero et al. 2004). Chytridiomycosis is associated with most of the frog species known to have declined in parts of Latin America including Costa Rica, Ecuador, Panama and Puerto Rico (Lips 1999, Young et al. 2001, Lips et al. 2003, Ron et al. 2003, Burrowes et al. 2004). Chytridiomycosis is also associated with amphibian deaths and/or population declines in Africa (Hopkins and Channing 2003, Lane et al. 2003a), Canada (Ouellet et al. 2003), Europe (Mutschmann et al. 2000, Bosch et al. 2001), Mexico (Lips et al. 2004), New Zealand (Waldman et al. 2001, Bell et al. 2004) and 12 states in the USA (Carey et al. 1999, Fellers et al. 2001, Bradley et al. 2002, Green et al. 2002, Carey et al. 2003). The wide occurrence and range of amphibian taxa from which *B. dendrobatidis* has been detected, regardless of the degree of habitat conservation, suggests that any amphibian species can become infected. A distinction can, however, be made between species regarding susceptibility to disease. A difference in the severity of the disease among species has been attributed to ecological niche and life history strategy of the species (Daszak et al. 1999), as well as resistance to disease (Daszak et al. 2004). Susceptibility to chytridiomycosis may even vary within a species (Davidson et al. 2003). At the population level these differences may lead to death in only some individuals, mass deaths followed by population recovery or mass deaths resulting in population declines. Population declines are particularly associated with stream breeding amphibians at high elevations in Australia (Berger et al. 1998, Alford et al. 2001), Latin America (Lips 1999, Young et al. 2001, Lips et al. 2004) and Spain (Bosch et al. 2001). In addition to breeding in streams, Lips et al. (2003) found that the most susceptible species have restricted elevational ranges and have large body sizes. Diagnostic findings of amphibian declines in eastern Australia as well as trials on captive frogs have demonstrated that prevalence and mortality rate due to chytridiomycosis is highest at lower temperatures (Berger et al. 2004, Retallick et al. 2004). Bradley et al. (2002) observed that the breeding behaviour and over wintering strategy (congregation in high densities) of native Arizona frogs influenced disease transmission that led to die-offs during the cooler months. During the warmer months, temperatures may rise to the range within which the organism cannot effectively reproduce in adequate numbers to cause disease (Bradley et al. 2002). In the case of the common midwife toads (*Alytes obstetricans*) in central Spain, Bosch et al. (2001) suggests that an extended larval period and small clutch size contributed to this being the only species in the area that experienced mass mortality. Following a decline event in wild

populations and in captivity, *B. dendrobatidis* can become a stable infection in a previously susceptible species that has since recovered (Mazzoni et al. 2003, Retallick et al. 2004). It was suggested that such recoveries could be because of evolution of resistance to the pathogen or evolution of less pathogenic strains of the fungus.

A subject of debate is how *B. dendrobatidis* persists in the environment between outbreaks because no resting spore stage has been found for this organism. Explanations for several survival mechanisms have been offered. The amphibian chytrid can survive in different types of water (sterilised tap-, distilled- and lake water) between 3 and 7 weeks during which time sporangia may attach to plankton (Johnson and Speare 2003). However, the ability of zoospores to infect a host is constrained by a short swimming distance (20 mm) and limited time (24 h) before they encyst (Piotrowski et al. 2004). It can also survive in soil for 7 days and up to 21 days when nutrient is added (Porter 2004). Between seasons *B. dendrobatidis* survives in the keratinised mouthparts of tadpoles until the next outbreak (Berger et al. 1998). Healthy frogs of exotic and/or native species may also serve as a reservoir of infection (Daszak et al. 2004, Retallick et al. 2004, Weldon et al. 2004) that transmits the disease to susceptible species.

It has been hypothesised that infectious diseases are only the proximate cause of declines in wildlife and that the process is driven by anthropogenic environmental changes such as increased UV-B, chemical pollution and habitat change (Carey et al. 1999, Pounds et al. 1999, Daszak et al. 2000). The evidence suggests that chytridiomycosis is capable of directly causing mortality in amphibians. However, global climate change, drought, pH, land use change and alien species have been hypothesised as drivers of chytridiomycosis emergence (Daszak et al. 1999, Bosch et al. 2001, Daszak et al. 2003, Burrowes et al. 2004). It is important to distinguish between factors that have an effect regardless of chytridiomycosis presence (co-factors) and factors that worsen the effect in the presence of chytrid (synergistic factors) (Hero et al. 2004) when searching for the most probable cause of decline events. These synergistic factors can, by acting alone or together, stress amphibians sufficiently to compromise their immune system that in turn increases disease susceptibility (Pounds 2001, Blaustein et al. 2003, Carey and Alexander 2003, Carey et al. 2003).

The quick rate at which populations decline where chytridiomycosis is involved and the distinct geographic and temporal progression of the epidemic wave as witnessed in Australia (Laurance et al. 1996, Mahony 1996) is characteristic of an introduced virulent pathogen dispersing through a previously unexposed population (Daszak et al. 1999). Similar directional progress of the pathogen is occurring in amphibians from New Zealand and intercontinentally

from western North America southward through Mexico and into lower Central America (Lips 1998, 1999, Bell et al. 2004, Lips et al. 2004). The pattern of amphibian declines is supported by molecular evidence that suggests anthropogenic introduction is involved in the spread or emergence of chytridiomycosis (Daszak et al. 2003, Morehouse et al. 2003). If chytridiomycosis was recently introduced (within the past 2-4 decades) into these affected areas, then a common source exists from which the disease originally spread and might still be spreading. A compilation of epidemiological evidence derived from a histological survey of archived and live *Xenopus* spp. indicates southern Africa as the origin of the amphibian chytrid fungus (Weldon et al. 2004). According to the "out of Africa" hypothesis the sub-clinically infected African clawed toad, *Xenopus laevis*, has carried the disease around the world via the scientific trade. The emergence of chytridiomycosis in new regions has in part been the result of anthropogenic introduction. The national and international movement of amphibians, including tadpoles, in the pet trade has widely been implicated in the spread of chytridiomycosis (Bishop 2000, Mutschmann et al. 2000, Waldman et al. 2001, Pasmans et al. 2004). There is growing concern about the role the food industry plays in the emergence or spread of chytridiomycosis (Mazzoni et al. 2003, Hanselmann et al. 2004), a risk that is highlighted by the large numbers of amphibians involved. Other mechanisms of introduction include the importation for zoo collections (Pessier et al. 1999) and exotic species used in biocontrol such as cane toads in Australia (Berger et al. 1998). Amphibians that have some sort of resistance to chytridiomycosis and are part of an anthropogenic mechanism of introduction can be regarded as high-risk carrier species. *Xenopus laevis* fills this role in the scientific trade (Weldon et al. 2004). The bullfrog, *Rana catesbeiana* has been identified as a reservoir host in the food trade (Cunningham et al. 2003, Mazzoni et al. 2003, Daszak et al. 2004).

1.2.4 Applied Chytridiomycosis Research

The event that paved the way for research aimed at better understanding *B. dendrobatidis* (aspects of physiology, pathology, immunology etc.) was the successful isolation and culture of the fungus. Zoospores can be harvested from pure cultures for use in transmission experiments to achieve specific outcomes. A protocol for counting zoospores in inocula and techniques for inoculating handling and housing experimental animals for chytridiomycosis research was provided by Daszak et al. (2004). Experimental transmission involving *B. dendrobatidis* has been used to show that chytridiomycosis can be a primary pathogen (Nichols et al. 2001), indicate within species susceptibility variation (Davidson et al. 2003), demonstrate the role of reservoir hosts (Daszak et al. 2004), investigate the effect of temperature on mortality rate (Berger et al. 2004) develop diagnostic tests (Berger et al. 2002,

Boyle et al. 2004) and for the development of curative treatments (Marantelli et al. 2001, Nichols et al. 2001).

Molecular Analysis

The first amphibian chytrid fungus DNA that was sequenced enabled scientists to identify the then unknown pathogen of amphibians as a fungus belonging to the Chytridiomycota (Berger et al. 1998). The ssu-rDNA was amplified from DNA extracted from skin scrapings from two wild *L. caerulea* of Australia and aligned with that of 44 other eukaryotes. While more cases of chytridiomycosis were being reported, the next step was to determine if one or more species existed. In the first attempt to investigate the relationship of isolates the rDNA internal transcribed spacers (ITS gene) in strains from Australia, Central America and North America were used (Morgan 2000). Results with the ITS gene were variable and at conflict with epidemiological data. Subsequently, it was discovered that *B. dendrobatidis* had multiple copies of the ITS and that this gene was unsuitable for taxonomic work ([3]R. Speare pers. com. 2004). Early comparison of the 18S rDNA of chytrid strains from Australian and American frogs did not show significant differences (see Berger et al. 1999a). *Batrachochytrium dendrobatidis* strain JEL 197 from the chytridiomycete collection at the University of Maine was one of 54 chytrids included in a phylogenetic study of the class Chytridiomycota (James et al. 2000). The phylogenetic trees that were generated placed *B. dendrobatidis* in an isolated position. This outcome supported what was already known about the zoospore ultrastructure because *B. dendrobatidis* contains unique character states among the chytrids (Longcore et al.1999, James et al. 2000). The most authoritative work to date regarding the population genetics of the amphibian chytrid was the use of multilocus sequence typing by Morehouse et al. (2003) to examine genetic diversity among 35 amphibian chytrid strains from Africa, Australia, Central America and North America. A low genetic variation of five variable nucleotide positions was detected among 10 loci. This study provides evidence to support the hypothesis that chytridiomycosis is an introduced emerging disease and implicates anthropogenic introduction as a mechanism of spread.

Diagnostic Test Development

Accurate diagnosis of infected amphibians is important when determining the cause of death in wild and captive amphibians as well as for quarantine regulations concerned with the import and export of amphibians. There have been three generations of diagnostic techniques for chytridiomycosis since the initial discovery of the fungus. The first tests relied on the morphology of the fungus. These were followed by tests that are based on antigen detection.

[3] Prof. Rick Speare, James Cook University, Townsville, Queensland, Australia

The third generation of diagnostic tests make use of DNA technology. With every new generation the level of sensitivity and specificity increased and the test becomes less invasive. Third generation tests have the added benefit of delivering a result in rapid time. On the down side, advanced diagnostic tests are more expensive to use and less accessible due to the high level of expertise involved in the respective disciplines. For this reason and due to the length of time they have been in practice, first generation diagnostic tests have been used far more often than second and third generation tests combined.

1998: In the original paper on chytridiomycosis Berger et al. (1998) detected the fungus through examining haematoxylin and eosin (H&E) stained sections of toe clips prepared by standard histology techniques. Morphology, position in the skin and host tissue response of the *stratum corneum* are used to make a positive diagnosis. A clear and conscious account of the use of the histological technique is provided by Berger et al. (1999b) that allows investigators that are unfamiliar with histopathology to positively diagnose chytrid infection. Skin from the pelvic patch, toes and ventral thighs of specimens as well as tadpole mouthparts are usually prepared for examination.

Berger et al. (1998) also examined wet mounts of skin scrapings from fresh specimens using a light microscope. Longcore (2001) provided the first detailed step-by-step instructions for using sloughed skin to identify chytridiomycosis. The use of skin sloughs or scrapings requires training and more skill than H&E histology in identifying *B. dendrobatidis*, but saves a lot of time once the technique has been mastered. To increase differentiation between fungus and skin tissue, skin smears can be stained with a few drops of cotton blue and 10% aqueous potassium hydroxide (1:1 solution) (Mazzoni et al. 2003) or with Congo red (Biggs and Bergin 2003).

In addition, tissue samples were examined by Berger et al. (1998) with a scanning transmission microscope (SEM, for external ultrastructure morphology) and transmission electron microscope (TEM, for internal ultrastructure morphology and function). Although the use of electron microscopy can accurately identify the amphibian chytrid, its primary function remains the study and description of ultrastructure morphology.

Nichols et al. (1998) and subsequently Longcore et al. (1999) succeeded in isolating and growing cultures of *B. dendrobatidis* on TGhL (tryptone, gelatin hydrolysate, lactose) agar. Although *B. dendrobatidis* can be identified through culture, it is not used as a primary diagnostic technique and is usually the means to an auxiliary objective such as molecular and pathogenicity studies.

The co-occurrence of healthy tadpoles with damaged mouthparts in streams with dead frogs with chytridiomycosis prompted Lips (1999) to realise that the lesions are due to chytridiomycosis and therefore an indication of infection. Damaged or missing mouthparts are

only an indication of possible infection (Fellers et al. 2001, Lips et al. 2004) because an incomplete oral disc could be due to mechanical damage (Rachowicz 2002) and should therefore be confirmed with specific techniques.

2001: An enzyme-linked immunosorbant assay (ELISA) was developed that uses an antibody detection system that binds to the specific antigen of *B. dendrobatidis* (Hyatt et al. 2001). ELISA is distinctive in that organisms from a broth culture are used instead of histological sections.

2002: An immunoperoxidase test was developed after polyclonal antibodies to *B. dendrobatidis* were produced (Berger et al. 2002). Antiserum from rabbit reacts strongly with all developmental stages of *B. dendrobatidis* and stains the walls, cytoplasm, rhizoids and zoospores. The test stains *B. dendrobatidis* tissue dark brown to red that contrasts strongly with host tissue (blue). Although the immunostaining is a more elaborate process than H&E, it is superior in its sensitivity for diagnosing chytridiomycosis (Berger et al. 2002) and therefore a valuable test for confirming infection status in suspicious cases. The value of the test for inexperienced diagnosticians was increased by addition of a stain for keratin (Olsen et al. 2004).

2004: A sensitive and specific polymerase chain reaction (PCR)-based assay was recently developed by Annis et al. (2004). The assay makes use of small skin samples (1-3 mm^2) and is able to detect as few as 10 zoospores (less than the average number per sporangium) in a sample. Large samples can be processed in a short time; an advantage for chytridiomycosis surveys of wild populations.
Boyle et al. (2004) developed a real-time PCR Taqman assay that is not only able to identify *B. dendrobatidis* with a high degree of sensitivity in skin samples, but is also able to quantify the infection (can detect 1 zoospore). The ability to detect early or low levels of infection allows for the prevention of spread between enclosures and for possible treatment of infected animals (Boyle et al. 2004).

The next advancement in diagnostic test development could be DNA-based assays that are able to detect *B. dendrobatidis* in pond water and even fish and reptiles (Annis et al. 2004).

Sterilisation

Simple sterilisation regimes based on the physiological characteristics of *B. dendrobatidis* as well as the application of chemical disinfectants were derived through experimental trial on *in vitro* cultures by Johnson et al. (2003). These physical techniques can be used to sterilise

equipment, protective clothing and enclosures contaminated with *B. dendrobatidis* during fieldwork, in the laboratory and in captive husbandry. Techniques recommended on the basis of their sensitivity to the environment and practical application include treatment by heat, desiccation, 70% ethanol, 1% Virkon (Antec International), 2% sodium hyopchlorite, didecyl dimethyl ammonium chloride, 1% formaldehyde, 10% sodium chloride (salt) and 2% potassium permanganate.

Treatment

Successful treatment of chytridiomycosis can prevent deaths of not only the animals being treated, but prevent the disease from spreading to other species and regions. An effective and safe treatment could be used as part of official requirements for movement of amphibians within countries and between countries. In the case of frogs with commercial value (food-, scientific- and pet trade) early detection and treatment can prevent huge economic losses.

Documented accounts on investigations into the cure of chytridiomycosis through the application of chemotherapeutic agents have been few and never tested on a range of taxa. Marantelli et al. (2001) were able to reduce the prevalence of *B. dendrobatidis* in great barred frogs, *Mixophyes fasciolatus* tadpoles through benzalkonium chloride and fluconazole baths. The topical application of itraconizole successfully treated chytridiomycosis in adult yellow poison dart frogs, *Dendrobates tinctorius* (Nichols et al. 2001), however, the treatment has not been tested on tadpoles. Treatment of captive *Xenopus tropicalis* with formalin/malachite green baths eliminated infection and cured sick animals that survived the epizootic (Parker et al. 2002). An elevation of temperature to 37ºC was effective against *B. dendrobatidis* in captive Australian red-eyed tree frogs, *Litoria chloris* (Woodhams et al. 2003). Benzalkonium chloride treatment, a recognised therapy for infections with non-hyphal fungi, was not able to reduce mortality rates in infected *Rana catesbeiana* from a frog farm in Uruguay (Mazzoni et al. 2003). No immunisations are currently available for chytridiomycosis or for other significant infectious diseases of amphibians.

Berger (2001) was able to demonstrate that antifungal products from the skin of amphibians (*L. caerulea*) could kill *B. dendrobatidis in vitro.* It has been suggested that the inherent peptide defence mechanism in amphibian skin may play a role in preventing and limiting infection by the amphibian chytrid and other pathogens associated with amphibian declines (Rollins-Smith et al. 2003). This follows after it was demonstrated that antimicrobial peptides, of which each species has a unique set, inhibit the growth of *B. dendrobatidis* and *Basidiombolus ranarum* (Rollins-Smith et al. 2002). The ability to disrupt the cell membrane and resistance to fungal proteases, may explain differences in peptide potency (Rollins-Smith et al. 2003).

1.3 Study Proposal

1.3.1 Research Problem

It is not known where *B. dendrobatidis* initially originated. The most likely hypothesis holds that *B. dendrobatidis* escaped from a host and locality in which it evolved, into host species and environments novel to the pathogen. Alternatively, it may be a widespread organism that has emerged as a pathogen to amphibians because of either an increase in virulence or an increase in host susceptibility caused by unknown factors (Berger et al. 1998). In Australia epidemiological evidence suggests that *B. dendrobatidis* was introduced into southeast Queensland in the late 1970s and spread north along the Queensland coast at 100 km per year (Laurance et al. 1996).

Three records of the amphibian chytrid have emerged from Africa since 1999. These include the tropical platanna *X. tropicalis* from West Africa (Reed et al. 2000), Anchieta's ridged frog *Ptychadena anchietae* from Kenya (Speare and Berger 2000) and the African clawed toad *X. laevis* from the Western Cape, South Africa (Speare and Berger 2000). Because massive amphibian declines have not been reported in Africa, there is a possibility that chytridiomycosis is endemic to this continent and has spread to other parts of the world by way of trade in amphibians such as *X. laevis* (Speare and Core Working Group 2001, Weldon et al. 2004).

Whether and how chytridiomycosis arrived in South Africa and what its effect is on wild populations is uncertain. We know that *Xenopus* from the Cape is a high-risk species for dissemination of chytridiomycosis through the scientific trade in amphibians. This puts amphibians both nationally and internationally at risk of acquiring chytridiomycosis through transmission via *X. laevis*. An international conference in Cairns, Australia recommended that risk assessments be carried out in all countries to determine the occurrence of chytridiomycosis, its geographical distribution and the species of amphibians infected (Speare 2001). Given that South Africa is a major supplier of *X. laevis* for scientific research in many countries, there is an urgent need to quantify the risks of infected frogs being exported and to protect the trade. Because the majority of the exported frogs are drawn from natural populations there is also a need to determine the status of chytridiomycosis in this region. In addition, the Office International des Epizooties (OIE), the equivalent in the animal field of the World Health Organization, has recently accepted amphibian chytridiomycosis on its wildlife list as a disease requiring quarantine restrictions in movement of amphibians between countries. Given that South Africa is a member state of OIE, it is timely to pre-empt any quarantine or movement restrictions that may be recommended.

1.3.2 Objectives

- Objective 1: Occurrence of *B. dendrobatidis* in Africa

  1a) Determine which species of *Xenopus* from Africa are infected with *B. dendrobatidis* by examining specimens from archived collections.

  1b) Determine also the extent of infection in other South African frogs from archived collections as well as from field surveys.

- Objective 2: Dissemination risk of *B. dendrobatidis* via the *X. laevis* trade

  2a) Determine the role of the *Xenopus* trade in the historical and current dissemination of chytridiomycosis locally and globally.

  2b) Develop strategies to reduce this dissemination.

- Objective 3: DNA analysis of South African strains

  3a) Isolate and cultivate *B. dendrobatidis* from South African frogs from a wide range of localities.

  3b) Determine the level of genetic variation in *B. dendrobatidis* strains from South Africa through multilocus sequence typing. Relationships with isolates from other parts of the world (e.g. Australia, Panama and USA) will be determined to investigate geographical patterns and the origin of the fungus.

- Objective 4: Electron microscopy on *B. dendrobatidis*

  Examine the surface and macro molecular characteristics of *B. dendrobatidis* with the use of conventional scanning electron microscopy and laser scanning confocal microscopy.

- Objective 5: Quantification of infection

  Develop a technique that will enable investigators to quantify an infection of chytridiomycosis in live amphibians. Naturally infected Cape river frogs, *Afrana fuscigula* will be used in the investigation.

- Objective 6: Investigation of the Kihansi spray toad decline

  6a) Investigate the circumstances that lead to the disappearance of the Kihansi spray toad, *Nectophrynoides aspergenis* from the Udzungwa Mountains, Tanzania.

  6b) Develop a pathogen hygiene protocol for Kihansi Gorge.

1.3.3 Phases of Research

The project can be divided into four phases; the outcome of each phase determines the course of the next (Figure 1-3).

Phase A centres around the formulation of research questions applicable to studying chytridiomycosis in Africa through a comprehensive study of available literature and by connecting with the international network of scientists involved in *B. dendrobatidis* research.

Phase B involves determining the host species and geographical distribution of *B. dendrobatidis* in Africa by conducting surveys of both archived and live frogs.

Phase C includes investigations into the epidemiology of chytridiomycosis by conducting a risk assessment of the *Xenopus* trade, investigating the role of chytridiomycosis in population declines, and by comparing South African strains of *B. dendrobatidis* (attained through isolation and cultivation) to global strains.

Phase D involves the drafting of strategies to control chytridiomycosis and reduce the risks of disseminating the disease in the trade sector, environmental science practices and wildlife management.

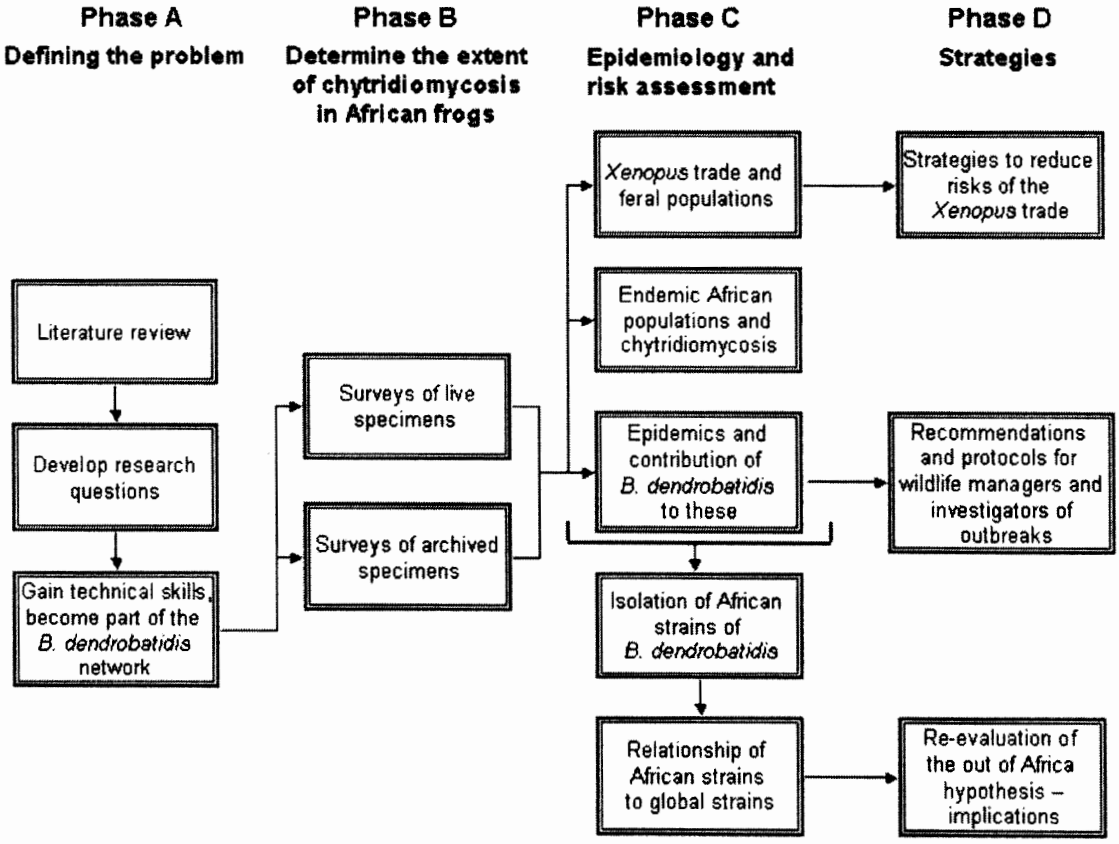


Figure 1-3 Flowchart of research design showing phased approach.

1.3.4 Thesis Chapters, Collaboration and Research Outputs

At the start of this project in 2002 no one else was actively involved in amphibian chytrid research in Africa and little was known about this amphibian pathogen among the herpetological community in Africa. Achieving the objectives set out in this thesis therefore required that the candidate become part of the international network of scientists involved in *B. dendrobatidis* research. The nature of collaboration either involved the acquisition of expert advice or technical skills needed for *B. dendrobatidis* research, or making use of specialised services provided by world experts in the relevant field of research.

Three publications in accredited journals (Lane et al. 2003a, Weldon et al. 2004, Harkewicz et al. 2005), a book chapter (Weldon and Du Preez 2004a) and a number of newsletters (Weldon 2002a, b, Weldon 2003, Lane et al. 2003b, Weldon and Du Preez 2004b) have stemmed from this research. Four more manuscripts are in various stages of preparation for publication. Since the onset of this project, the findings have received maximum exposure at scientific conventions. Various aspects of this project have been delivered as oral presentations at six national and two international symposia. Scientific posters have also been presented at two national and one internal symposium. Invitations have been received to present aspects of this project at two symposia (Declining Amphibia-Symposium, *Xenopus*-Symposium) at the 5th World Congress of Herpetology in June 2005. Our research on the threat that the amphibian chytrid fungus poses to the global amphibian biodiversity received exposure in a number of local and national newspapers.

The thesis is divided into ten chapters including an introduction and literature review, a methodology chapter, seven text chapters dealing with various aspects of chytridiomycosis research in Africa, and a final discussion chapter. Examples of data sheets and major publications by the candidate that are related to this project have been included as annexures following the last chapter. A complete list of references that have been used throughout this thesis has been placed at the back of the thesis. The format for references provided by the scientific journal *Biodiversity and Conservation* was used in this thesis.

Chapter 1 deals with the literature review of amphibian declines and specifically the amphibian chytrid fungus and contains the study proposal that serves as an introduction to the rest of the thesis.

Collaboration

- Prof. Rick Speare, James Cook University (JCU), Townsville, Queensland, Australia was involved with the project from the planning phase and consistently throughout its

duration in an expert advisory capacity on chytridiomycosis biology, pathology and epidemiology. Prof. Speare was appointed co-promoter for the project in January 2002.

Publications

- At the onset of the project the candidate wrote a memo to the newsletter of the African Herpetological Association of Africa as a notification of his intended research and as a call for the involvement of fellow herpetologists in the event of them finding dead or sick frogs during fieldwork (Weldon 2002a).
- The candidate co-authored a roundtable manuscript in *Journal of Herpetological Medicine and Surgery* that deals with aspects of the biology, pathology and epidemiology of *B. dendrobatidis* (Harkewicz et al. 2005).

Chapter 2 is a synthesis of general materials and methods that are applicable to most of the contents chapters that follow. The chapter includes sections on permits, frog collection methods, field hygiene protocol and chytridiomycosis diagnostic techniques.

Collaboration

During November/December 2002 the candidate spent five weeks in Australia with the purpose of developing and advancing skills that would aid in South Africa's competence to recognise and manage amphibian diseases, especially chytridiomycosis.

- Training in histological techniques was arranged by Prof. Speare and provided by Dr Diana Mendez, (JCU), Townsville, Queensland, Australia.
- On-hands experience was gained at the Animal Health Laboratory, Geelong, Victoria under the guidance of Dr Alex Hyatt regarding the operation of two diagnostic tests specific for chytridiomycosis: immunoperoxidase test (polyclonal antibodies are used in the diagnoses) and an ELISA test (enzyme linked immunosorbant assay that uses an antibody detection system that binds to the specific antigen of *B. dendrobatidis*).
- The candidate also participated in the annual Frog Search at Eungella National Park, Queensland organised by the Queensland Parks and Wildlife Service. The aim was to search and monitor some of central Queensland's most threatened montane frog species such as the tusked frog, *Adelotus brevis* that is now critically endangered after suffering serious population declines due to chytridiomycosis. During fieldwork a strict pathogen hygiene protocol was followed to prevent potential spread of diseases between streams and between frogs.

Chapter 3 is a study of the morphology and development of *B. dendrobatidis* with the use of scanning electron microscopy and laser scanning confocal microscopy. The data are supported with a comprehensive set of high-resolution digital images.

Collaboration

- Dr Lourens Tiedt from the Laboratory for Electron Microscopy, North-West University provided expert assistance with the examination of *B. dendrobatidis* using scanning electron microscopy.
- Me. Anne Grobbler from the School of Pharmacy, North-West University provided expert assistance with the examination of *B. dendrobatidis* using confocal microscopy.

Chapter 4 deals with the occurrence of chytridiomycosis in African frogs and includes species lists and distribution maps. In this chapter is presented evidence to support the hypothesis that *B. dendrobatidis* originated in Africa.

Collaboration

- In April 2002 Dr Lee Berger (JCU), Townsville, Queensland, Australia visited our facility at North-West University. Dr Berger provided training in the histological diagnosis of chytridiomycosis as well as diagnosis using skin sloughs prepared on wet mounts.
- The candidate collaborated with Dr Emily Lane, a veterinary surgeon from the University of Pretoria, Onderstepoort to diagnose chytridiomycosis in dead frogs that were submitted to the animal health facility at Onderstepoort.
- Prof. Speare helped to formulate the "out of Africa" hypothesis that was proposed for the origin of *B. dendrobatidis*.
- Dr Hyatt provided antibodies for the specific immunoperoxidase test (IpX) and helped to diagnose chytridiomycosis in archived specimens of *X. laevis* collected prior to 1945 using IpX.

Publications

- After detecting chytridiomycosis during a survey of frogs from the Western Cape, the initial results were posted in the newsletter of the Declining Amphibian Population Task Force (DAPTF) (Weldon 2002b). This served to expose our research activities from the North-West University to the international herpetological community concerned with the study of declining amphibians and as a call for collaboration with researchers involved in the rest of Africa.
- These preliminary results were also presented in the newsletter of the School of Environmental Sciences and Development of the North-West University to inform our colleagues of our progress (Weldon 2003).
- In a joint effort with scientists affiliated with the University of Pretoria, Onderstepoort to identify possible pathogens in a dead frog, the first report of chytridiomycosis in a free-ranging frog from South Africa was published (Lane et al. 2003a, Annexure A).

- A summary of this publication, including a call for collaboration, was distributed among veterinary surgeons in the newsletter of the Veterinary Association of South Africa (Lane et al. 2003b).
- Histological evidence supporting the hypothesis that *B. dendrobatidis* originated in Africa, was presented in a multi-authored publication in the December 2004 edition of *Emerging Infectious Diseases* (Weldon et al. 2004, Annexure B). This paper generated a lot of attention from the international herpetological community. It lead to a radio interview with an environmental correspondent (John Nielsen) with NPR, a Washington, D. C. -based radio station. A PDF-version of the paper has since been circulated on the list servers of two international herpetological associations (RANA – Network of Scientist for Threatened Neotropical Amphibians, and AZA - The American Zoo and Aquarium Association).
- A chapter contribution dealing with the role of infectious diseases on amphibian declines in South Africa was included in the *Atlas and Red Data book of the Frogs of South Africa, Lesotho and Swaziland* (Weldon and Du Preez 2004a).

Chapter 5 concerns the trade, both domestic and international in *X. laevis* and related conservation issues are discussed.

Collaboration

- Atherton De Villiers from the Western Cape Nature Conservation Board (WCNCB) provided information on the numbers and export destinations of *X. laevis* from South Africa.

Chapter 6 is a critical evaluation of the movement of chytridiomycosis through the *Xenopus* trade in the form of a risk assessment.

Collaboration

- Strategies to reduce risks of disseminating chytridiomycosis through the trade in *X. laevis* was submitted to the WCNCB and discussed with officials (Dr Ernst Baard, Atherton De Villiers) at the WCNCB head office in Jonkershoek during November 2003.
- Prof. Richard Tinsley, Bristol University UK and Dr John Measey, University of Antwerp, Belgium, provided specimens of feral *X. laevis* for determining the prevalence of chytridiomycosis.

Chapter 7 presents a new technique that was developed for the quantification of *B. dendrobatidis* infection on sloughed skin from live amphibians.

Chapter 8 deals with the molecular biology of the South African strains of *B. dendrobatidis* and discusses the implications of the analyses on the "out of Africa" hypothesis.

Collaboration

- In August 2004 Dr Joyce Longcore from the University of Maine, Orono, USA and Prof. Rick Speare, visited our laboratories at the North-West University during which time we received extensive training in the isolation and cultivation of *B. dendrobatidis* from infected frogs collected in Namaqualand, Northern Cape Province.
- Infected archived specimens were sent to Dr Hyatt for molecular analysis of *B. dendrobatidis* using the Taqman real-time PCR assay.
- Dr Tim James and Dr Rytas Vilgalys from Duke University, Durham USA performed multilocus sequence typing on the South African isolates.

Chapter 9 presents a case study of a chytridiomycosis related decline and presumed extinction of the Kihansi spray toad from Tanzania.

Collaboration

- The investigation into the decline of the Kihansi spray toad was performed under the auspices of the Lower Kihansi Environmental Management Project (LKEMP), Dar es Salaam, Tanzania. The project manager, Dr Wilfred Sarunday, provided logistical support and the technical advisor, Dr Jeremy Thompson, lead the expedition and provided assistance during fieldwork.

Publications

- The first report on chytridiomycosis associated population declines in Africa was posted in the newsletter of the DAPTF following the investigation (Weldon and Du Preez 2004b).

Chapter 10 is a synthesis of the most significant conclusions that were derived at in the contents chapters. Included in the chapter is a section on recommendations for future research.



Chapter 2

GENERAL MATERIALS AND METHODS



2.1 Ethical Clearance and Permits

Clearance by an ethical committee is mandatory for conducting research involving vertebrates at the North-West University. A proposal for obtaining ethical clearance for studying chytridiomycosis in anurans was therefore submitted to the ethical committee and ethical clearance for the project was subsequently granted (Reference no.: 02D02).

Before any wild frogs were collected permits were obtained from the relevant nature conservation authorities responsible for the regions in which fieldwork was intended (Table 2-1). A declaration form granting permission to collect frogs was completed for each site and signed by the respective land owner (Annexure C).

Table 2-1 Permits applied for during the project for the collection of wild frogs.

Permit No.	Issuing Body	Date Valid	Region	Specifications
354/2001	Western Cape Nature Conservation Board	2000-2002	Western Cape Province	*Xenopus laevis* (unlimited)
0209/04	Northern Cape Nature Conservation Service	2004	Namaqua region	*Xenopus laevis* (50) *Afrana fuscigula* (50)
0663/04	Northern Cape Nature Conservation Service	2004	Goegap Nature Reserve	*Afrana fuscigula* (10 live, as well as any dead animals)

2.2 Anuran Sampling Techniques

2.2.1 Visual Encounter Sampling

A visual encounter approach was used to collect mainly river frogs and toads that are associated with streams and ponds. Animals that were spotted on land hiding in vegetation or in the water clinging to the base of reeds, drifting logs etc., were individually collected by hand. The duration of search effort was not limited like when collecting qualitative data for monitoring amphibian assemblages, but was determined by the time it took to collect a sample of a certain size that would enable the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* to be

detected at a certain prevalence. The only prevalence data for chytridiomycosis in South African frogs at the start of the study was infection in 60% of a sample of African clawed toads, *Xenopus laevis* from the Western Cape that were collected in 1999 ([4]R. Speare pers. com. 2002). The assumption was made that for the range of infection within a sample to vary between 10 and 60%, a minimum of 50 frogs would have to be examined to detect a minimum number of 5 infected animals. Active sampling has the benefit of allowing the observer to assess the activity patterns of the frogs for abnormal behavior that may be present as a result of chytridiomycosis.

2.2.2 Dip-net Sampling

After a site was searched for frogs, a dip net (300 x 250 mm opening) with mesh size of 2 mm was used to sample aquatic larvae by sweeping among aquatic vegetation and in open water (Figure 2-1). Dip-netting is an invasive technique that disturbs the habitat and might chase frogs away and should therefore not immediately precede visual encounter sampling at the same site.



Figure 2-1 Dip-netting in a shallow roadside pond in the Kammiesberg near Kammieskroon.

2.2.3 Aquatic Traps

Aquatic bucket traps were constructed from 20 L plastic buckets fitted with a funnel near the bottom and air holes drilled at the top (Figure 2-2). These traps were exclusively used to collect post-metamorphic *X. laevis*, and they make use of the aquatic nature and feeding behaviour of the species in order to trap them. Strongly odoured bait (liver and marrow bones) were used to lure frogs into the traps because *Xenopus* strongly rely on their olfactory sense for locating food. The sampling method is based on the assumption that animals that enter the trap are unable to escape or escape is delayed because of the difficulty in locating the inward-directed funnel opening. Traps were placed and left overnight near the water's edge with one third protruding above the water level allowing the trapped animals to surface for air. Trapped animals were immediately transferred to a container half-filled with water from the specific locality after traps were lifted because *X. laevis* (especially young individuals) can suffocate from the froth produced by the beating of skin secretion when they are crowded. Data from

[4] Prof. Rick Speare, James Cook University, Townsville, Queensland Australia

field sampling was recorded onto data sheets (Annexure D) and transferred into spreadsheets generated in Microsoft Excell upon return from fieldwork.



Figure 2-2 Setting an aquatic trap in a pond for the collection of *Xenopus laevis*.

2.2.4 Pathogen Control Protocol

Because the subject of fieldwork involved detecting an infectious disease of amphibians care was taken to prevent becoming a vector for transmitting chytridiomycosis between study sites. The Fieldwork Code of Practice developed by the Declining Amphibian Population Task Force provides a standard pathogen control protocol that should be followed when conducting amphibian related fieldwork (available from URL: http//ventura.fws.gov/es/protocols/dafta.pdf). By using this protocol as a guide, the following precautions and procedures were implemented during fieldwork.

- When not in use footwear, nets and aquatic traps were cleaned and stored in a dry place.
- A portable disinfection station was taken along during field visits. The station included adequate supplies of disinfectant solution (sodium hypochlorite) and tap water, two large square buckets (at least 30 L volume), a scrub brush and a pair of protective gloves.
- Footwear and equipment was disinfected before entering a site. Disinfection involved scrubbing boots and traps and soaking nets in a 2% sodium hypochlorite solution for 2 min., followed by rinsing with tap water.

- Upon leaving a site, mud and other organic and inorganic debris was removed from all footwear and equipment followed by disinfection.
- Disposable gloves were worn at all times during handling of live frogs. Gloves were changed between species and between sites. Care was taken to dispose of gloves and all other disposable equipment such as tissue wipes sensibly by using a dedicated refuse bag.
- Frogs that were collected from different sites were kept separate. Terrestrial frog species were not transported in water in order to reduce the risk of transmitting disease organisms through accidental splashing. A damp paper towel was used instead. When aquatic species and larvae were transported containers were covered with tight-fitting lids. On long trips the lids were regularly opened for a few seconds to allow for aeration of the containers. Once again, disposable gloves were used for this procedure.
- Collected frogs were examined for chytridiomycosis at the first opportunity after capture (on site, at base camp or in laboratory). All equipment used to gather specimen data or to remove tissue samples such as measuring instruments and dissecting equipment were sterilised with 70% alcohol between each specimen.
- When tissue samples were taken for molecular studies dissecting equipment was flamed between individuals in addition to sterilisation.
- Frogs that were kept for voucher specimens were anaesthetised by immersion in 0.1 % MS222 (tricaine methane sulphonale), fixed in 70% alcohol and provided with a label.

2.2.5 Identification of Frogs

Post-metamorphic frogs were identified in the field on their morphological traits and on the advertisement calls of males. Because captive frogs very seldom call after two to three days in enclosures, the identification of donated frogs (frogs collected by other workers and brought to our laboratory for examination) mostly relied on morphology only, like with fixed specimens. The species descriptions and taxonomic keys in Passmore and Carruthers (1995) and Channing et al. (2003) were used to identify frogs. Van Dijk (1966, 1971) and Lambiris (1989) were used to identify tadpoles. A useful clue to the identity of specimens in addition to morphology is information on site description and locality as most anuran species in South Africa are fairly restricted in type of habitat resulting in limited ranges.

2.3 Tissue Preparation for Diagnosis of Chytridiomycosis

2.3.1 Histology

Skin tissue fixed in 10% formalin was rinsed in running tap water for 90 min. and dehydrated in an alcohol series (30%, 50%, 70%). Toe clippings still containing bone were decalcified with Perreni's fixative by placing the tissue in the fixative for 18 h. The skin tissue was dehydrated further, elucidated with xylene and infiltrated with paraffin wax at 60°C (Figure 2-3). Following the wax infiltration the pieces of tissue were embedded in paraffin wax blocks using a SLEE MPS/P2 embedding center and allowed to harden overnight.

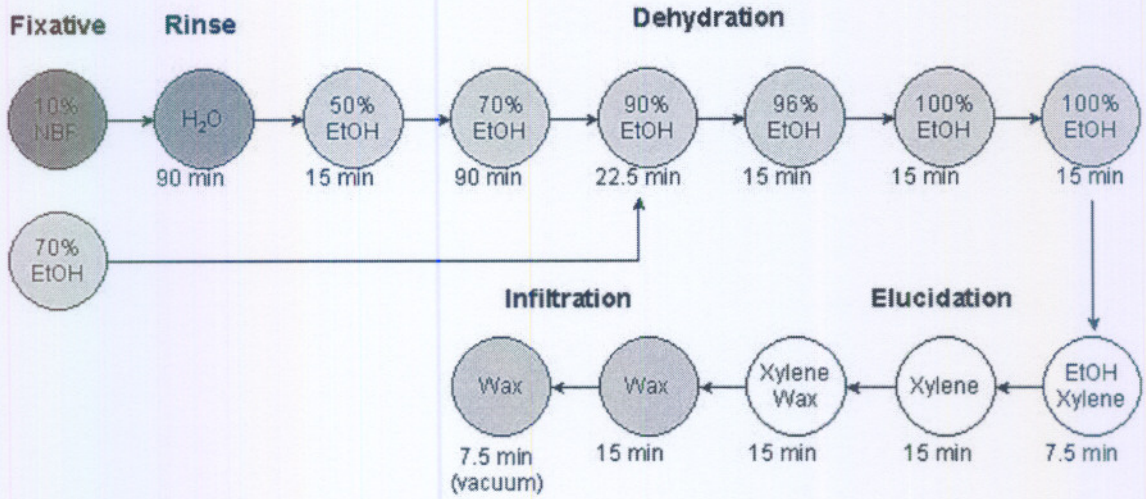


Figure 2-3 Tissue processing procedures for preparation of histological slides.

The skin tissues were sectioned at 6 µm with a Reickert-Jung 2050 automated microtome. Sections were stretched on a heated water bath (45°C) and adhered to microscope slides. Slides were dried overnight in an oven at 35°C. Three slides containing two ribbons of 4-8 sections each were prepared per specimen. Slide labels included date, species, tissue type and a reference number. Mayer's haematoxylin was used as staining solution and eosin as counter stain (Figure 2-4). Slides were examined under a Nikon Eclipse E800 compound microscope for the presence of *B. dendrobatidis* using the criteria described in Berger et al. (1999b).

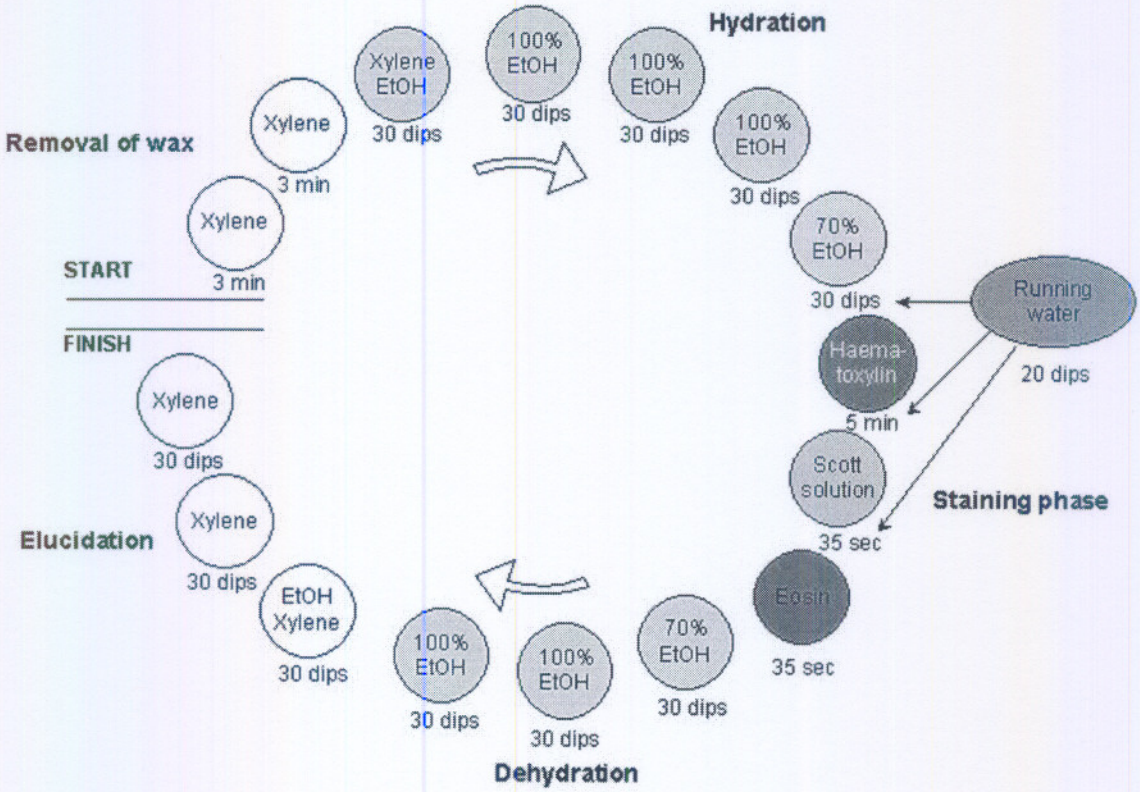


Figure 2-4 Steps in the staining of paraffin wax sections with haematoxylin and eosin.

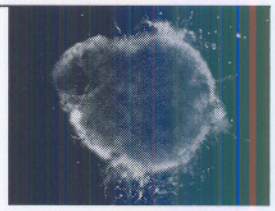
2.3.2 Wet Mounts

Skin sloughs from the feet, hind legs and ventral pouch can be used to diagnose chytridiomycosis without staining or tissue processing (Longcore 2001). Skin sloughs webbing, de-boned toe clippings, as well as tadpole mouth discs were placed on microscope slides in a drop of sterile water and covered with a coverslip. The wet tissue mounts are immediately ready for examination under a compound microscope. Wet mounts were used in conjunction with histological examination during prevalence surveys and to screen frogs for the purpose of isolating *B. dendrobatidis* into cultures.



Chapter 3

SCANNING MICROSCOPY EXAMINATION OF *Batrachochytrium dendrobatidis*



3.1 Introduction

Members of the phylum Chytridiomycota were until recently referred to as protoctistan fungi, because of their microscopic existence and often unicellular thalli (Kendrick 1992). Molecular phylogenetic studies, however, place the phylum with the true fungi (Forster et al. 1990, Bowman et al. 1992). Within the Chytridiomycota there exists some variation in the morphology of the vegetative phases, reproductive patterns and adaptations to parasitic or saprobic lifestyles, but they all have in common a zoospore with a single, posteriorly directed, whiplash flagellum and the cell wall is comprised of the polysaccharide, chitin (Sparrow 1960, Kendrick 1992).

Although chytrids are ubiquitous in water as well as soil, all members of this group are considered to be aquatic, because zoospores require water for dispersal. Many zoosporic fungi decompose a number of refractory substrates including pollen, keratin, chitin and cellulose (Sparrow 1960) thereby functioning as primary biodegraders of the ecosystem. Others have a parasitic mode of life involving other fungi, algae, plants, nematodes and insects (Barr 1990, Powell 1993). At least two chytrids are considered to be of economic importance: *Synchytrium endobioticum* causes wart disease of potato (Kendrick 1992) and *Batrachochytrium dendrobatidis* that is responsible for rapid population declines of wild and captive amphibians (Berger et al. 1998, Mazzoni et al. 2003).

The vegetative thallus of chytrids is either single celled (monocentric development) or may develop many sporangia (colonial development) through the formation of septa and may be entirely converted into a reproductive sporangium or be differentiated into a sporangium with rhizoids. The sporangium produces the zoospores, whereas the rhizoids function as an anchoring and absorptive apparatus. Filamentous chytrids produce a more extensive system of rhizomycelia with multiple nuclei. Asexual reproduction through mitotic nucleic division is the norm with chytrids. Although not common, sexual reproduction does occur in chytrid fungi and may occur by fusion of zoospores with each other or with somatic portions of the thallus, and is

associated with the production of a resistant, thick-walled resting spore (Sparrow 1960, Barr 1990).

The phylum has only one class, the Chytridiomycetes that comprises five orders: Blastocladiales, Chytridiales, Monoblepharidales, Neocallimastigales and Spizellomycetales, that are separated by the ultrastructural features of the zoospores (Barr 1980, 1990, Li and Heath 1992, Alexopoulos et al. 1996). The utility of ultrastructural features as a taxonomic character is supported by molecular phylogenetics (James et al. 2000). These taxonomically important features are revealed through transmission electron microscopy (TEM). Within-order classification may also depend on other reliable characters such as type of zoospore discharge and type of thallus development (Sparrow 1960, Karling 1977, James et al. 2000).

The amphibian chytrid fungus *B. dendrobatidis* is placed within the Chytridiales because of the zoospore features such as aggregated ribosomes and lack a connection between the nucleus and the kinetosome (Longcore et al. 1999, Pessier et al. 1999). The combination of the above mentioned zoospore ultrastructural features, together with numerous small lipid globules partially surrounded by the microbody and the microtubule that extends anteriorly into the ribosomal mass, are taxonomically distinct in *B. dendrobatidis* (Berger et al. 1998, Longcore et al. 1999). Berger (2001) in her examination of cultures and infected skin with conventional and cryo scanning electron microscopy (SEM) and high-pressure freeze-substitution TEM amplified the existing knowledge about *B. dendrobatidis* development. In addition, Berger supplements her findings with an extensive collection of high-resolution micrographs from all developmental stages. Among the observations Berger (2001) made were that the flagellum is rapidly resorbed shortly after the zoospore encysts, germlings may parasitise mature sporangia, entrapped zoospores are able to develop inside the sporangium and rhizoids from adjacent sporangia become entangled to form a strongly adhering colony. Numerous papers on *B. dendrobatidis* have since appeared that enhanced their findings with images from histochemical (Nichols et al. 2001, Bradley et al. 2002, Muths et al. 2003, Olsen et al. 2004) and electron microscopical (Bosch et al. 2001, Davidson et al. 2003, Mazzoni et al. 2003) examination. These images, however, primarily serve a diagnostic purpose and do not contribute to new information. There is no account in the published literature of the use of laser scanning confocal microscopy (LSCM) to study *B. dendrobatidis*.

Scanning confocal techniques are widely used to detect fungi in clinical and experimental specimens in all sorts of tissue types such as body fluids, internal organs, skin, nail scrapings and hair (Calich et al. 1979, Rüchel and Margraf 1993, Monod et al. 2000, Rüchel et al. 2000, Nishikaku and Buger 2003). Quite often a fluorescent stain is used that has a high affinity for

fungal cell wall components such as chitin and glucan. In mycological research, the use of LSCM enables the observation of biochemical processes such as cytoplasmic cleavage in living zoosporangia, the relationship between cytoplasmic calcium and hyphal growth, and the molecular architecture of fungal cell surfaces (Von Sengbusch et al. 1983, Fisher et al. 2000, Lilje 2004). A combination of fluorescent dyes with different staining properties and different microscopical techniques can be most effective when describing attributes of the morphology and development of certain lichenicolous fungi, due the difficulties in discerning the parasitic hyphae from that of their fungal hosts (Grube and De Los Ríos 2001).

In the present study, the observation of *B. dendrobatidis* with scanning microscopy is not a re-description of the organism, but rather a review of the molecular characteristics pertaining to morphology and development as revealed by confocal microscopy. The staining efficiency of the fluorescent dyes on *B. dendrobatidis* is discussed and recommendations are made on how LSCM can be used to study aspects of the biology and pathology of the amphibian chytrid.

3.2 Material and Methods

3.2.1 Scanning Electron Microscopy

Infected skin samples and tadpole mouthparts were obtained from wild *Afrana fuscigula* and *Xenopus laevis* from Namaqualand (see Chapter 4 for details). The samples were fixed in 70% ethanol, 10% neutral buffered formalin, Todd's fixative and vapour fixed in 4% osmium tetroxide. After critical-point drying, specimens were sputter-coated with a 20 nm layer of gold/palladium (80/20). Tissue samples were examined with a FEI Quanta 200 ESEM scanning electron microscope at 10 kV.

The following tissue preparation procedure were followed for specimens fixed in Todd's fixative:

Steps 1-3 were done in a fume cabinet.

1) Tissue were fixed in Todd's fixative for 2-8 hours,
2) rinsed for 15 min. in 0.05 M cacodilate buffer (x3) and
3) post-fixed in 2% osmium tetroxide (in distilled water) for 1 h.
4) Tissue were then rinsed for 15 min. in distilled water (x3) and
5) dehydrated in an acetone series (70%, 90%, 100%, 100% for 15 min. each).
6) Following the dehydration tissue were critical-point dried,
7) sputter-coated and
8) mounted on SEM stubs.

For specimens fixed in formalin, steps 4-8 above were followed and for ethanol fixed specimens steps 5-8 above were followed.

For vapour fixation with osmium:

1) Specimens were placed in a petri dish along with three drops of 4% osmium tetroxide around the tissue. Care was taken to prevent the fixative from coming in direct contact with the tissue.
2) The osmium tetroxide was allowed to vaporise in an oven at 40°C.
3) Tissues were then sputter-coated and mounted.

3.2.2 Laser Scanning Confocal Microscopy

Specimens to be imaged with a LSCM must be labelled with a fluorescent probe or be inherently fluorescent. When labelled with fluorescent probes macromolecular structures such as proteins, lipids, carbohydrates and nucleic acids or physiological ions such as calcium and pH can be imaged with LSCM. Performing a raster sweep of the specimen at that focal plane generates a two-dimensional image of the specimen centered on the focal plane. While the laser scans across the specimen, the analogue light signal, detected by a photomultiplier, is converted into a digital signal, contributing to a pixel-based image displayed on a computer monitor attached to the LSCM. It is important to remember that one does not view the tissue or organism on the monitor but a digitally generated image based on signals detected by the system.

Various tissue types and preparations were prepared for LSCM. Histological sections of *Nectophrynoides asperginis* from Kihansi Gorge, Tanzania (see Chapter 9 for details) and *A. fuscigula* were stained with haematoxylin and eosin (H&E). Skin tissue from a 70% ethanol fixed *A. fuscigula* specimen (A960612C14, National Museum Bloemfontein), collected in 1996 from Gharies, was labelled with 1 mM Acridine Orange (Molecular Probes) for 30 min. Nile Red (2 mM, Sigma) was applied to fresh interdigital webbing of *X. laevis* from Namaqualand for 30 min. For the examination of live *B. dendrobatidis*, broth culture from isolate CW36 (see Chapter 8 for details) was prepared in the following way:

1) Zoospores were separated from sporangia by centrifuging 1 ml of broth culture for 10 min. at 3,000 rpm.
2) Supernatant with zoospores was decanted into a 1 ml centrifuge tube. The pellet containing sporangia was mixed with 100 µl tryptone broth.
3) Supernatant from step 1 was centrifuged at 8,000 rpm for 30 min. and the pellet containing zoospores was mixed with 100 µl tryptone broth.

Sporangia and zoospores were labelled with four different dyes: 1 µl each of 1 mM Acridine Orange, 2 mM Nile Red, 2 mM Tetramethylrhodamine methyl ester (Molecular Probes) and 2 µl of 1 mM LysoTracker (Molecular Probes). All dyes were applied for 15 min.

Specimens and sections were examined with a Nikon PCM 2000 confocal scanning microscope, equipped with Argon (Ar) and Helium/Neon (He/Ne) Spectra-physics lasers. Excitation and emission used were selected according to the specific fluorochrome or staining protocol used. Typically, excitation at 475 nm and 505 nm, and emission at between 505 nm and 568 nm (green), as well as long pass (> 565 nm, red) were employed. A 5 µm pinhole was employed to prevent dye bleaching and light scattering. Image processing and analysis were performed on supplied Nikon software. Various ApoPlan objectives were used, with most of the images captured by magnification with a Nikon 60x/1.40 Apo Planar oil objective and bars in the micrographs indicate size. A scan speed of 3 µs/scan was typically used and capturing was averaged to obtain representative micrographs.

Properties of Dyes

Haematoxylin and Eosin

Although haematoxylin will stain tissue components such as myelin, elastic and collagen fibres, muscle striations, mitochondria etc. it is most commonly used as a nuclear dye in the standard H&E and staines nuclei either blue or purple. In addition haematoxylin is autofluorescent. Eosin is most often used as a counterstain to haematoxylin in H&E staining. When used with haematoxylin, eosin stains cytoplasm orange-pink.

Acridine Orange

Acridine orange exhibits metachromatic fluorescence that binds to nucleic acid. DNA usually fluoresces in the red spectrum and RNA fluoresces green. Yellow light is emitted when RNA is being transcribed.

Nile Red

Nile Red associates with phospholipid bilayer membranes and is therefore used as a membrane and lipid probe. When bound to lipids, Nile Red fluoresces in the red to orange spectrum.

Tetramethylrhodamine methyl ester (TMRM)

The accumulation of TMRM in mitochondria, endoplasmic reticulum and other membranes is driven by membrane potential. TMRM is also used as a mitochondrion-selective dye. The intensity of fluorescent light in the red spectrum is related to the membrane potential.

LysoTracker

LysoTracker is a fluorescent acidotropic probe for labelling and tracking acidic organelles such as lysosomes and vacuoles in live cells. Depending on which fluorescent molecules are used in the labelling of LysoTracker, it will emit fluorescence in either the green or red spectrum. In this case, LysoTracker Green was selected, allowing bright green fluorescence when associated with acidic organelles.

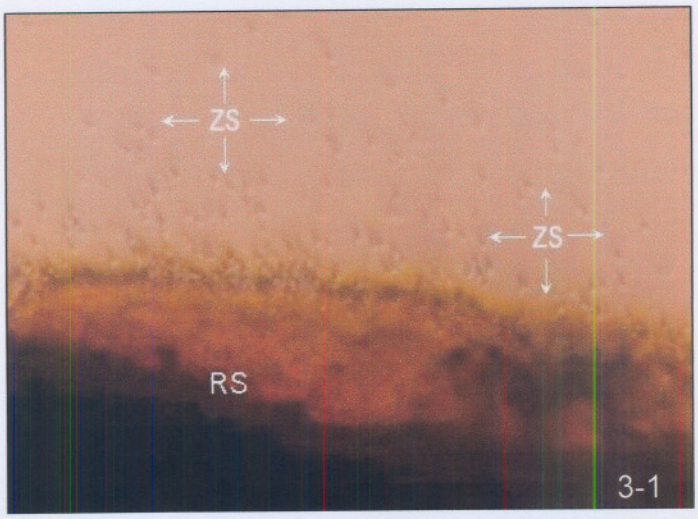
3.3 Results

3.3.1 Development of the Thallus in Culture

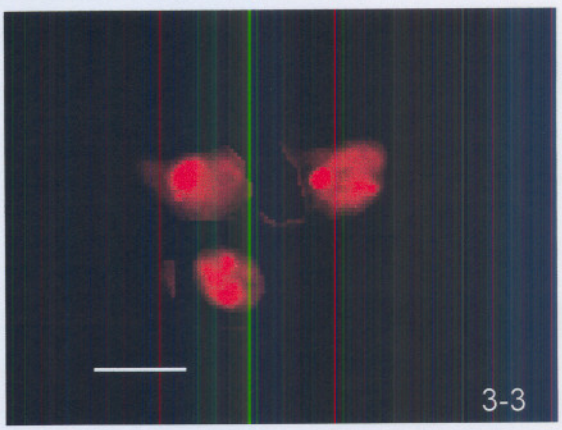
Zoospores

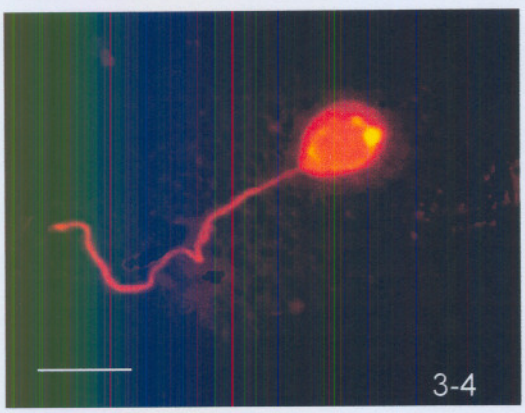
Within a day of transferring host tissue containing active sporangia to culture plates, the sporangia start to release their zoospores. In the case of heavy infections zoospores appear in dense masses surrounding the entire tissue, but limited to a thin film of water enclosing the tissue sample (Figure 3-1). Upon their release the zoospores appear amoeba-shaped, but shortly thereafter obtain a round shape of uniform size (approximately 3-5 µm in diameter). Zoospores swim with the aid of a posterior directed flagellum (Figure 3-2) away from and towards the tissue sample and eventually encyst on and near the edge of the tissue.

Ultrastructural characteristics of zoospores and the combinations in which they occur are among the features used in the taxonomic classification of Chytridiomycota (Barr 1990). The zoospore of *B. dendrobatidis* contains a nucleus that is nested on the outer perimeter of a central mass of aggregated ribosomes, which is surrounded by an endoplasmic reticulum (Longcore et al. 1999). Microbodies that partially surround lipid globules, as well as mitochondria are scattered on the periphery of the central core. The cytoplasm contains a single Golgi apparatus. The kinetosome and associated components are situated in the posterior section of the zoospore. The zoospore is encapsulated within a cell membrane, however, a cell wall starts to develop after the zoospore has encysted and the single flagellum has been resorbed. Selective staining with fluorescent probes revealed some of these above-mentioned features (Figures 3-3 and 3-4). The acidotropic probe produced fluorescent light from the zoospore, suggesting the presence of lysosomes and vacuoles (Figure 3-5). These organelles may be the equivalent of what Longcore et al. (1999) in their original description of *B. dendrobatidis* referred to as "clear and dense-cored vesicles" contained in the cytoplasm of the zoospore.









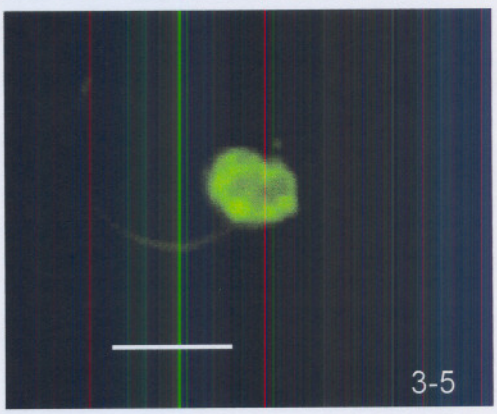


Figure 3-1 Active zoospores of *B. dendrobatidis* in culture, one day following isolation. RS = rostrodont, ZS = zoospores.

Figure 3-2 Light micrograph of live zoospores showing the posterior flagellum (scale bar = 5 µm).

Figure 3-3 Nile Red LSCM of zoospores. The bright red vesicles represent lipid globules that are arranged in single or multiple groups around the ribosomal core not visible in this image (scale bar = 5 µm).

Figure 3-4 TMRM LSCM of zoospore. Membrane potential was detected for the flagellum, cell membrane, endoplasmic reticulum (yellow halo surrounding the central core) and the perinuclear situated mitochondria (visible as bright yellow spots). A single outside membrane indicates that the cell wall has not yet formed (scale bar = 5 µm).

Figure 3-5 LysoTracker LSCM of zoospore. The pattern of the bright green emission, suggests that acidic organelles such as lysosomes and vacuoles probably occur in the cytoplasm surrounding the ribosomal mass and nucleus (scale bar = 5 µm).

Zoosporangium and Rhizoids

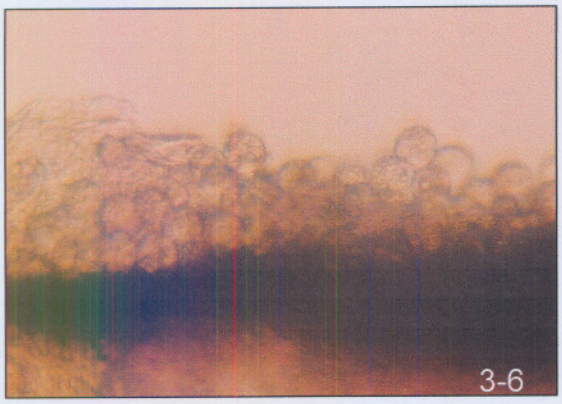
The zoospores develop into sporangia that become visible as cell-walled, spherical vesicles after 2-4 days in culture (Figure 3-6). At the same time fine branching rhizoids extend outwards from one or more positions on the sporangium (Figure 3-7). The rhizoids function as attachment organs and are lined with numerous mitochondria (Figure 3-8) that are presumably responsible for providing the energy required by their growth.

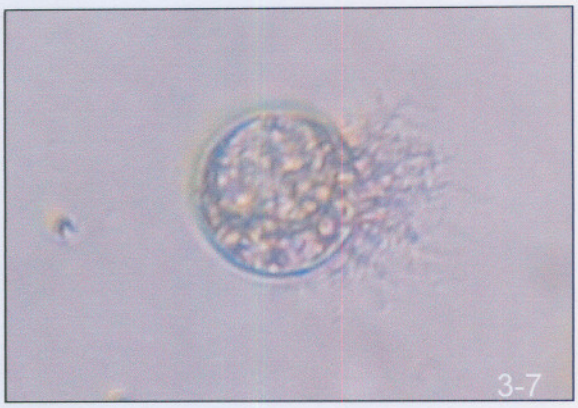
As the sporangium continues to develop, the thallus becomes multinucleate by repetitive mitotic division (Figure 3-9). The entire content of the sporangium then cleaves and encapsulates a nucleus and multiple mitochondria to form the flagellated zoospores (Figures 3-10 and 3-11). Mature sporangia vary greatly in size and may reach sizes of up to 60 µm.

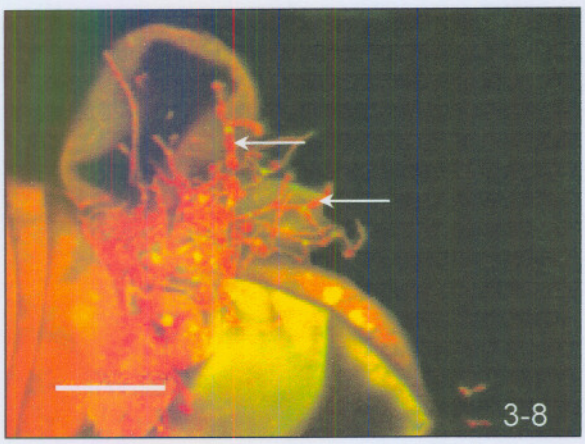
While zoospore formation is taking place, tube-like processes called discharge papillae form on the surface of sporangia (Figure 3-12). The number of discharge papillae is not fixed, but generally varies in accordance to the size of the sporangium and may protrude in any direction when grown in culture. Up to seven discharge papillae protruding in all directions were counted on a single sporangium. The discharge papillae are so called because they are the exit ways for mature zoospores. The discharge papillae are inoperculate and are blocked by a plug that reveals moderate membrane potential (Figure 3-13). Labelling of sporangia with a lipid probe indicates that the plug consists of lipid molecules, but of a higher density than the phospholipid bilayer of the cell membrane (Figure 3-14). The cell wall is discontinued in the distal section of the discharge papillae; a characteristic that may ease the release of zoospores after the plug has been dissolved.

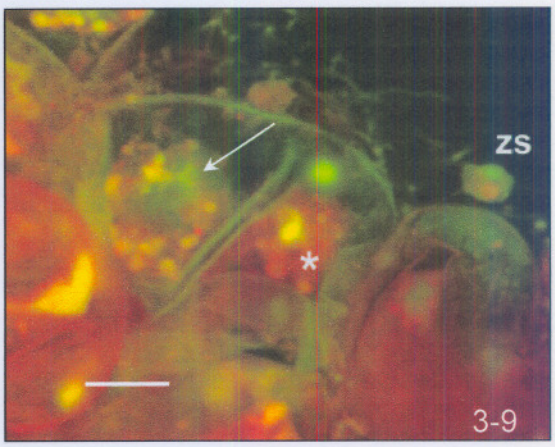
The sporangium may develop as a monocentric thallus or may become divided into two or more compartments by the formation of a septum (Figures 3-15 – 3-17). The septum appears to develop along a central axis of the sporangium. The flaps of the septum then extend towards the wall, thus allowing free movement of the content of the sporangium until they make contact with the wall. The flaps may part to form a cavity free of any sporangium content. The septum fluoresces in the same spectrum as the cell wall, which indicates that it consists of a similar macro molecular structure.

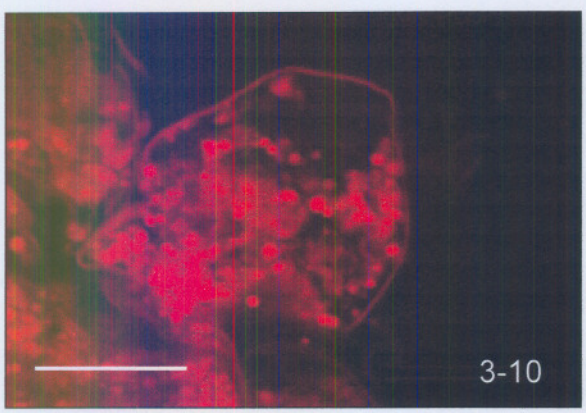
Labelling with the acidic probe LysoTracker indicates a concentration of acidic organelles (lysosomes and vacuoles) near the outer perimeter of the sporangium and within the cell membrane (Figure 3-18). Lysosomes also fluoresce within the rhizoids, suggesting that the rhizoids are involved in the absorption of nutrients for the maturing sporangium.











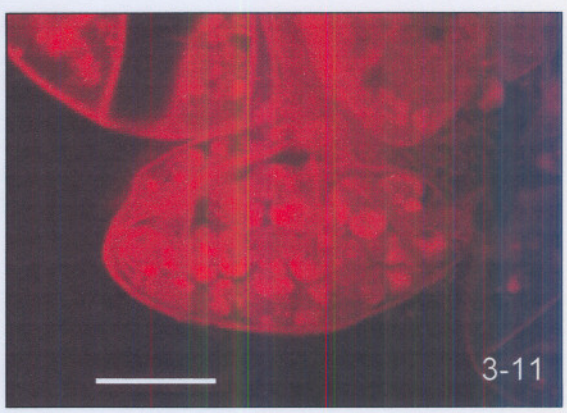


Figure 3-6 Light micrograph of first-generation, immature *B. dendrobatidis* sporangia in culture.

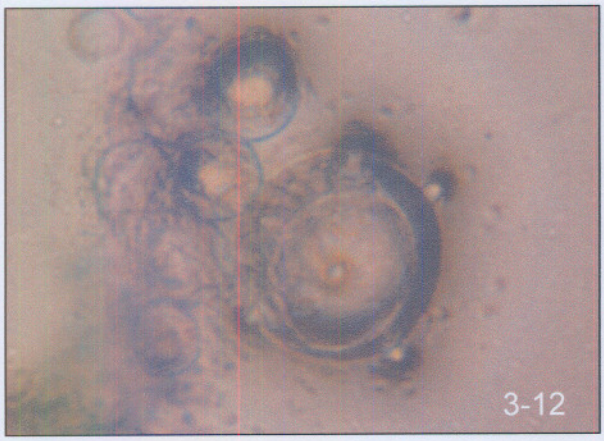
Figure 3-7 Light micrograph of an immature sporangium with a dense aggregation of rhizoids spreading into the nutrient agar.

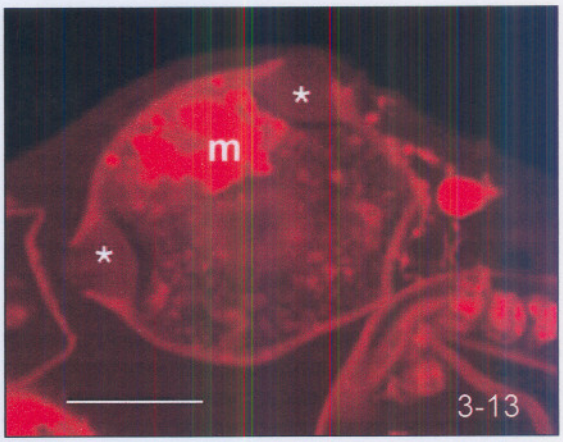
Figure 3-8 Acridine Orange LSCM of rhizoids. Mitochondria contain DNA and are visible as red spots along the shafts of rhizoids (arrows) that resemble beads on a string (scale bar = 10 µm).

Figure 3-9 Acridine Orange LSCM of developing sporangium. Somatic DNA (asterisk) can be seen transcribing RNA (first step in zoospore formation). A mass of RNA, possibly ribosomal RNA, is visible at a second site within the thallus (arrow) and is surrounded by multiple sites where RNA transcription is in progress. The nucleus (red) and ribosomal mass (green) are discernible in a released zoospore (ZS) (scale bar = 5 µm).

Figure 3-10 TMRM LSCM of a sporangium in late development. Clusters of mitochondria are associated with maturing zoospores (scale bar = 10 µm).

Figure 3-11 Acridine Orange LSCM of a mature sporangium that is filled to the limit with zoospores containing genetic material (scale bar = 10 µm).





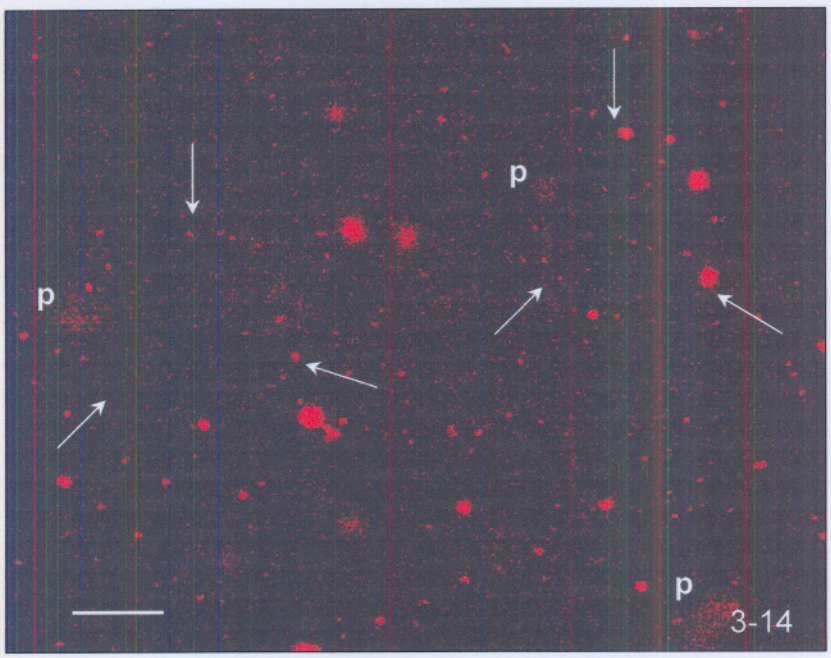
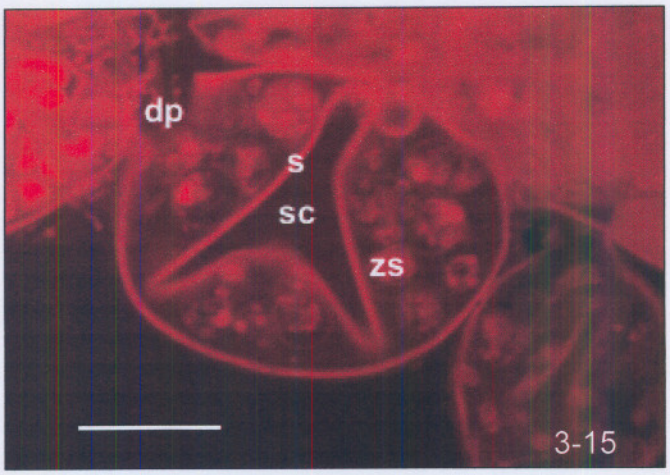
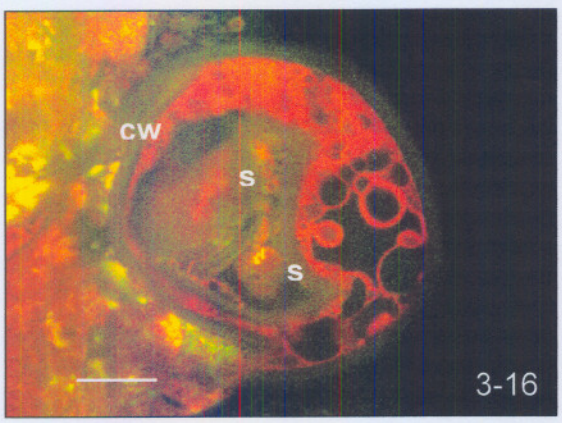


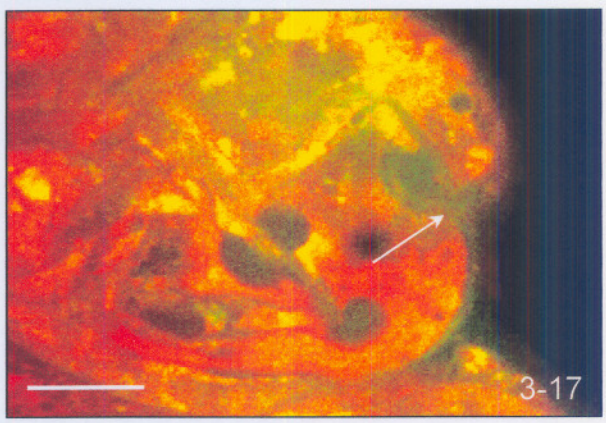
Figure 3-12 Light micrograph of a mature sporangium in culture with five, possibly six, discharge papillae.

Figure 3-13 TMRM LSCM of an immature sporangium with two discharge papillae. The cell wall is discontinued over the tip of discharge papillae. A plug with membrane potential (asterisk) blocks the opening. A dense mass of mitochondria (m) is visible inside the sporangium (scale bar = 10 µm).

Figure 3-14 Nile Red LSCM of a cluster of sporangia. The arrows delineate the circumference of two sporangia. The lipid fraction of the cell membrane is visible as bright red spots. The diffuse areas (P) in line with the wall correspond to the plugs of discharge papillae (scale bar = 10 µm).







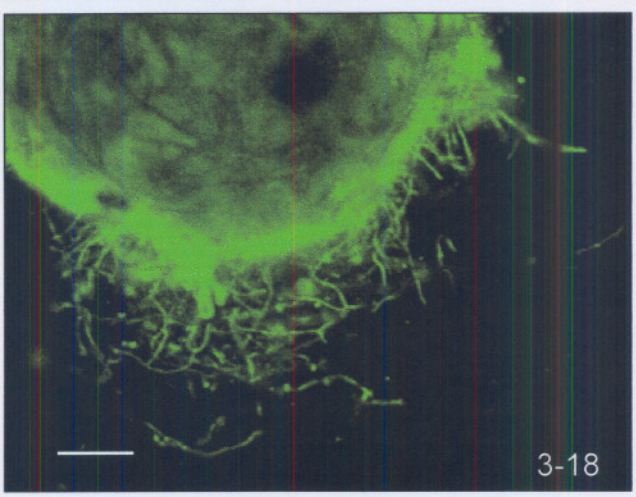


Figure 3-15 Acridine Orange LSCM showing the form and position of a developing septum (s). dp = discharge papilla, sc = septum cavity, zs = zoospore (scale bar = 10 µm).

Figure 3-16 TMRM LSCM of sporangium with septum. Fluorescence of cell wall (cw) and septum (s) in the same spectrum and with the same intensity indicates that these two components have the same molecular structure (scale bar = 10 µm). The fact that the cell membrane of the sporangium is not as distinct as that of the zoospores could be due to inhibition of its membrane potential caused by the cell wall.

Figure 3-17 TMRM LSCM of sporangium showing part of a septum. The arrow indicates the connection between the cell wall and the septum (scale bar = 10 µm).

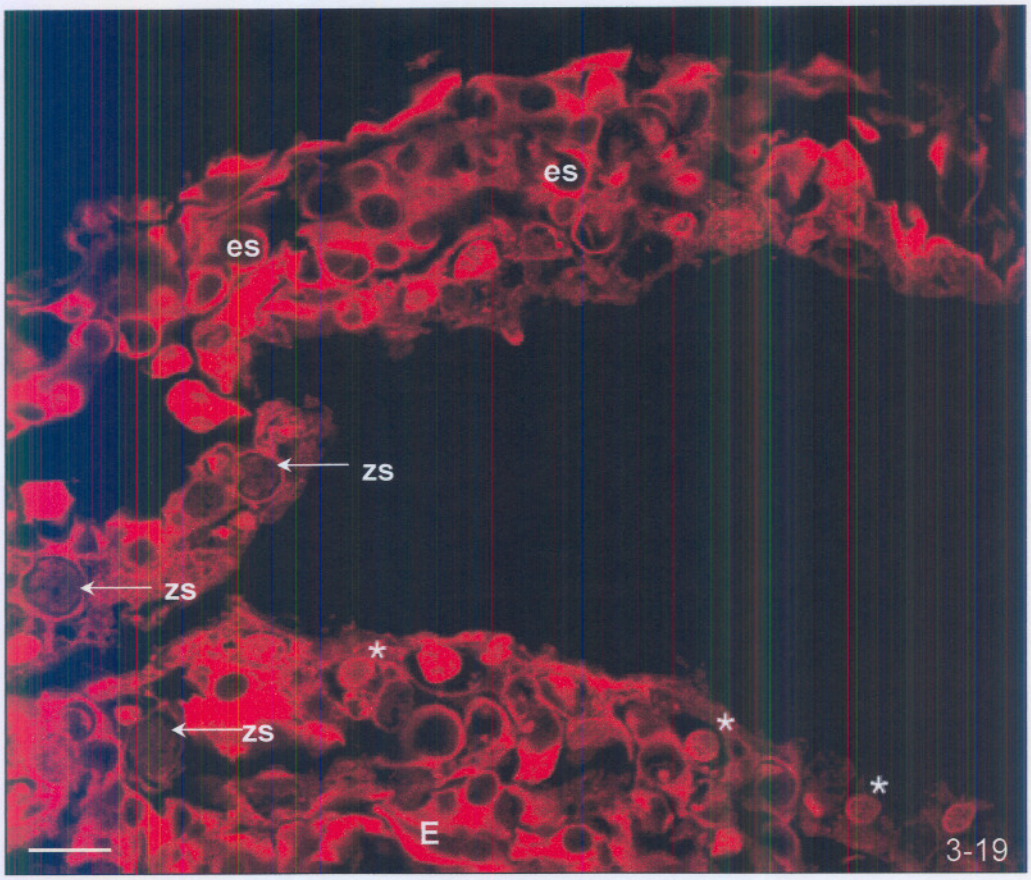
Figure 3-18 LysoTracker LSCM of a giant sporangium indicates that the cell membrane and surrounding environment has a lower pH than the rest of the sporangium content. Distinct vesicles with low pH inside the rhizoids suggest that acidic enzymes may be secreted by the rhizoids (scale bar = 10 µm).

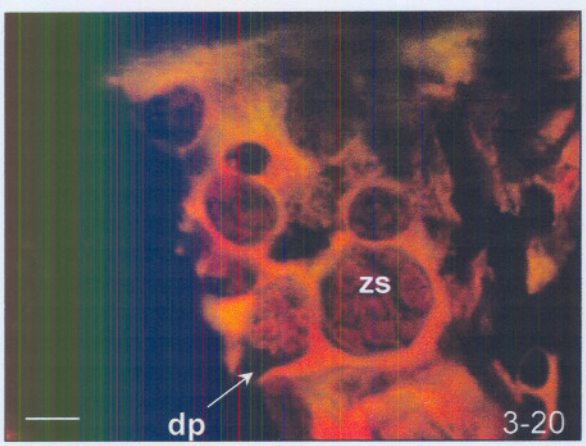
3.3.2 *Batrachochytrium* in Amphibian Skin

Sporangia start their development in the deepest layers of the *stratum corneum* or the outside layers of the *stratum granulossum* of the epidermis within the keratinous cells (Figure 3-19). Although it has not been documented how the DNA from encysted zoospores on the amphibian skin surface reach the deeper layers, it is suspected that a germ tube could facilitate this transfer (Longcore et al. 1999). Growth of the thallus in skin differs from that in culture in that the sporangia do not grow as large in skin and discharge papillae are less abundant and are usually directed towards the outside surface (Figure 3-20). These differences arise from the restrictions in growth imposed by intracellular development. Typically, the epithelium layers closest to the surface contain the largest proportion of empty sporangia (sporangia that have released their zoospores). When different excitation wavelengths and emission filters were combined, subtle differences in the staining patterns were observed between connective tissue, infected epidermis and the outer cornified layer (Figure 3-21). While the sporangia develop, they are pushed toward the surface though the natural process of transitional epithelium regeneration. When the infected cell reaches the surface, it bulges out due to the growing sporangium within its cytoplasm (Figure 3-22 and 3-23). The discharge papilla eventually punctures the cell membrane to expose its tip to the free surface of the skin (Figure 3-24). After sporangia have released their zoospores they sometimes collapse (Figure 3-25) before being shed with the superficial epidermal layer. Fixation of samples in Todd's fixative, 70% ethanol and 10% neutral buffered formalin for SEM gave satisfactory results of the skin surface and *B. dendrobatidis'* external morphology. On the other hand, vapour fixing with 4% osmium tetroxide caused excessive shrinking, preventing meaningful interpretation of samples.

Gross lesions on the surface of amphibian skin are localised to the areas of infection. The smooth, squamous pattern of the epithelium is disrupted due to a hyperkeratotic reaction and the continuous flaking of infected cells (Figure 3-26). Melinisation of infected areas is often observed in fresh skin scrapings of *X. laevis*. In the keratinised rostrodonts of tadpoles, that are usually strongly pigmented, the reaction seems to be reversed resulting in depigmentation. Sometimes *B. dendrobatidis* infection leads to a loss of kerotodonts on tadpole mouths (Figure 3-27).

Labelling of fixed skin samples with a nucleic acid probe allows a distinction between active and empty sporangia to be made, because the DNA from developing and mature sporangia fluoresces at a high intensity (Figure 3-28). It is also possible to trace *B. dendrobatidis* by labelling skin samples with a lipid probe because the phospholipid bilayer in the cell membrane and the presence of lipid globules in the zoospores (Figure 3-29).





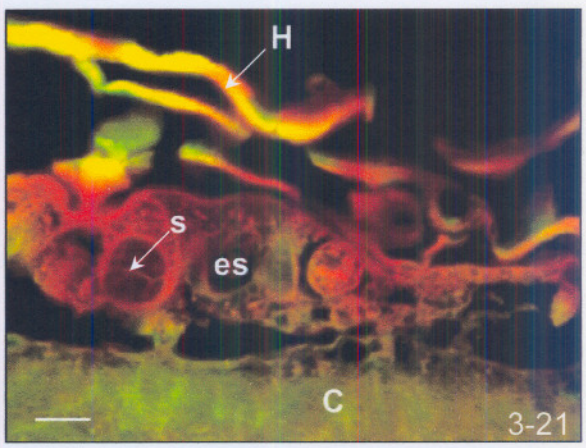
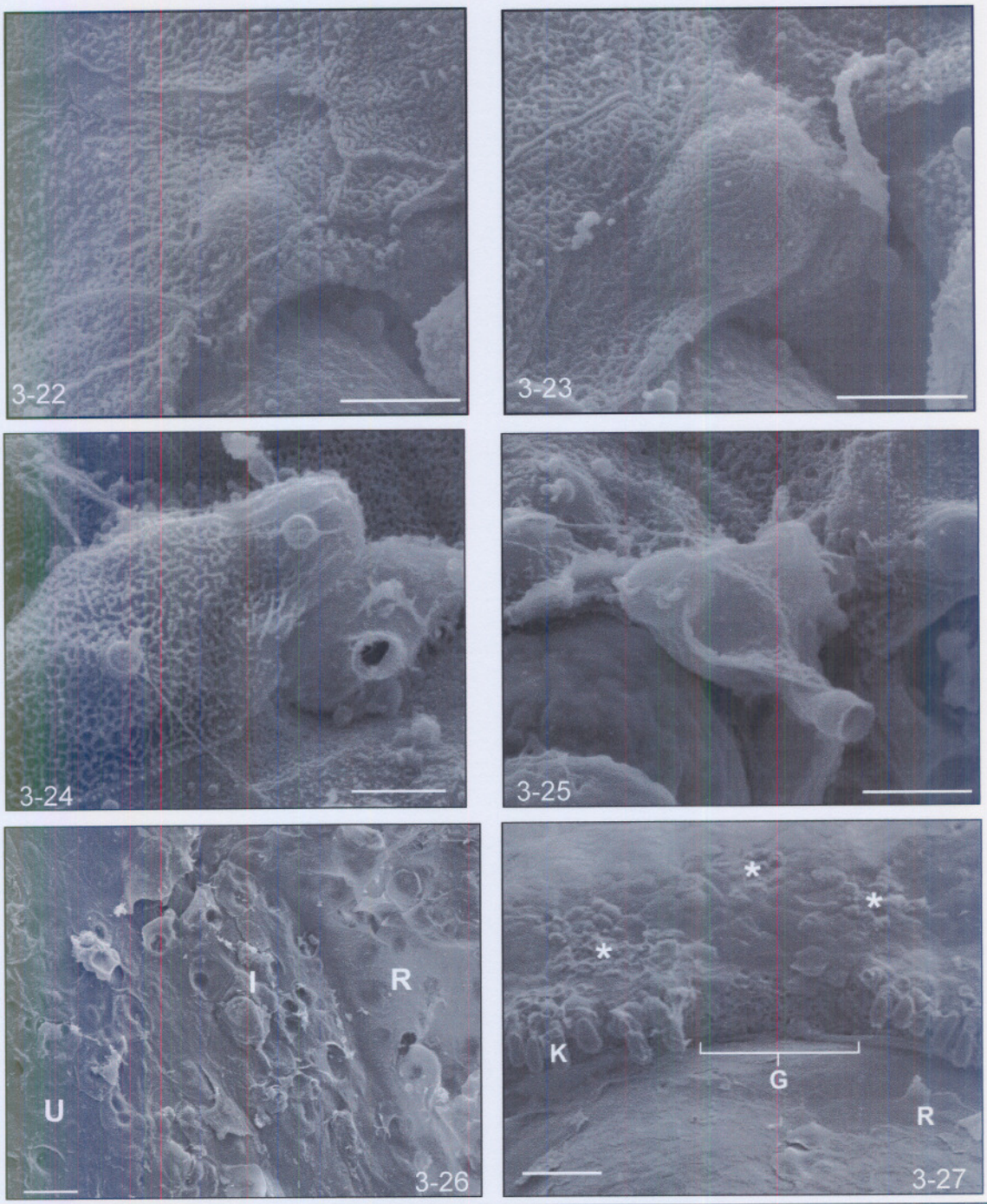


Figure 3-19 LSCM of an H&E stained cross section through heavily infected skin of *Afrana fuscigula*. Various developmental stages of *B. dendrobatidis* are visible in this section. Immature sporangia are embedded in the deeper layers of the *stratum corneum* (asterisk). The older, empty sporangia (es) are located near the surface of the skin. E = epidermis, zs = zoospores (scale bar = 10 µm).

Figure 3-20 LSCM of an H&E stained cross section through *Nectophrynoides asperginis* skin. The discharge papilla (dp) of a mature sporangium opens towards the free surface of the epidermis. zs = zoospore (scale bar = 5 µm).

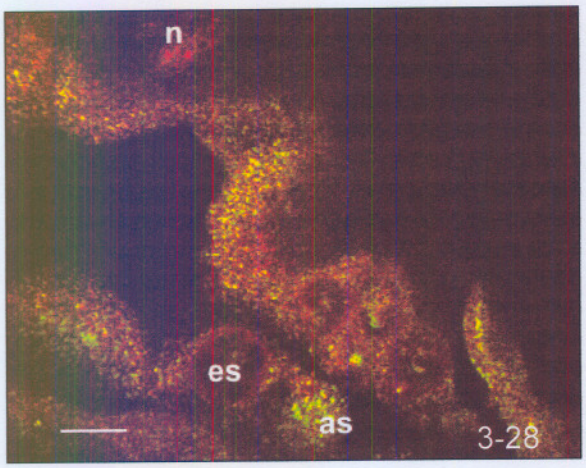
Figure 3-21 LSCM of an H&E stained section of *X. laevis* skin. The combination of different excitation wavelengths and emission filters results in differences in the emission spectrum of infected epidermal cells, collagen fibres (C) and hornified epithelium (H). es = empty sporangium, s = septum (scale bar = 5 µm).



Figures 3-22 – 3-25 SEM series of different stages of *B. dendrobatidis* development on the mouth of an *A. fuscigula* tadpole fixed in Todd's fixative (scale bar = 5 µm). **3-22** When an infected epithelial cell reaches the surface it can be identified by a tiny bulge in the surface. **3-23** The shape of the sporangium is clearly visible and the position of the discharge papilla can be identified. **3-24** Open and closed discharge papillae protruding though the surface of the epithelial cells. **3-25** A collapsed sporangium that is about to be shed with a hornified skin flake.

Figure 3-26 SEM of *X. laevis* webbing. Infected parts of the skin (I) have an abrasive appearance due to the flaking of the superficial cell layer and indentations left by sporangia compared to uninfected parts (U) that have a smooth appearance. In recovering skin (R), faint indentations left by sporangia is the only evidence of the infection (scale bar = 20 µm).

Figure 3-27 SEM of the upper jaw of an *Afrana fuscigula* tadpole. A loss of kerotodonts has resulted from *B. dendrobatidis* infection. Protruding discharge papillae and flaking of superficial cells give infected areas a coarse appearance (asterisk). G = gap in kerotodont row, K = kerotodont, R = rostrodont (scale bar = 50 µm).



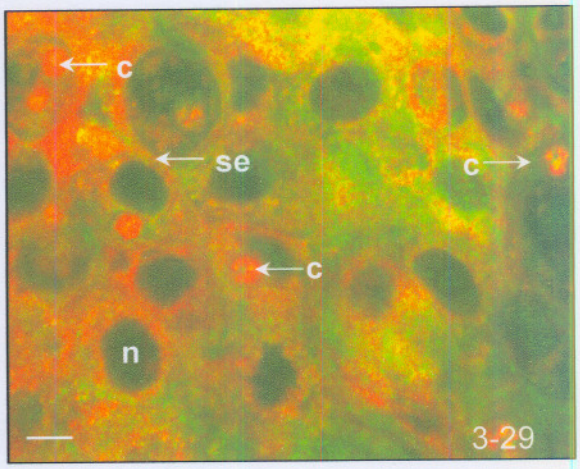


Figure 3-28 Acridine Orange LSCM of *A. fuscigula* skin fixed in 70% ethanol. The contents of active sporangia (as) fluoresce, whereas empty sporangia are visible as dark spherical structures. The wave like pattern is created by numerous mitochondria in the host tissue. n = nucleus of squamous epithelium cell (scale bar = 10 µm).

Figure 3-29 Nile Red LSCM of fresh *X. laevis* skin. Lipids contained in the cell membrane and zoospores enable chytrid thalli (c) to be observed in the cytoplasm of squamous epithelium cells (se). n = nucleus of squamous epithelium cell (scale bar = 10 µm).

3.4 Discussion

Through selective staining every component of the thallus of *B. dendrobatidis* can be viewed with LSCM. The surface and septa of sporangia react strongly with TMRM and Acridine Orange. The cell wall is not expected to associate with Acridine Orange and might therefore contain a molecule that intercalates with the nucleic acid dye (i.e. chitin). Nile Red effectively illustrated the configuration of the lipid bilayer of the cell membrane and the lipid globules of zoospores. On the other hand, the acidotropic dye, LysoTracker, specifically highlighted areas and organelles with an acidic pH in the sporangium, zoospores and rhizoids. The approximate shape and length of rhizoids can be discerned by its mitochondria fraction revealed by Acridine Orange or TMRM labelling, and by the presence of acidic enzymes through LysoTracker labelling. It is possible to distinguish between developing and mature sporangia in two ways. Firstly, when labelled with TMRM, mitochondria tend to be randomly distributed within the content of the developing sporangium, whereas a more clumped distribution is eminent in mature sporangia. Secondly, when labelled with Acridine Orange, RNA transcription is clearly visible in actively growing sporangia, whereas the shape of individual zoospores is visible in mature sporangia through the reaction of nucleic and mitochondrial DNA with the dye.

As far as the application of LSCM as a diagnostic tool for *B. dendrobatidis* goes, the dyes used on fresh and fixed skin samples, Acridine Orange and Nile Red, are not the best choices. Although it was possible to observe the fungus in host tissue, the DNA and other organelles of host were also labelled, making it slightly more difficult to make a positive diagnosis. When diagnosis is the primary objective it would be better to use dyes that associate with unique fungal characteristics such as Blankophor and Calcofluor that have a high affinity for beta-glycosidically linked polysaccharides, such as chitin and glucan, found in the fungal cell wall (Monod et al. 2000, Nishikaku and Burger 2003). The morphology, size and position in the skin of fluorescent objects would have to be taken into account in order to distinguish *B. dendrobatidis* from other possible fungal infections. Confocal microscopical observation of H&E stained skin sections proved to be most effective in visualising cell walls, septa and nuclei of zoospores. Although no additional characteristics were observed, the distinction between the nucleus and cytoplasm of zoospores is more apparent than with conventional light microscopy.

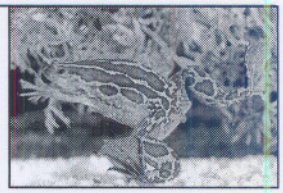
LSCM could be very useful when attempting to answer specific questions regarding the biology of *B. dendrobatidis* that are not yet fully understood. There exists some debate regarding whether or not *B. dendrobatidis* utilises keratin as a nutrient. The amphibian chytrid fungus is localised to keratinised tissue (tadpole mouthparts and post-metamorphic skin), however, a recent study did not find proof that keratin is indeed degraded by the extracellular proteases produced by the fungus (Daszak et al. 1999, Piotrowski et al. 2004). Selective staining of keratin with fluorescent dyes may be an alternative avenue to pursue this question. Calcium changes in encysted zoospores have been studied through labelling with calcium-sensing dyes fluo-3 and calcium green (Deacon 2005). A similar approach could be followed to investigate the germination of live encysted zoospores on amphibian skin to establish whether or not a germ tube is indeed formed, as is currently believed to be the most likely mechanism for the migration of the nucleus to the deeper layers of the epidermis (Longcore et al. 1999). Another field of study that can be investigated through the use of LSCM relates to the pathology of *B. dendrobatidis*. Because it has been suggested that *B. dendrobatidis* impairs cutaneous respiration (Berger et al. 1998, Pessier et al. 1999), labelling of physiological ions responsible for gaseous exchange in amphibian skin could allow the quantification of these vital ions in the vicinity of infected skin.

As this study and previous work have shown, through the combination of technological advances provided by qualitative and quantitative analytical techniques, we continue to expand our knowledge of morphology and development of microscopic organisms. *Batrachochytrium* is fairly new to science and there remains a vast number of aspects of especially the ecology and pathology, including host-pathogen relationships that await their discovery.



Chapter 4

GEOGRAPHICAL ORIGINS OF CHYTRIDIOMYCOSIS IN AFRICAN ANURA, WITH THE EMPHASIS ON SOUTH AFRICA



4.1 Introduction

Worldwide amphibians are under severe threat from anthropogenic influences as well as global factors. Over the past three decades especially, reports of declines have increased in the Neoarctic, Neotropical and Australasian realms (Drost and Fellers 1996, Laurance et al. 1996, Houlahan et al. 2000, Stuart et al. 2004). Of even greater concern is that declines have not only occurred in disturbed habitats, but also in pristine environments. Evidence for a countrywide decline in frog populations in South Africa is lacking (Channing and Van Dijk 1995, Sparling et al. 2003). Amphibian declines in South Africa have only been observed at the local population level and are often confined to an area that is directly affected by the concerned threat (Weldon and Du Preez 2004a).

South Africa has a relatively rich amphibian biodiversity. Only frogs and toads are represented and comprise almost 120 species and 33 genera from 9 families (Carruthers 2001). Frogs occur throughout South Africa and their distribution is largely influenced by rainfall and temperature. The highest number of species is found in the eastern regions where high rainfall combines with warm year-round temperatures. The highest level of endimicity, however, occurs in the Fynbos biome (particularly the Cape Fold Mountains) of the Western Cape. Although some species are widely dispersed in a variety of localities and climate regions, most species are fairly restricted in type of habitat resulting in limited ranges. Of the 115 recognised species in South Africa roughly one sixth are threatened (4 critically endangered, 8 endangered, 8 vulnerable) and a further 5 are near threatened according to the IUCN criteria for the conservation status of wildlife (Harrison et al. 2001). Threatened taxa are spread across most families, however, the more specious families tend to contain relatively fewer threatened taxa than less specious families. An analysis of the distribution of threatened species was conducted by Branch and Harrison (2004) in which richness/count of threatened and near threatened species as well as categories of different weight in terms of their priority for conservation were used as criteria. According to this analysis six hotspots of threatened frogs emerged, namely, and in order of decreasing rank, Western Cape lowlands, Western Cape

uplands, KwaZulu-Natal coastal, KwaZulu-Natal midlands, Amatola Mountains (Eastern Cape) and Other (a combination of all the cooler hotspots).

Few threats to amphibians in South Africa have been studied in detail and reports have been largely anecdotal. Habitat loss is cited as the most pervasive threat for South African amphibians and was listed as a possible threat to all threatened species (Harrison et al. 2001). Agricultural development, afforestation, invasive plants and urban development are the main drivers of habitat loss. The effects of pollutants on South African frogs have not yet been addressed, but are considered important threats to populations. Other lesser threats include natural and anthropogenic fire regimes and mortalities due to road traffic. Climate change is predicted to become an increasingly important threat to amphibian assemblages because annual mean temperature is predicted to increase and rainfall to decrease (Midgley et al. 2001).

One of the biggest threats facing amphibian species and population survival worldwide is the disease, chytridiomycosis, caused by the chytrid fungus, *Batrachochytrium dendrobatidis* (Daszak et al. 1999, Speare et al. 2001). Chytridiomycosis was proposed as the cause of mortality in frog populations in the rain forests of Australia and Panama, and associated with declines in North, Central and South America, New Zealand, and Spain (Berger et al. 1998, Lips 1999, Bosch et al. 2001, Bonaccorso et al. 2003, Muths et al. 2003, Bell et al. 2004). Chytridiomycosis is known in South Africa from *Xenopus laevis, Afrana fuscigula* and *Strongylopus grayii* (Weldon 2002b, Hopkins and Channing 2003, Lane et al. 2003a) and is suspected to have been involved in a mass mortality event of *Amietia vertebralis* (Weldon and Du Preez 2004a). Although the amphibian chytrid is widespread in the rest of Africa, having been detected in frogs from Ghana, Kenya, and Western Africa (Reed et al. 2000, Speare and Berger 2000, Morehouse et al. 2003), records are few and for most countries there is no data.

Because *B. dendrobatidis* has been recognised as an emerging pathogen whose spread is facilitated by the international and intranational movement of amphibians (Daszak et al. 1999), a useful piece of the epidemiological puzzle is to identify its origin. Some emerging infectious diseases are caused by pathogens localised to a single host or small geographic region escaping from that confinement (Morse 1995). We hypothesise that for *B. dendrobatidis,* if this was the case, the source would meet the following criteria:

1) the hosts would show minimal or no apparent clinical effects,
2) the earliest known global occurrence would be found,

3) the date of this occurrence would precede any amphibian declines in pristine areas (i.e., late 1970s),
4) the prevalence in the source host or hosts would be stable over time,
5) no geographical spreading pattern would be observed over time in the region,
6) a feasible means of global dissemination of *Batrachochytrium* from the region of origin would be identified.

- Through surveys of extant and archived specimens, *Batrachochytrium* has been found in every continent that has amphibians, except Asia (Carey et al. 1999, Lips 1999, Speare and Berger 2000, Bosch et al. 2001, Weldon 2002b). Given that only regional surveys dominated by a single species have been carried out in South Africa (Weldon 2002b, Hopkins and Channing 2003) and because surveys are lacking from any other African country, one of the objectives of this study was to determine the extent of chytridiomycosis in Africa by surveying mainly archived *Xenopus* spp. Secondly, I wished to produce a more detailed map of chytridiomycosis in South Africa by surveying additional species with the attributes of high-altitude, stream breeders or a wide distribution, as well as, examining any specimens that were submitted to the laboratory.
- Owing to the widespread occurrence of *B. dendrobatidis* in African frogs and poorly documented population declines in Africa combined with the global trade in *X. laevis* and *X. tropicalis*, a further objective was to investigate the likelihood that Africa was the origin of *Batrachochytrium* and that the trade in *Xenopus* spp. was involved in its global dissemination. Within the *Xenopus* genus, *X. laevis* is distributed over the greatest area in sub-Saharan Africa. It occupies most types of water bodies in savannah habitat from the Cape to Nigeria and Sudan (Tinsley et al. 1996, Channing 2001).

This chapter presents the geographical distribution and hosts of chytridiomycosis in Africa, the earliest case of the amphibian chytrid yet found in any amphibian, as well as epidemiological evidence to support the hypothesis that *B. dendrobatidis* originated in Africa.

4.2 Material and Methods

4.2.1 Source of Material

Retrospective Survey

A retrospective survey of chytridiomycosis was conducted on archived *Xenopus* from various African countries. Six more species from South Africa were also included in the survey. Species were selected on the basis of their relevance to chytridiomycosis using the following criteria: wide spread species (to provide information on the geographical distribution of

chytridiomycosis), or high altitude, stream breeding species because chytridiomycosis has an affinity to these species (Alford et al. 2001, Young et al. 2001). The survey included members of the Pipidae, Ranidae and the South African endemic family, Heleophrynidae, housed in five southern African institutions; Bayworld Port Elizabeth (BW), Natal Museum Pietermaritzburg (NM), National Museum Bloemfontein (NMB), South African Museum Cape Town (SA), and Transvaal Museum Pretoria (TM), as well as the Natural History Museum, Paris (MNHP) and the private collection of Prof. Richard Tinsley (University of Bristol, UK) (Table 4-1). These specimens had been collected for archiving by a large number of people for various purposes and had not been selected for a systematic survey of amphibian disease. A data sheet was completed for each source of material stating the species, collection number and date, and locality information (Annexure E).

Live Specimens

Two field trips to the Boland region of the Western Cape were undertaken in September 2001 and February 2002 specifically aimed at determining the population prevalence of chytridiomycosis in *X. laevis*. Disease data that resulted, forms part of an in depth investigation into the spread of chytridiomycosis via the frog trade and is discussed in more detail in chapter 6 of this thesis. During May and August 2004 collections were made in the Namaqualand region to attain material for the development of a quantification technique for chytridiomycosis; the subject of discussion in chapter 7 of this thesis. In addition the candidate received frogs and toads from various scientists that conducted fieldwork in the Cape Fold Mountains, Eastern Cape, Bela-Bela region and Lesotho. The animals were collected during monitoring and parasite surveys or coincidentally, between September 2001 and December 2004. These specimens are listed in Table 4-2.

Table 4-1 Archived specimens from museum and private collections examined for chytridiomycosis.

Host species	Criteria for selection	No. of specimens	Source of material	Countries
Heleophryne natalensis	high altitude, stream breeder	68	NM	South Africa
Heleophryne regis	high altitude, stream breeder	52	BW	South Africa
Heleophryne rosei	high altitude, stream breeder	7	NM	South Africa
Heleophryne purcelli	high altitude, stream breeder	31	BW	South Africa
Afrana fuscigula	wide spread, stream breeder	5	NMB	South Africa
Amietia vertebralis	high altitude, stream breeder	85	BW	Lesotho
Pseudohymenochirus sp.	related to *Xenopus*	37	RT	Guinea
Xenopus borealis	wide spread genus, high altitude	24	MNHP, RT	Kenya
Xenopus fraseri	wide spread genus	52	MNHP, RT	Cameroon, Central African Republic, DRC
Xenopus gilli	wide spread genus	64	NM, NMB, SA, TM	South Africa
Xenopus laevis	wide spread genus	614	BW, NM, NMB, SA, TM	South Africa
Xenopus laevis petersii	wide spread genus	76	BW, MNHP, RT, SA, TM	Botswana, Zambia
Xenopus laevis victorianus	wide spread genus	98	RT	DRC, Kenya, Rwanda
Xenopus meulleri	wide spread genus	97	MNHP, NM, RT, TM	Central African Republic, Kenya, South Africa, Swaziland, Zanzibar
Xenopus meulleri (west)	wide spread genus	75	RT	Ghana, Ivory Coast, Togo
Xenopus tropicalis	wide spread genus	163	MNHP, RT	DRC, Guinea, Ghana, Ivory Coast, Liberia, Nigeria, Sierra Leone, Togo
Xenopus vestitus	wide spread genus	36	RT	Uganda
Xenopus wittei	wide spread genus	59	RT	DRC, Rwanda, Uganda
		1,643		

Abbreviations: (BW) Bayworld Port Elizabeth, (DRC) Democratic Republic of the Congo, (NM) Natal Museum Pietermaritzburg, (NMB) National Museum Bloemfontein, (SA) South African Museum Cape Town, (TM) Transvaal Museum Pretoria, (MNHP) Natural History Museum Paris, (RT) = private collection of Prof. Richard Tinsley, University of Bristol.

Table 4-2 Live frogs examined from South Africa and Lesotho for chytridiomycosis.

Host species	Locality	Collection date	No. examined
Afrana angolensis	SA, Limpopo Province, Bela-bela	04-Oct	2
Afrana dracomontana	SA, Free State, Meiringskloof	04-Dec	5
Afrana dracomontana	Lesotho, near Katsi Dam	04-Dec	6
Afrana fuscigula	SA, Eastern Cape, Stutterheim	02-Sep	1
Afrana fuscigula	SA, Northern Cape, Kammieskroon	04-May	50
Afrana fuscigula	SA, Northern Cape, Kammieskroon	04-Aug	17
Afrana fuscigula	SA, Northern Cape, Kammiesberg	04-Aug	3
Afrana fuscigula	SA, Northern Cape, Springbok	04-Aug	2
Afrana fuscigula	SA, Northern Cape, Goegap NR	04-Aug	7
Afrana fuscigula	SA, Eastern Cape, Port Elizabeth	04-Oct	3
Amietia vertebralis	Lesotho, near Katsi Dam	04-Dec	4
Cacosternum boettgeri	SA, Eastern Cape, Kenton on Sea	04-Oct	6
Strongylopus fasciatus	SA, Eastern Cape, Kenton on Sea	04-Oct	3
Tomopterna cryptotis	SA, Limpopo Province, Bela-bela	04-Oct	2
Tomopterna natalensis	SA, Limpopo Province, Bela-bela	04-Oct	1
Bufo gariepensis	Lesotho, near Katsi Dam	04-Dec	5
Bufo rangeri	SA, North West, Potchefstroom	04-Aug	5
Bufo robinsoni	SA, Northern Cape, Springbok	04-Aug	5
Kassina senegalensis	SA, Eastern Cape, Kenton on Sea	04-Oct	1
Xenopus laevis	SA, Western Cape, Stellenbosch	01-Sep	99
Xenopus laevis	SA, Western Cape, Klapmuts	01-Sep	50
Xenopus laevis	SA, Western Cape, Strand	02-Feb	43
Xenopus laevis	SA, Western Cape, Wellington	02-Feb	48
Xenopus laevis	SA, Western Cape, Botrivier	02-Feb	49
Xenopus laevis	SA, Western Cape, Paarl	02-Feb	48
Xenopus laevis	SA, Northern Cape, Kammieskroon	04-Aug	29
			494

4.2.2 Chytridiomycosis Diagnosis

A piece of the interdigital webbing and/or one phalanx were removed from one hind foot of each specimen. Tissue was prepared for histological examination using routine techniques (Culling 1963). Sections were cut at 6 µm and stained with haematoxylin and eosin (H&E). Chytridiomycosis was diagnosed using criteria described by Berger et al. (1999b). Sections from two specimens before 1971 (one collected in 1938, the other in 1943) and diagnosed as

positive for chytridiomycosis with H&E were confirmed with the more specific immunoperoxidase test to increase the confidence of the diagnosis. Histological slides prepared from archived specimens were examined "blind" without reference to dates that the frogs were collected to decrease any opportunity for bias in diagnosis. In addition to histological slides, wet mounted skin smears were prepared from some of the live specimens, for quick diagnosis of large samples, using methods described by Longcore (2001).

4.2.3 Statistical Analysis

Exact versions of Chi-square tests were used to analyse bivariate associations between chytridiomycosis prevalence and the host *Xenopus* in South Africa (*X. laevis*, *X. gilli*, *X. meulleri*), region (South-West, Eastern and Central South Africa), and season (wet and dry). Bivariate time trends of prevalence were analysed by exact Chi-square tests for trend. Multivariate logistic regression models were applied to assess the potential confounding effect of species, region and season on the time trend of chytridiomycosis prevalence. Confidence intervals (CI) were calculated using exact binomial probabilities. Longitudinal and latitudinal historical patterns of spread in South Africa were analysed with linear regression models.

The relationship between the detection of one infected individual in a population and the prevalence of infection in that population is important in interpreting the findings and is described in the following equation from DiGiacome and Keopsell (1986):

$$n = \frac{\log(1-C)}{\log(1-P)}$$

where n = number of animals examined

C = desired probability of detecting one infected animal

P = prevalence of infection

4.3 Results

4.3.1 Categorisation of Hosts

In total 1,643 specimens fixed in 70% alcohol or 10% formalin and 494 live animals were examined for chytridiomycosis. Collectively, the specimens consisted of 27 species of frogs and toads from 10 genera, representing five families (Bufonidae, Heleophrynidae, Hyperoliidae, Ranidae, Pipidae). The distribution of dates of collection of specimens was greatly skewed to the latter half of the 20th century (Figure 4-1). Considerably more specimens were collected during the time interval 2001-2004 than during any other decade since 1864.

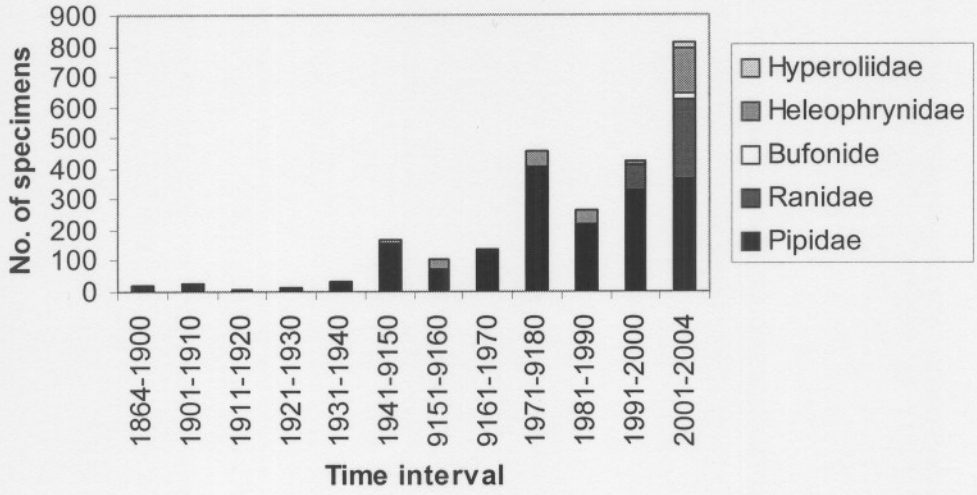


Figure 4-1 Distribution of anuran families screened for chytridiomycosis by time intervals. A total of 2,137 specimens representing five anuran families and collected between 1864 and 2004 were examined.

4.3.2 Infection Data

Chytridiomycosis Incidence

Chytridiomycosis was detected in one toad species, 11 frog species and four clawed frog species (57% of species examined), representing all five families for which specimens were collected (Table 4-3). Roughly one half of the cases resulted from examining live specimens, however, three times as many archived specimens were examined to detect the same number of cases. Although archived specimens from 19 countries were examined, chytridiomycosis was detected in only four southern African countries (Botswana, Lesotho, South Africa, Swaziland). Infected frogs were also found in both countries from which live specimens were examined (South Africa and Lesotho). The distribution of the search effort by country is reflected in the outcome of chytridiomycosis cases; 80% of the cases are from South Africa from which the majority of specimens were examined. Data from the survey indicated that the geographical distribution of chytridiomycosis in southern Africa covers the coastal lowland of the Northern Cape, Western Cape, Eastern Cape and KwaZulu-Natal, as well as Namaqualand, KwaZulu-Natal Midlands, Swaziland, montane Lesotho and the Lowveld (Figure 4-2). In every region from which live specimens were examined, new localities and host species for chytridiomycosis were added to the localities from historic records (predominantly *Xenopus*, *Heleophryne* and *Amietia*).

Table 4-3 Host species and localities of chytridiomycosis in Africa. Most of the records were detected in post-metamorphic frogs, unless otherwise indicated.

Host species	Locality	Collection date	No. infected/ no. examined
Bufonidae			
Bufo robinsoni	SA, Northern Cape, Springbok	Aug-04	5/5
Heleophrynidae			
Heleophryne purcelli	SA, Western Cape, Grootwinterhoek	Sep-98	3/3
Heleophryne regis	SA, Western Cape, Oudtshoorn	Nov-81	1/14
Hyperoliidae			
Kassina senegalensis	SA, Eastern Cape, Kenton on Sea	Oct-04	1/1
Ranidae			
Afrana dracomontana	SA, Free State, Meiringskloof	Dec-04	1/5 tadpoles
Afrana dracomontana	Lesotho, near Katsi Dam	Dec-04	5/6
Afrana fuscigula	SA, Northern Cape, Gharries	Jun-96	5/5
Afrana fuscigula	SA, Eastern Cape, Port Elizabeth	Oct-04	3/3 tadpoles
Afrana fuscigula	SA, Eastern Cape, Stutterheim	Sep-02	1/1
Afrana fuscigula	SA, Northern Cape, Kammieskroon	May-04	25/41, 7/9 tadpoles
Afrana fuscigula	SA, Northern Cape, Kammieskroon	Aug-04	13/17
Afrana fuscigula	SA, Northern Cape, Kammiesberg	Aug-04	3/3 tadpoles
Afrana fuscigula	SA, Northern Cape, Springbok	Aug-04	2/2
Afrana fuscigula	SA, Northern Cape, Goegap NR	Aug-04	6/7 tadpoles
Afrana angolensis	SA, Limpopo Province, Bela-Bela	Oct-04	1/2
Amietia vertebralis	Lesotho, Makheleng River	Sep-00	1/2 tadpoles
Amietia vertebralis	Lesotho, Matsoku River	Sep-00	1/2 tadpoles
Amietia vertebralis	Lesotho, Redi River	Sep-00	1/2
Amietia vertebralis	Lesotho, Sani tributary	Oct-00	1/4
Amietia vertebralis	Lesotho, near Katsi Dam	Dec-04	2/4
Cacosternum boettgeri	SA, Eastern Cape, Kenton on Sea	Oct-04	4/6
Strongylopus fasciatus	SA, Eastern Cape, Kenton on Sea	Oct-04	3/3
Tomopterna cryptotis	SA, Limpopo Province, Bela-Bela	Oct-04	2/2
Tomopterna natalensis	SA, Limpopo Province, Bela-Bela	Oct-04	1/1
Pipidae			
Xenopus gilli	SA, Western Cape	1943	1/13

Table 4-3 Continued

Host species	Locality	Collection Date	No. infected/ no. examined
Pipidae			
Xenopus gilli	SA, Western Cape, Zeekoeivlei	Jun-76	1/10
Xenopus laevis	SA, Western Cape, Zeekoeivlei	Jun-38	1/5
Xenopus laevis	Botswana, Kanye Youth Centre	Apr-69	1/1
Xenopus laevis	SA, Eastern Cape, Moordenaarshoek	Dec-72	1/13
Xenopus laevis	SA, Free State, Harrismith	Sep-72	1/1
Xenopus laevis	SA, KwaZulu-Natal	Dec-73	1/1
Xenopus laevis	SA, Free State, Rosendal	Feb-74	1/7
Xenopus laevis	SA, Western Cape, Touw River	Aug-74	1/3
Xenopus laevis	SA, Western Cape, Phillipi	1982	1/2
Xenopus laevis	SA, KwaZulu-Natal, Sudwana Bay	Jan-85	1/1
Xenopus laevis	SA, Free State, Florisbad	Oct-87	1/1
Xenopus laevis	SA, Free State, Koffiefontein	Sep-91	1/1
Xenopus laevis	SA, Free State, Sannaspos	Sep-91	1/6
Xenopus laevis	SA, KwaZulu-Natal, Mooi River	Dec-95	1/2
Xenopus laevis	SA, Free State, Kommissiepoort	Nov-96	1/2
Xenopus laevis	SA, Northern Cape	Sep-96	1/4
Xenopus laevis	SA, Northern Cape, Windsorton Road	Nov-98	1/1
Xenopus laevis	SA, Western Cape, Stellenbosch	Sep-01	14/48
Xenopus laevis	SA, Western Cape, Klapmuts	Sep-01	17/50
Xenopus laevis	SA, Western Cape, Strand	Feb-02	17/43
Xenopus laevis	SA, Western Cape, Wellington	Feb-02	4/48
Xenopus laevis	SA, Western Cape, Botrivier	Feb-02	14/49
Xenopus laevis	SA, Northern Cape, Kammieskroon	Aug-04	26/29
Xenopus l. petersii	Botswana	Apr-65	1/1
Xenopus meulleri	Swaziland	Feb-91	1/1
Xenopus meulleri	Swaziland	Feb-98	1/3

From the chytridiomycosis cases in South Africa in live frogs only two instances were associated with host mortality. The first outbreak involved *Afrana fuscigula* from Gharries on the southern extreme of Namaqualand. In the winter of 1996 five dead frogs were collected in a well that was being used as a drinking hole for small stock (sheep and goats). Almost 50 live frogs that displayed unusual behaviour were counted in the sedges surrounding the well. *Afrana fuscigula* are predominantly nocturnal and usually squat on the banks of a water body or are partially submerged in shallow water and leap into the water or plunge at the first sign of approaching danger. The frogs were perched on the sedges up to 1 m above the surface

during daylight hours and did not respond to human interference ([5]L. Du Preez pers. com. 2002). When the site was revisited in August 2004, the well and surrounding riverbed was dry and silted up.



Figure 4-2 Geographical distribution of *B. dendrobatidis* in five South African anuran families from archived material and live specimens; Bufonidae (1 region), Heleophrynidae (2 regions), Hyperoliidae (1 region), Pipidae (6 regions) and Ranidae (4 regions). Source of map: Microsoft Encarta 2002.

The second fatal case also involved *A. fuscigula* from Stutterheim in the Eastern Cape. A dead juvenile was collected near a stream in September 2002 (Lane et al. 2003a). No other carcasses or live frogs were encountered at this site. A third outbreak is suspected only with chytridiomycosis associated with the death of *A. fuscigula* at a drinking well in Goegap Nature Reserve in northern Namaqualand in May 2004. Three dead and heavily decomposed frogs were floating in the water and although live frogs were found, permit restraints prevented the collection of specimens. Northern Cape Nature Conservation was notified of the mortality event and the candidate returned with the necessary collecting permit in August 2004. During this survey not a single adult frog was found, but six of the seven tadpoles collected were infected with chytridiomycosis.

[5] Prof. Louis du Preez, North-West University, Potchefstroom, South Africa

Because the majority of frogs with chytridiomycosis were from specimens from incidental collections and other donated specimens that were mostly of small sample size, they can provide only basic epidemiological information. Local population prevalence data could be obtained for only two species *A. fuscigula* from Namaqualand (70.2%, one population, 2 samples) and *X. laevis* from Namaqualand (89.7%, one population, one sample) and the Boland (27.9%, 6 populations, one sample each).

Histopathology

In all of the infected specimens chytridiomycosis was identified by the presence of chytrid thalli in the *stratum corneum* and host reaction of the epidermis. Species belonging to the genus *Xenopus* were only lightly infected with few zoosporangia (1-2 layers of mostly empty sporangia), mild, focal hyperplasia of the epidermis and hyperkeratosis of the *stratum corneum* and sloughed skin containing chytrid thalli usually occurred (Figure 4-3A). In contrast, specimens of the Cape river frog, *A. fuscigula* were consistently heavily infected. A heavy infection was characterised by multiple layers (up to 10 layers thick) of sporangia in various stages of development, severe hyperplasia and hyperkeratosis and associated sloughing of the epidermis (Figure 4-3B). A range of infections between these two extremes was observed in other species, however, the majority of cases demonstrated only mild infections. The chytrid structures stained brown (positively) in the immunoperoxidase test using the specific anti-*Batrachochytrium* antibody (Figure 4-4), thus verifying infection in the old specimens.

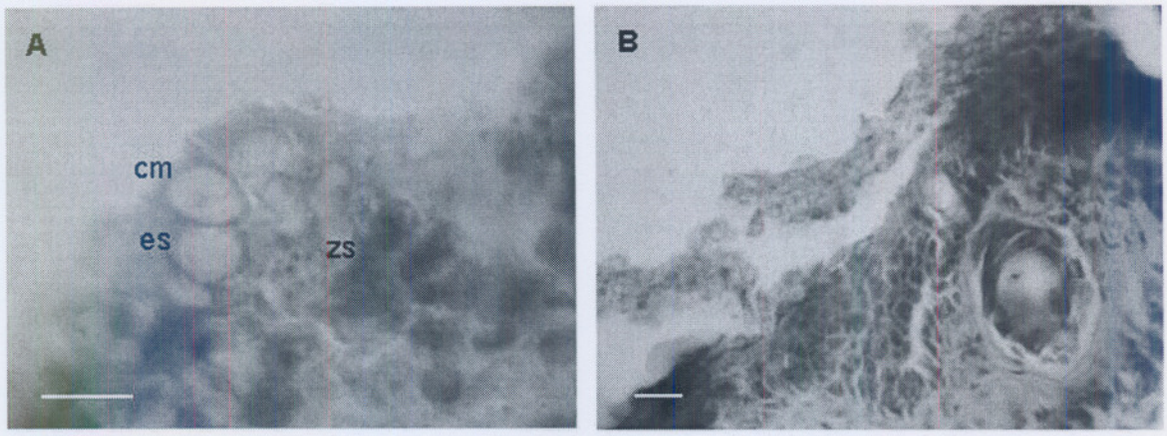


Figure 4-3 Micrographs of haematoxylin and eosin stained sections through frog skin infected with *Batrachochytrium dendrobatidis*. A) Interdigital webbing of a lightly infected *Xenopus laevis* showing the morphology and size of zoosporangia with focal hyperkeratosis (600 X, scale bar = 10 µm). B) Toe from a heavily infected *Afrana fuscigula*. Zoosporangia are visible as clear, circular structures in *stratum corneum* being sloughed (200 X, scale bar = 20 µm).

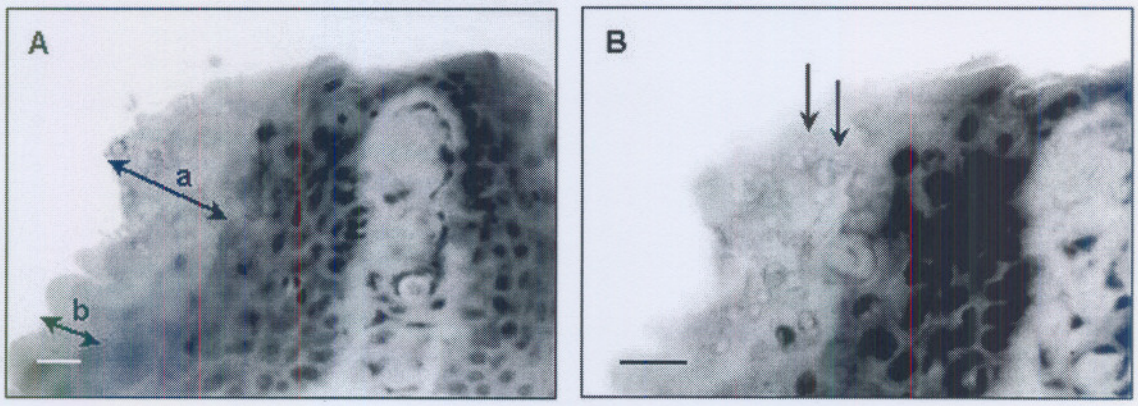


Figure 4-4 Micrographs of immunoperoxidase stained sections through the interdigital webbing of *Xenopus gilli* showing the morphology and size of zoosporangia consistent with *Batrachochytrium dendrobatidis* (scale bar = 20 µm). A) Arrow "a" indicates localised hyperplastic epidermal response compared to uninfected region of epidermis indicated by arrow "b". B) Mostly empty, circular sporangia are visible in the *stratum corneum*. Arrows indicate two zoosporangia that have developed internal septa.

Infection Correlation Data in Xenopus

Overall, chytridiomycosis prevalence in *Xenopus* from South Africa, Lesotho and Swaziland was 2.7% (19 positives out of 697 specimens of *X. gilli*, *X. laevis* and *X. meulleri*) and did not differ significantly between the species ($p = 0.7$; Table 4-4). The earliest date for a chytridiomycosis positive specimen was 1938 in an *X. laevis* collected from the Western Cape coastal lowland. This specimen is housed in the South African Museum, Cape Town (SAMZR 18927). The next earliest positive detected was *X. gilli* from 1943 (specimen number NMBA 112, National Museum, Bloemfontein). The majority of positive records, however, were detected since 1970 (Table 4-5). There is no statistically significant change of chytridiomycosis prevalence over the decades since the 1940s ($p = 0.36$), nor when the broader interval of pre-1971 is used as the baseline for the calculations ($p = 0.22$; Figure 4-5). No evidence for any trend in prevalence over time could be found using multivariate modelling where the odds ratios for the time intervals were adjusted for the potential confounders of "species", "season" and "region". The multivariate odds ratios in these models were not significant and very similar to the bivariate findings, thus indicating no confounding effects. Thus there is strong evidence that chytridiomycosis has been a stable endemic infection in southern Africa before any positive specimen was found outside this region.

Table 4-4 Prevalence of chytridiomycosis in archived *Xenopus* spp. from southern Africa (p = 0.7).

Species	Number examined	% positive (95% CI)	Earliest positive detected	Country
Xenopus gilli	61	3.3 (0.4-11.4)	1943	South Africa
Xenopus laevis	583	2.6 (1.5-4.2)	1938	South Africa
Xenopus meulleri	53	3.8 (0.5-13.0)	1991	Swaziland
	697	2.7		

Table 4-5 Prevalence of chytridiomycosis in archived *Xenopus* from southern Africa, by time intervals (p = 0.36). P = minimum prevalence needed to detect one infected animal in numbers examined.

Time interval	No. examined	Number of positives	% positive (95% CI)	P (%)
1871-1940	56	1	1.8 (0.0-9.6)	5.2
1941-1950	16	1	6.3 (0.2-30.2)	17.1
1951-1960	63	0	0.0 (0.0-5.7)	4.6
1961-1970	17	0	0.0 (0.0-19.5)	16.1
1971-1980	230	6	2.6 (1.0-5.6)	1.3
1981-1990	145	3	2.1 (0.4-5.9)	2.0
1991-2001	170	8	4.7 (2.0-9.0)	1.7
	697	19	2.7 (1.7-4.2)	

The prevalence of chytridiomycosis in South Africa showed no significant change over time after 1940. No significant change of the geographic distribution of chytridiomycosis was detected after 1973. By 1973 the distribution of chytridiomycosis - as proved by positive specimens - covered already the area between 27-34 degrees latitude and 18.25-32.5 degrees longitude. This implies that positive specimens were detected from all regions of southern Africa by 1973. Infected frogs were found in five of the nine provinces in South Africa including the Western Cape (5 of 171), Northern Cape (2 of 22), Free State (6 of 141), KwaZulu-Natal (3 of 152) and Eastern Cape (1 of 137), as well as in Swaziland (2 of 42). Prevalence of *B. dendrobatidis* did not differ (p = 0.24) between the designated three broader regions with a

prevalence of 3.0% in the South West, 3.8% in the Central region and 1.5% in the Eastern region. Overall, the seasons ("wet" versus "dry") when the specimens were collected were not significantly associated with prevalence (p = 0.22). Only in the Eastern region, was a significantly higher prevalence found in the "wet" season as opposed to "dry" season.

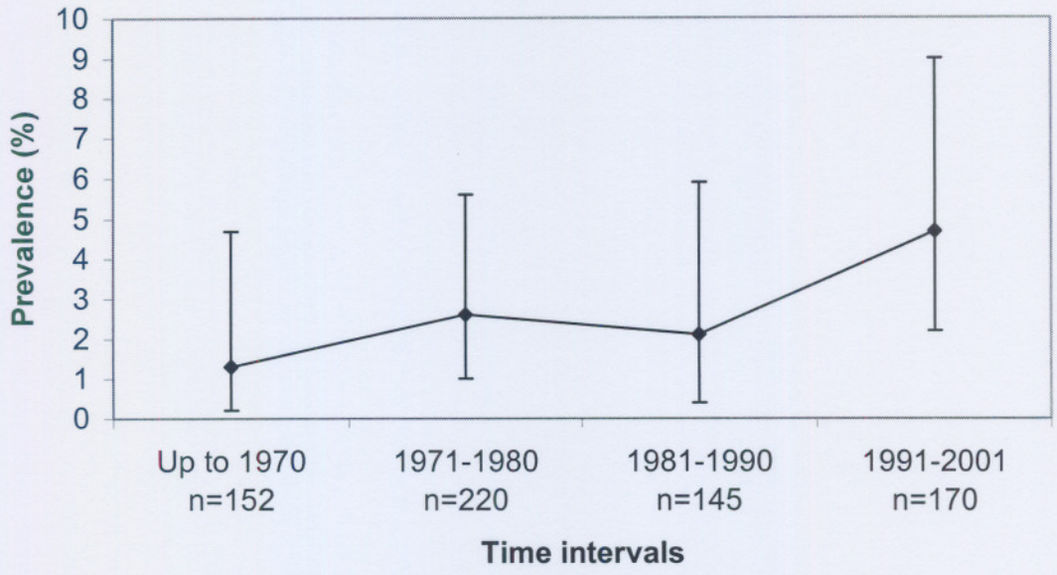


Figure 4-5 Historical time-trend of chytridiomycosis prevalence in *Xenopus* from southern Africa. There is no significant change in the prevalence over time (p = 0.22, 95% CI).

4.4 Discussion

4.4.1 Synopsis of Chytridiomycosis Data

Histopathological examination of archived specimens is a valuable method for determining whether or not *B. dendrobatidis* was introduced into a region/country and how long it has been present. Temporal and geographical interpretation of historic records may provide information on routes of entry and spread through affected areas. To formulate a clear and precise re-enactment of the events does, however, require examination of large quantities of specimens, especially when reports of mass mortality events that can guide the investigation are few or absent. In addition retrospective surveys can help to determine proximate causes of population declines where disease agents are suspected. Surveys of live specimens on the other hand may help to identify diseases as relevant potential threats to amphibian populations. The identification of shared traits among amphibian populations that promote susceptibility can help diagnose causes of declines and direct conservation actions (Lips et al. 2003). In countries where amphibians do not appear to be declining, initial chytridiomycosis surveys can be directed at species that share traits with species that are susceptible to disease in other parts

of the world. Amphibian population declines in Australia, Latin America and Spain are particularly associated with stream breeding amphibians that have restricted elevational ranges (Berger et al. 1998, Bosch et al. 2001, Young et al. 2001, Lips et al. 2003). A further advantage of live frog surveys is that it provides population prevalence data that becomes more reliable with increase in sample size.

The equation of Digiacome and Koepsell is important in interpreting the findings. The earliest record of chytridiomycosis in *Xenopus* from southern Africa was 1938 and after this date the average prevalence was 2.7%. Twenty seven specimens collected prior to 1938 were examined. Using the equation above, this number would allow the detection of at least one infected animal with 95% probability only if the prevalence was 10,5% or greater. Therefore failing to find any infected frogs prior to 1938 may mean that the infection was truly not present or that the prevalence was too low to be detected by the number of frogs examined. In the decades when chytridiomycosis was found, average prevalence was 3.2%. For both decades that chytridiomycosis was not detected, 1950s and 1960s, the minimum prevalence needed to detect one infected animal with 95% probability (4.6% and 16.1% respectively) is higher than the average prevalence (Table 4-4). Failure to find any infected individuals in these two decades is most likely related to too small sample sizes. For the same reason and as mostly only archived *Xenopus* spp. were examined from the rest of the African countries for which surveys were conducted, the known geographical distribution of chytridiomycosis in Africa is likely to be an under-representation of the true distribution. So far, chytridiomycosis has been detected in southern, western and eastern Africa, but large gaps separate these regions (Figure 4-6). The incidence of chytridiomycosis in Kenya involves *Ptychadena anchietae* and was diagnosed by Speare and Berger (2000). In Tanzania, chytridiomycosis was detected in three species (*Nectophrynoides asperginis*, *Arthroleptidis yakusini*, *P. anchietae*) during an investigation into an amphibian decline event in Kihansi Gorge, south-western Tanzania (Weldon and Du Preez 2004b, Chapter 9 of this thesis). Chytridiomycosis was also detected in *Xenopus tropicalis* on two occasions; once from Ghana (Morehouse et al. 2003) and once from an unknown locality in western Africa (Reed et al. 2000).). However, both these records are from frogs imported into the USA and the record extrapolated to the country of origin. Because no details were given on measures taken within the importing institution to prevent cross-infection after arrival in USA, the reports should be regarded as suspect only for Africa and requiring confirmation by survey in Africa.

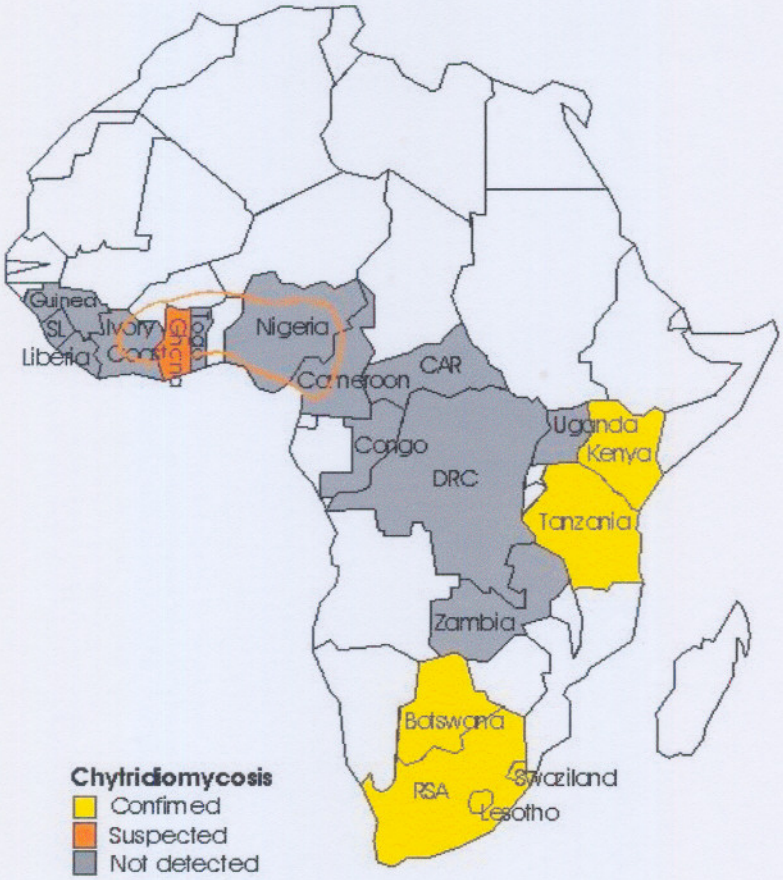


Figure 4-6 Map of Africa, showing the known (yellow) and suspected (orange) distribution of chytridiomycosis. The outlined area in western Africa represents a suspected incidence in *Xenopus tropicalis* for which the country of origin is not known. Countries from which pipid frogs (*Xenopus* spp., *Pseudohymenochirus* sp.) were examined during this survey, but no chytridiomycosis detected, are indicated in grey.

4.4.2 Threatened Species

Only two confirmed and one suspected host mortality events in South Africa were detected during this study that are associated with chytridiomycosis; all three cases involving *A. fuscigula*. This does not imply that this species is always susceptible to disease, because an infected population near Kammieskroon appeared healthy and displayed breeding activity on two consecutive visits during the same breeding season. Moreover, the small population at Goegap Nature Reserve has carried the infection at least since 2002 (Hopkins and Channing 2003) and persists in spite of sporadic deaths. Recurrence of disease related die-offs at the same sites over consecutive years may be an indication that carrier animals are involved or that the pathogen persists outside of the host in the environment (Green et al. 2002). Based on the outcome of the survey and the lack of evidence for a general decline of any species in southern Africa it appears as though chytridiomycosis does not directly threaten any species

from this area. Furthermore, the detection of chytridiomycosis in predominantly healthy animals fits the description of an endemic disease. Because the synergistic effect of various co-factors (climate change, habitat alteration, pollution etc.) can cause chytridiomycosis to be a fatal infection (Daszak et al. 1999, Bosch et al. 2001, Daszak et al. 2003, Burrowes et al. 2004), the disease should be regarded as a potential threat. Those species that have already been assigned a threatened conservation status, especially due to extent of occurrence or area of occupancy, are particularly threatened by becoming extirpated from certain areas in the event of an epidemic. Not only disease, but also other catastrophic events such as global climate change and introduced predators are more likely to cause declines in isolated, endemic species as apposed to common species with numerous subpopulations (Margules et al. 1994, Davies et al. 2000, Lips et al. 2003).

The only infected species from South Africa detected by this survey with a threatened status is the Cape platanna, *X. gilli*. Endangered status was assigned to this, the second most threatened lowland frog in South Africa, based on a severely fragmented habitat, continuing decline in the extent of occurrence and quality of habitat (Harrison et al. 2001, De Villiers 2004). Urban development on the Cape Flats resulted in the disappearance of *X. gilli* from this region whereas sand mining activities, alien vegetation encroachment and general habitat degradation along the south-west coast of the Western Cape are responsible for population declines. (De Villiers 1997, Picker and De Villiers 1989). The remaining habitat of *X. gilli* is restricted to a few acidic black-water bodies (minimum pH 3.4) in the coastal lowlands and seepages in the Fynbos Biome (Picker 1985, Baard et al. 1999). The remaining number of individuals of this species would therefore be greatly reduced should one of the remaining populations be extirpated due to chytridiomycosis. Those populations that occur in water bodies with a pH close to the minimum tolerable range may benefit from a reduced likelihood of infection because chytridiomycosis does not tolerate pH levels below 4 (Piotrowski et al. 2004, Johnson and Speare 2005).

4.4.3 Evidence for the Origin of Chytridiomycosis

This study has extended the date for the earliest case of chytridiomycosis in wild amphibians by 23 years. The next earliest case outside South Africa is for *Rana clamitans* from Saint-Pierre-de-Wakefield, Québec Canada in 1961 (Ouellet et al. 2003). After the case in Canada, the earliest cases from other countries follow sequentially over a period of 38 years from 1961 to 1999 (Figure 4-7).

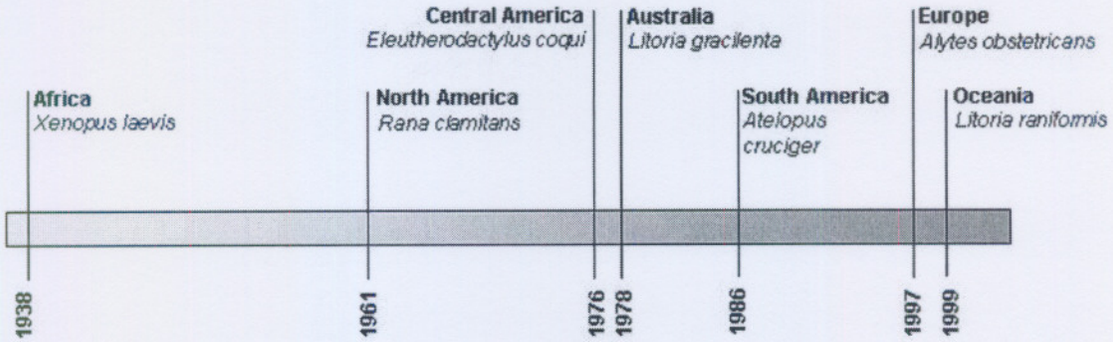


Figure 4-7 Time bar indicating when chytridiomycosis first appeared in the major centres of occurrence in relation to each other. Following a 23-year interruption in occurrences after the *Xenopus laevis* infection in 1938, records outside Africa appear with increasing frequency up until the present; North America (Ouellet et al. 2003), Australia (Berger et al. 1999a, Speare et al. 2001), South America (Bonaccorso et al. 2003), Central America (Burrowes et al. 2004), Europe (Bosch et al. 2001), Oceania (New Zealand) (Waldman et al. 2001).

Xenopus laevis in the wild does not show clinical signs nor has it experienced any sudden die-offs. Moreover, only sub-clinical chytrid infections have been observed among in-house colonies of *X. laevis* (Reed et al. 2000, Parker et al. 2002). The related species, *X. tropicalis*, died in captivity from chytridiomycosis, and it was suspected to have contracted the agent from *X. laevis* (Parker et al. 2002). An ideal host for transmission of chytridiomycosis through international translocation would be a species of amphibian that does not become diseased or die from the infection, and hence *X. laevis* could take on the role of a natural carrier.

The sudden appearance of chytridiomycosis can best be explained by the hypothesis that *B. dendrobatidis* was recently introduced into new regions and subsequently infected novel host species (Daszak et al. 1999). Dispersal of *B. dendrobatidis* between countries is most likely through the global transportation of amphibians (Berger et al. 1999a, Daszak et al. 1999, Mutschmann et al. 2000, Speare et al. 2001, Mazzoni et al. 2003). The World Organization for Animal Health has recently placed amphibian chytridiomycosis on the Wildlife Diseases List in recognition of this risk. If Africa is the source of *B. dendrobatidis*, a feasible route of dissemination via infected amphibians needs to be identified. Some members of the family Pipidae have been exported, in particular *Hymenochirus curtipes* and *X. laevis* to North America and Europe (Hey 1986).

In terms of a most likely candidate for spread from Africa, the weight of numbers of frogs and geographic dissemination favours *X. laevis*. Large-scale exploitation of *X. laevis* started soon

after the discovery of the pregnancy assay for humans in 1934 (Hey 1986) when enormous quantities of the species were caught in the wild in southern Africa and exported around the world. The pregnancy assay is based on the principle that ovulation in *X. laevis* is induced by injection with urine from pregnant women because of high levels of gonadotrophic hormones in the urine (Hogben et al. 1931, Shapiro and Zwarenstein 1934). Discovery of the pregnancy assay followed after *X. laevis* was selected as the most suitable amphibian for investigating the mechanism of the mating reflex because of the relative ease with which the animal can be maintained in captivity (Shapiro 1936a). For 34 years the trade in *X. laevis* in South Africa was controlled by the then Cape of Good Hope Inland Fisheries Department (Western Cape Nature Conservation Board) at the Jonkershoek Fish Hatchery. As an indication of the numbers involved in this trade, 10,866 frogs were distributed in 1949 of which 3,803 (35%) were exported and of the 20,942 frogs distributed in 1970, 4,950 (24%) were shipped abroad (PACGH 1949, PACGH 1970). After the introduction of non-biological pregnancy tests, *X. laevis* became important as a model for the scientific study of immunity and later embryology and molecular biology. It is likely that *X. laevis* could have carried the disease globally, particularly if the prevalence was similar to that seen in wild-caught *X. laevis* today. In the importing country escaped frogs and/or the water they lived in (Johnson and Speare 2003) could have come into contact with local amphibian species, followed by subsequent transmission of the disease. The establishment of feral populations of *X. laevis* in Ascension Island, UK, USA and Chile in 1944, 1962, 1960s and 1985 respectively shows that the opportunity for transmission could have become ongoing if these feral populations were infected (see Tinsley and McCoid 1996).

Although we have demonstrated that *B. dendrobatidis* was present in southern Africa since 1938, this study provides no indication whether this region was the original source within Africa. This study and other sources have found that *B. dendrobatidis* is present in wild frogs in Tanzania and Kenya, and is possibly present in wild-caught frogs in western Africa (Reed et al. 2000, Speare and Berger 2000, Parker et al. 2002), indicating that *B. dendrobatidis* is widely disseminated in Africa. *Xenopus* consists of 17 species that are found in sub-Saharan Africa with a varying degree of sympatry between species (Tinsley et al. 1996). The overlap in the distribution and in some cases the sharing of habitats could facilitate transmission of *B. dendrobatidis* between these species. This would imply that chytridiomycosis could have originated elsewhere in Africa and spread within multiple host-region combinations. More detailed historical studies of archived African amphibians other than *Xenopus*, as well as, selected surveys of wild populations may indicate whether *B. dendrobatidis* was originally present in only a small area of Africa from which it emerged to occupy large areas of the continent. Until the deficit in distribution data and comparative genetic studies is remedied,

locating the source of origin within Africa of *B. dendrobatidis* remains speculative. It does, however, appear as though the relationship to co-evolve within an anuran host, as well as the opportunity to disseminate across the globe, existed for *B. dendrobatidis* in southern Africa.

If *X. laevis* did carry *B. dendrobatidis* out of Africa as proposed, other amphibian species subsequently could have distributed it between and within countries. The American bullfrog, *Rana catesbeiana*, has been proposed as a significant vector, mainly through international trade as a food item, but also within countries because populations established for the food trade escape and spread (Mazzoni et al. 2003). The earliest current record for the occurrence of chytridiomycosis in *R. catesbeiana* is 1978 in South Carolina (Carey et al. 2003), 40 years after the first record in southern Africa, but details on the intensity of searching for chytridiomycosis in archived bullfrogs are not available. The most recent discovery of chytridiomycosis in introduced *R. catesbeiana* is in the UK, detected in late 2004 (Halliday 2004, Pasmans et al. 2005). Another informative case is the occurrence of chytridiomycosis in the dwarf African clawed frog (*Hymenochirus curtipes*) in the pet trade in USA (Groff et al. 1991). Although the pathogen was originally reported as *Basidiobolus ranarum*, a zygomycete fungus, retrospective analysis of specimens has confirmed that it was *B. dendrobatidis* (Carey et al. 2003). Because Groff et al. (1991) described an epidemic situation in the breeding stock used to supply frogs for the pet trade, the likelihood is high that frogs carrying *B. dendrobatidis* were disseminated commercially over USA in the late 1980s. The transmission of chytridiomycosis globally may involve a series of key steps: occurrence of *B. dendrobatidis* in an amphibian vector in southern Africa that is relatively resistant to disease (*X. laevis*), sudden rise in 1935 of export trade in this vector owing to technological advances (*Xenopus* pregnancy test), escape of the pathogen from the exported *Xenopus* to establish new foci in other countries (possibly expedited in some countries by establishment of feral populations of *X. laevis*), transmission into other vector amphibians (food and pet trade), further transmission to other countries along different trade routes in key amphibian vectors that move in high numbers and establish commercial populations with close interaction with wild frogs and high possibility of feral populations (food frogs *R. catesbeiana*). Spread through native amphibian populations with epidemic disease in some species could have occurred at any point after *B. dendrobatidis* entered a naive native species.

4.5 Conclusion

This study has improved our knowledge concerning the geographical distribution of chytridiomycosis in Africa and the species infected. It appears from the general lack of chytridiomycosis associated population declines, that most species are relatively resistant to

the disease. This does not, however, exempt any infected populations from sporadic deaths or declines due to the synergistic effect of multiple co-factors as demonstrated by *A. fuscigula* from Namaqualand and *N. asperginis* from Tanzania (Chapter 9 of this thesis).

Epidemiological evidence for Africa as the origin of the amphibian chytrid fungus has been provided. Support for all six of the criteria proposed for the source of *B. dendrobatidis* has been demonstrated; 1) the major host (*X. laevis*) shows minimal or no apparent clinical effects, 2) the earliest global occurrence (1938), 3) this date precedes any amphibian declines in pristine areas, 4) the prevalence in the source host or hosts (*Xenopus* spp.) has been stable over time, 5) no geographical spreading pattern could be observed over time, and 6) a feasible means of global dissemination exists via the international trade in wild-caught *X. laevis*, which commenced in 1935 and continues today. The next avenue to pursue is the investigation of African strains of *B. dendrobatidis* at molecular level, because one would expect to find a greater genetic variation in the original host region than in more recently invaded regions. A low level of genetic variation was shown for 35 strains of *B.* dendrobatidis, suggesting that the pathogen is a recently emerged clone (Morehouse et al. 2003). The strains had been collected in North America, Australia, Panama and Africa from wild and captive amphibians. Three strains isolated from captive *X. tropicalis* in USA had been imported from Ghana, however, these showed no significant differences from the strains isolated from USA (Morehouse et al. 2003). The assignment of these strains to Africa assumes no cross-infection had occurred within the importing facility. Future work on the genetic diversity of *B. dendrobatidis* in Africa compared with strains from regions outside Africa will add weight to the hypothesis if a greater genetic diversity is found among African strains.



Chapter 5

TRADE IN THE AFRICAN CLAWED TOAD, *Xenopus laevis*, WITH IMPLICATIONS FOR CONSERVATION

> Ask anybody what uses a frog can be put to and you'll get replies ranging from a meal for a Frenchman to pets for small boys. Ask a hospital laboratory worker, though and the first thing she'll think of is the frog test for pregnancy.
>
> Marian Kuhl (1970)

5.1 Introduction

Despite a diversity of 115 recognised species in South Africa, the frog trade has been largely centred on a single species, the African clawed toad, *Xenopus laevis*. The commercial *Xenopus* trade in South Africa is an established business that has been operational for more than six decades. In South Africa, the species is very common and apart from northern KwaZulu-Natal and the far eastern parts of the Mpumalanga and Limpopo Provinces its distribution covers nearly the entire country. The species tolerates a range of environmental conditions, which enables it to inhabit all types of freshwater bodies within the region. When favourable conditions persist, sexual maturity is reached in eight months from the egg stage (Tinsley et al. 1996). The breeding season usually starts with the onset of rain in spring or winter, depending on the region, and can last up to six months (Balinsky 1969). Natural breeding may occur up to three times per season for some individuals (Hey 1949).

During the 1990s *Xenopus* surpassed *Rana* as a laboratory animal, thereby becoming the most widely-used amphibian in research (Major and Wassersug 1998). The history of the international trade in *X. laevis* is associated with its suitability for use as a laboratory animal. The relative ease with which this aquatic frog can be maintained in captivity lead to its selection for investigating the mechanism of the mating reflex. Progress was made on the understanding of reproduction in *X. laevis* during the 1930s by a series of observations and experiments on seasonal changes in ovaries and anterior pituitary function (Shapiro 1936a, b, Berk 1938). In 1934 it was discovered that *X. laevis* could be used to accurately detect early pregnancy in humans (Shapiro and Zwarenstein 1934). The new demand for *X. laevis* that followed soon after this discovery saw the start of an ongoing large-scale exploitation of the animal.

At first, animal dealers exploited this demand by catching large numbers of *X. laevis* without official control. Consequently, local populations in the vicinity of Cape Town (on the Cape Peninsula and Cape Flats) were rapidly depleted. The Cape Provincial Administration (CPA) intervened by introducing protective legislation regarding the collection of wild *X. laevis*. At the same time the CPA undertook research on the artificial cultivation of *X. laevis* at the Jonkershoek Fish Hatchery (JFH) near Stellenbosch, South Africa (Hey 1945). In August 1941, propagation of *X. laevis* for medical and scientific purposes was initiated. Most of the supplies of *Xenopus* were collected from farm dams mainly in the Stellenbosch vicinity and as far away as Paarl, Caledon, Malmesbury and Piketberg. In the same year local deliveries were made and the first shipment was sent to the USA. The collection, cultivation and selling of *X. laevis* from Jonkershoek was stopped in 1974, when operations were focused on the propagation of indigenous fish species like the scarce Clanwilliam yellowfish, *Barbus capensis*. The trade in *X. laevis* would henceforth be left to private businesses.

The biggest demand for these frogs today is from the various research facilities in genetics, molecular biology, embryology, biochemistry and ecotoxicology. The most significant purpose for *Xenopus* in molecular biology is for oocyte production. These are used as heterologous expression systems and for studying the molecular aspects of development, especially pattern formation in embryos (Weldon 1999). According to Hamilton (1976), the study of *Xenopus* development stimulates many inquiries and investigations, and can be taken as a model system for development because many of the problems faced throughout the vertebrates are met by solutions found in *Xenopus*. The exposure that this species gained through the scientific trade provided an opportunity for its introduction to the pet trade. *Xenopus laevis* quickly gained popularity as an aquatic pet around the world not only because it can be maintained in captivity with relative ease, but also because its unique features (aquatic existence, clawed feet, no tongue or eyelids) spur the curiosity of collectors. In the 1980s it became very popular among children in the USA to order a *Xenopus* tadpole, which was supplied with a small fish bowl, some food and husbandry instructions. In some parts of South Africa, *X. laevis* has for decades been sold in angling shops as bait for freshwater angling. Recent legislation makes it illegal to use any live bait in South Africa. Although *X. laevis* is not cultivated for the food market, an insignificant number is removed in South Africa from the wild for human consumption. Hey (1986) reports on *X. laevis* being eaten in the Western Cape and it is mentioned along with the giant bullfrog, *Pyxicephalis adspersus* and a large ranid, in a list of traditional foods eaten by the San people in the southern Kalahari (Steyn 1984). Although

some African tribes are superstitious and would not touch a *Xenopus*, others like tribes in Uganda regard it as a delicacy ([6]R. Tinsley pers. com. 2002).

Despite the long existence of the trade in *X. laevis* and the extent of the trade in terms of numbers exported and global distribution, no attempt has been made to monitor trends or to quantify the frogs being exported. Data pertaining to the trade were formerly summarised in annual reports of the CPA and more recently assimilated in electronic format by the nature conservation authority – Western Cape Nature Conservation Board (WCNCB).

Given the deficit in trade information in the literature, a survey was conducted involving suppliers and the export regulatory body (WCNCB) to determine the local and international demand for *X. laevis*.

5.2 Material and Methods

This study is largely based on the evaluation of literary and historical data pertaining to *X. laevis* exports from the Western Cape. Comparisons were made to testimonial data from suppliers on existing conditions within the trade. Information on the activities at the JFH regarding the cultivation and dispatching of *X. laevis* was retrieved from archived reports of the Inland Fisheries Department of the CPA (1941-1951, 6 reports), which became the Department of Nature Conservation in 1952 (1952-1976, 21 reports). Data on the numbers and destinations of *X. laevis* for the period 1998-2004 were extracted from the permit files of the WCNCB head office in Cape Town. Interviews and correspondence with four suppliers verified the data from the WCNCB and provided additional data for the period 1994-1997. Detailed information on collection sites, captive breeding and supply to local (South African) institutions were also obtained from the interviews and correspondence.

An inquiry was also conducted on the use of *X. laevis* in freshwater angling in South Africa. The owners of seven angling shops in the Free State Province were interviewed face to face on their involvement in the selling of *X. laevis*. A questionnaire was drawn and the same questions were presented to each shop owner (Annexure F).

[6] Prof. Richard Tinsley, University of Bristol, United Kingdom

5.3 Results and Discussion

5.3.1 Suppliers

During the past decade, the WCNCB has issued annual permits for the collection and export of *X. laevis* to three major suppliers. The registered companies of the three suppliers are African Reptile Park (Cape Town), Xenopus Express (Somerset-West) and African Xenopus Facility (Knysna). Each supplier is allocated one or more magisterial districts within which they are allowed to collect. The suppliers do not compete for the collection of *X. laevis* because the allocated districts do not overlap and each supplier is limited to 10,000 frogs per annum. The localities from which *X. laevis* may be collected are restricted to man-made water bodies (such as dams and sewage treatment ponds) and these may be visited only on an annual rotational basis. The number of localities differs between suppliers and from year to year. During 1998-2001 Xenopus Express made use of between 23 and 43 localities, whereas African Xenopus Facility and African Reptile Park utilised seven and 14 localities respectively during 1998. Frogs are usually collected on request and housed at the collector's facility until the date of export. All quotas are made up of wild-caught frogs only, because none of the facilities breeds *X. laevis*. The number of frogs that are collected during the year is determined by the demand of the import facilities. In 2003 Xenopus Express translocated their facility from Somerset-West to Vernassal, France. A permit was consequently issued to C.E. Loesch to collect in the magisterial districts vacated by Xenopus Express. The most frequently-used method to catch *X. laevis* is by means of baited aquatic traps that are set for overnight up to a week. Variations of the aquatic trap exist, but they all share the same basic components, namely, a baited bucket or basket fitted with a funnel entrance and a design that allows for breathing space.

5.3.2 Trade Numbers

Jonkershoek Fish Hatchery was the only official supplier of *X. laevis* in the Western Cape for 34 years until 1974. Records of frog sales are available for 23 of the years. During this period a total of 343,588 frogs were sold, the majority of which went to local institutions (Table 5-1). It was calculated from 11 year's data, that less than one fifth (19.6%) of the frogs were exported between 1949 and 1974. Mention of sex ratio is only made for six of the years and indicates that more females were consistently supplied. The preference for females may reflect the uses to which frogs are put. When the *Xenopus* pregnancy assay was in use, a disproportionate number of females were exported, because the assay did not involve the use of males. Males are just as important as females for the production of positive oocytes by induced spawning in especially embryology and molecular research. Females are reused for this purpose every two to six months (Weldon 1999); time needed for the maturation of oocytes is related to food supply, overcrowding and the availability of water (Alexander and Bellerby 1938, Bellerby and Hogben 1938). For this reason many facilities have their own in-house colonies that are

stocked from time to time when older females that produce smaller clutch sizes are replaced with young sexually mature individuals (Weldon 1999). Males are often sacrificed and the testes homogenised, which would suggest a high turnover of males.

Table 5-1 Particulars of *Xenopus laevis* sales from the Jonkershoek Fish Hatchery for the period 1941 to 1974. Data are only available for 23 years with details of exports available for only 11 of the years.

Year	Numbers dispatched		Total	Sex ratio
	South Africa	Exported (%)		Male:Female
1941			350	
1942			2,700	
1943			4,300	
1944			3,547	
1948			12,000	
1949	7,063	3,803 (35.0)	10,866	
1954	11,406	3,384 (22.9)	14,970	
1955			17,000	
1958			17,300	
1959			23,787	1:1.6
1960			17,565	1:1.4
1961			22,699	
1962	19,714	2,076 (9.5)	21,790	
1963			19,325	
1964	14,033	1,260 (8.2)	15,293	0.7:1
1965			14,766	0.9:1
1966	11,400	4,191 (26.9)	15,591	
1968	13,578	6,389 (32.0)	19,967	*0.6:1
1970	17,356	6,352 (17.4)	21,000	*1:1.6
1971	15,992	4,950 (23.6)	20,942	
1972	12,705	3,505 (21.6)	16,210	
1973	12,378	1,560 (11.2)	13,938	
1974	16,482	1,200 (6.8)	17,682	
			343,588	

* Sex ratios are representative of exported frogs only.

We were not able to retrieve any data on *X. laevis* sales for the decades after the JFH withdrew from the trade until the current suppliers started operating in the 1990s. Since 1998 more than 68,051 frogs have been exported from the Western Cape. This corresponds to an average of nearly 10,000 frogs per year. Data obtained directly from the suppliers for the period 1994-1997 were incomplete and therefore not suitable for inclusion in the results. Sales

of *X. laevis* gradually decreased since 1998 from 18,871 to 6,560 during 2002 (Figure 5-1). In 2003, however, numbers picked up again when 14,269 frogs were exported. During this year a large number of frogs, accounting for 70.1% of the annual export, were exported by Xenopus Express. These frogs were translocated to a sister facility of Xenopus Express in France to replenish its stock population. It can be deduced that the demand for *X. laevis* has declined in recent times if 1) we consider that for most of the years fewer frogs were exported than the previous year, 2) we acknowledge that an exceptional transaction took place between one supplier and one import facility in 2003 that resulted in an increase in annual sales and 3) we assume that the upward trend in 2003 will not repeat itself in 2004 because Xenopus Express is no longer in operation in South Africa. By July 2004, with only two major suppliers left, exports were below average at 1,566 for the first semester.

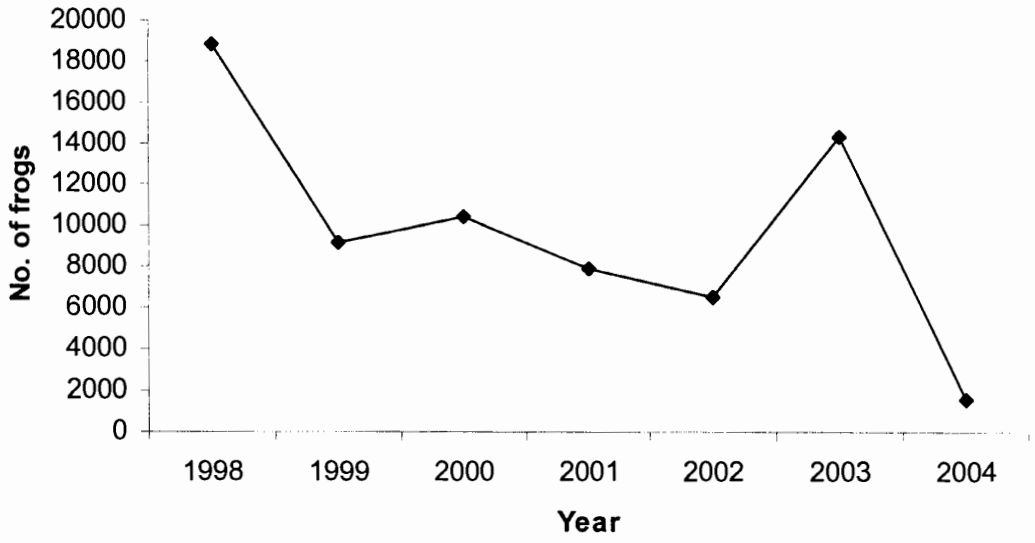


Figure 5-1 Trend in the demand for *Xenopus laevis* by foreign facilities for the period 1998 – 2004.

5.3.3 Import Facilities

Over the past seven years *X. laevis* from the Western Cape have been exported to 124 facilities situated in 31 countries from around the world (Figure 5-2). The majority of destinations are research facilities associated with academic and government institutions, whereas others include companies that breed and supply *X. laevis* directly to the end-user and the pet trade (Table 5-2).

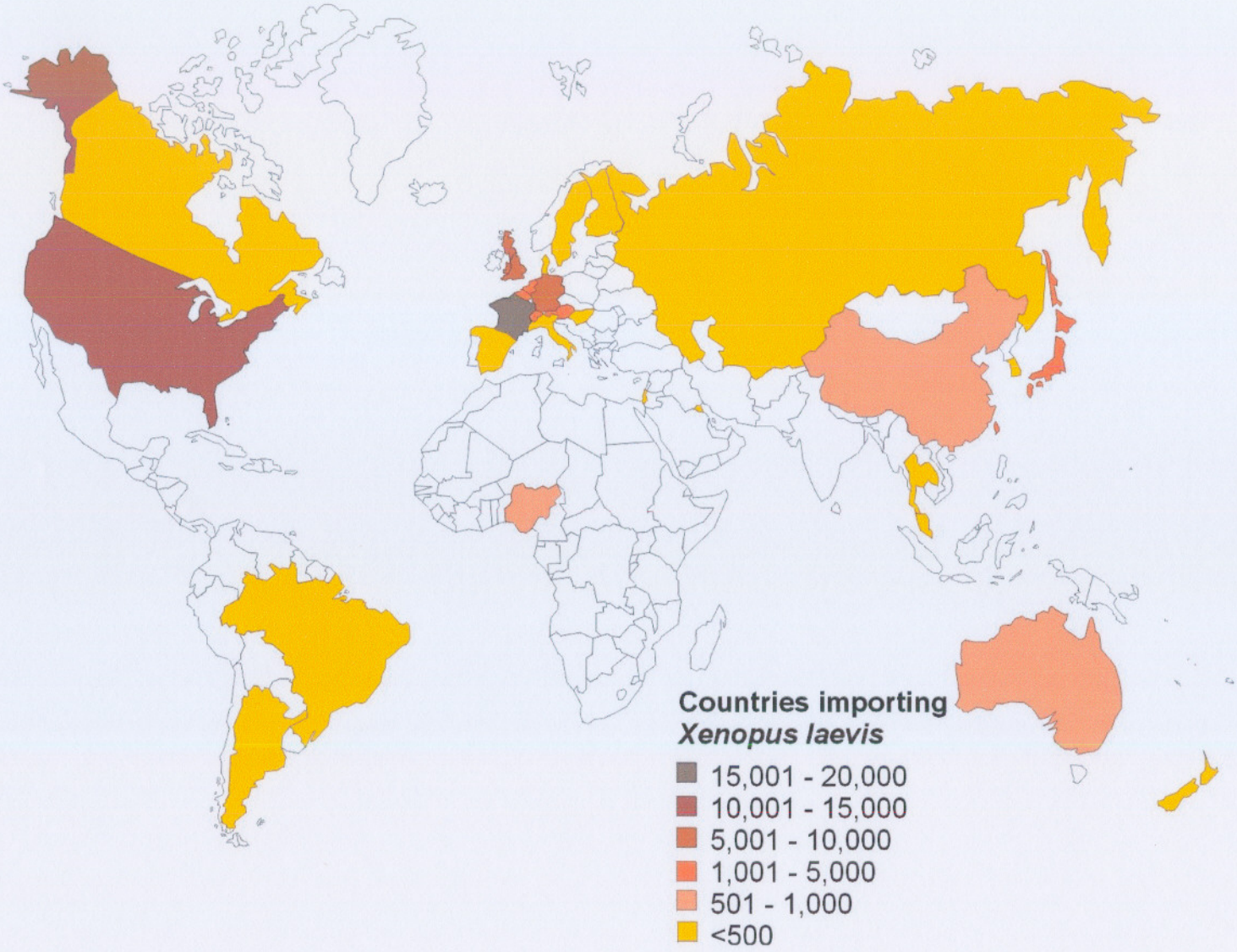


Figure 5-2 Map of the world showing which countries imported *Xenopus laevis* between 1998 and 2004. The numbers of frogs that were imported are colour coded.

Table 5-2 List of import facilities of *Xenopus laevis* sorted by countries and arranged alphabetically during 1998-2004.

Country	Recipient institution	No. of frogs
Argentina	National Research Council of Argentina	50
Australia	Australian National University	305
	Flinders University, Dept. Medical Biochemistry	25
	The University of Melbourne, Dept. of Physiology	50
	The University of Queensland, Dept. Physiology and Pharmacology	105
	The University of Queensland, School of Biomedical Sciences	40
	University of Sydney, Pharmacology	95
Austria	Institute of Molecular Biology, Austrian Academy of Sciences	600
	Research Institute/Molecular Pathology, Vienna	340
	University of Vienna, Dept. Cell Biology & Genetics	85
	University of Vienna, Dept. Cytology & Genetics	70
Belgium	Katholieke Universiteit te Leuven	370
	University of Antwerp, Dept. Biology	20
	University of Gent, Dept. Molecular Biomedical Research	110
	University of Gent, Dept. Molecular Biology	670
	University of Gent, Unit of Developmental Biology	80
	Vrije Universiteit Brussel, Laboratorium voor Cellulaire Genetica	50
	Janssen Pharmaceutica, Beerse	30
	Universitaires Notre-dame De La Paix	55
Brazil	FUNDEP, Av Antonio Carlos	50
Canada	CHUM (Centre Hospitalier De L'Universite De Montreal	12
China	Shangai Institute of Cell Biology, Chinese Acadamy of Sciences	600
Denmark	DENMARK Aquarium	50
	Copenhagen, Dept. Neurobiology	170
	NeuroSearch, Ballerup	20
	Royal Danish School of Pharmacy, Dept. Pharmacology	136
England	(MRC) of the National Institute for Medical Research, London	150
	Merck Sharp & Dohme, Neuroscience Research Centre	20
	Molecular Pharmacology Unit, Hertfordshire	20
	University of Newcastle upon Tyne	240
	University of Oxford	40
	University of Warwick, Dept. Biological Science	1,300

Table 5-2 Continued

Country	Recipient institution	No. of frogs
England	Leeds University, Central Biomedical Services	12
	University of Bath	2,100
	University of Bristol, School of Biological Sciences	110
Finland	University of Joensuu, Dept. Biology	25
France	Centre d'Elevage de Xenopus – CNRS	9,460
	Xenopus Express, Vernassa	10,000
Germany	Albert-Ludwigs-Universitat-Freiburg	20
	Biocenter of the University of Wurzburg	255
	Centre für Forshung und Lehre, Hamburg.	400
	Deutsches Krebsforschungszentrum (DKFZ)	905
	University of Hohenheim, Institute for Zoology	190
	Eberhard-Karls-University of Tuebingen	230
	European Molecular Biology Laboratory (EMBL)	3,670
	Forschungszentrum Karlsruhe	50
	Freie Universitat Berlin, Institut fur Pharmakologie	40
	Institute für Gewässerörkologie und Binnenfischerei (IGB), Berlin	400
	Johann Wolfgang Goethe-Universitat	140
	Johannes Gutenberg University, Dept. Pharmacology	85
	Martin-Luther University, Julius-Bermstein-Institute	10
	Justus-Liebig-Universität Giessen	320
	Ludwig-Maximilians-Universitat Munchen, Adolf-Butenandt-Institute	350
	Max-Planck-Institut fur Biophysik, Frankfurt	165
	Max-Planck-Institut für Entwicklungsbiologie, Tübingen	695
	Max-Planck-Institute Für Experimentelle Medizin, Göttingen	110
	Dr R Stick, Bremen	70
	Technical University of Munich	16
	Universität GH Essen, (Biologie)	255
	Universitat Rostock, Institut fur Zellphysiologie und Biosystemtechnnik	77
	Universitat Wuerzburg, Dept.Anatomy	100
	Universitatsklinikum Aachen, Dept.Molecular Pharmacology	100
	University of Karisruhe, Dept. Zoology II	225
	University of Mainz	70
	University of Tuebingen, Dept. Molecular Biology	110
	University of Ulm	190

Table 5-2 Continued

Country	Recipient institution	No. of frogs
Germany	University of Wurzburg, Institute of Anatomy & Cell Biology	40
	Westfalische Wilhelms-Universitat, Institut Fur Zoophysiologie	50
Hong Kong	The Chinese University of Hong Kong, Dept. Biochemistry	20
	The Chinese University of Hong Kong, Faculty of Medicine	10
	The Hong Kong University of Science and Technology	175
	The University of Hong Kong, Institute of Molecular Biology	515
Hungary	Budapest Zoological and Botanical Department	50
	Semmelweis University of Medicine, Dept. Physiology	100
Israel	Faculty of Medicine, Dept. Biochemistry	290
	Tel-Aviv University, Ramat-Aviv Campus	100
	DBSF Universita dell"Insubria (Varese)	80
	The University of Varese, Dept. Biology	200
	Universita degli Studi di Padova, Facolta di Medicina e Chirurgia	70
Japan	Hamamatsu Biological Research Service Inc.	1,800
	Kato-S-Science, Sumida-ku, Tokyo	1,000
	The Institute of Space and Astronautical Science, Kanagawa	6
Kuwait	Kuwait University, Faculty of Medicine	30
New Zealand	University of Canterbury, Dept. Zoology	50
	Victoria University of Wellington, School of Biological Sciences	20
Nigeria	McBod Aquarium Supply, Lagos	700
Republic of China	Chung Shan Medical University, Dept. Life Sciences	30
	Institute of Biomedical Sciences, Academia Sinica	400
	Institute of Molecular Biology, Academia Sinica	850
	National Cheng Kung University	160
	National Sun Yat-Sen University, Dept. Biological Sciences	300
	National Taiwan University, Dept. Physiology	500
	National Tsing Hua University, Dept. Live Science	145
Rep. of Korea	Sogang University, Dept. Life Science	126
Russia	Engelhardt Institute of Molecular Biology, Moscow	149
Scotland	University of Dundee, Biochemistry	272
	University of Dundee	1051
	University of Glasgow	20
Singapore	Institute of Molecular and Cell Biology (IMCB)	159

Table 5-2 Continued

Country	Recipient institution	No. of frogs
Spain	Unidad de Neurologia Experimental, Departemento de Investigacion	70
	Institutio Cajal, CSIC, Madrid	56
	Institutio Edificio de Servicios Generales, Madrid	50
	Universitat de Barcelona, Facultat de Biologia	56
Sweden	Uppsala Universitet, Evolutionary Biology Centre	25
Switzerland	Basel Institute for Immunology	550
	Eidgenossische Technische Hochschule Zurich, Dept. Applied Biosciences	200
	ETH Institute of Cell Biology	250
	Institut de Pharmacologie et de Toxicologie de L'Université	1,616
	RCC Ltd., Biotechnology & Animal Breeding Division, Fullensdorf	100
	University of Genéve, Station de Zoologie expèrimentale	225
	Swiss Federal Institute of Technology, Zurich	350
	Universitat Spital Zurich, Biologisches Zentrallabor	130
	Universite de Lausanne, Centre Integrative de Genomique	60
	University of Genéve, Sciences III, Faculty of Science	785
	University of Zurich, Institute of Physiology	75
Thailand	Srinakharinwirot University, Faculty of Medicine	20
The Netherlands	Universiteit van Amsterdam, E.C. Slater Instituut	65
	Katholieke Universiteit Nijmegen	1,750
	University of Nijmegen, Dept. Molecular Animal Physiology	600
USA	Animal Resource Center, Medical College of Wisconsin	32
	Merck & Co., Inc. Linden, New Jersey	25
	Merck & Co., Inc. Edison, New Jersey	30
	Xenopus 1, Dexter	6,500
	Xenopus Express, Homosassa	8,050
		68,051

In terms of number of import facilities per country, Germany is at the top of the list with 30. Germany is followed by Switzerland and England with 11 and nine facilities respectively. The majority of countries (12 countries), however, had only a single facility that imported frogs. There seems to be a high turnover of facilities that utilise *X. laevis* because 34.14% of facilities imported this species only once (Figure 5-3). An increase in the number of orders is concomitant with a decrease in import facilities. The highest numbers of orders, 32, were

ordered by only one facility (0.81% of facilities). This implies that only a few facilities have long-term research programmes that involve the use of *X. laevis*. Most other facilities are occasionally involved in research projects that require the use of this species or that facilities are developing their own breeding colonies. When we group the number of countries according to categories of imported frog numbers, it becomes apparent that the largest movement of frogs is towards a few countries only (Table 5-3). More than half of the importing countries (16) have imported fewer than 500 frogs, accounting for 3.2% of the total movement of frogs. Half of the total number of exports (50.1%) went to the two largest importing countries. The largest number of frogs was imported by France (19,460) followed by the USA (14,637) and Germany (9,338).

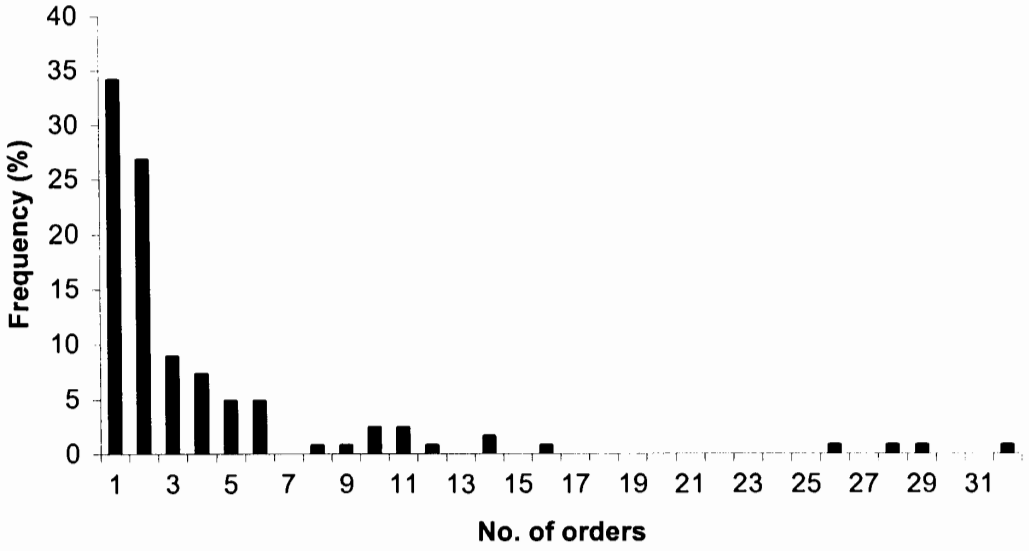


Figure 5-3 Frequency at which *Xenopus laevis* were ordered by the various international facilities, indicated as percentage of the total number of facilities.

Table 5-3 Number of frogs exported and import countries per numbers category for the period 1997- April 2004.

No. frogs category	No. countries	No. frogs imported	% of total
15001 - 20000	1	19,460	28.6
10001 - 15000	1	14,637	21.5
5001 - 10000	2	14,673	21.6
1001 - 5000	6	14,432	21.2
501 - 1000	4	2,640	3.9
< 500	16	2,194	3.2
	30	68,051	100

5.3.4 Local Utilisation

Locally the demand for the species follows two separate streams, namely teaching and the angling industry, and at a much smaller scale than the international utilisation. *Xenopus* is used in teaching as a model system for physiology and anatomy dissection and occasionally in scientific research at some tertiary academic institutions. The two companies, African Reptile Park and African Xenopus Facility, also supply *X. laevis* in South Africa, but the largest current supplier to the local market is C.E. Loesch, based in the Klapmuts/Stellenbosch area near Cape Town. During the past seven years almost 8,000 frogs were sold within the country to 11 academic and 3 scientific research institutes (Table 5-4). In 1998 sales were at a high of 3,500 frogs. After 1998 the local demand dropped below 1,500 varying with a few hundred frogs between the years (Figure 5-4). Although on a much smaller scale, juvenile *X. laevis* are used for bait by freshwater anglers. Numbers utilised at a national level are not available because most of the collecting is done without official control by independent collectors and the anglers. However this is an illegal activity in accordance with the Performing Animals Protection Act of 1935.

A large number of frogs are sold annually in angling shops in the Free State Province, but none of the shops keep records of the actual numbers of frogs that are sold. It is estimated by shop owners that the annual number of frogs sold ranges between 5,000 to as many as 32,000. Sales continue all year round, but it is especially during the angling season from September until April, that large quantities are sold. These shops often cannot keep up with the demand. They are totally dependent on outside sources to replenish their stock and are constantly on the lookout for persons who can deliver. These include a few people who occasionally collect from existing farm dams or farmers themselves who find the frogs by chance when cleaning dams or drinking troughs. *Xenopus laevis* is a much sought after bait when fishing for the sharptooth catfish (*Clarias gariepinus*). Only young frogs between the sizes of 35 mm and 50 mm (snout-vent length) are used for this purpose. Other fish that are known to take *X. laevis* are the endemic yellowfish species (*Barbus* spp.) and introduced bass and trout species.

Table 5-4 *Xenopus laevis* supplied to South African facilities from January 1998 to April 2004. The facilities are listed in alphabetical order. Frogs were supplied by African Reptile Park, African Xenopus Facility and C.E. Loesch.

Recipient institution	1998	1999	2000	2001	2002	2003	2004	Total
Biologic Hout Bay	20							20
Butterfly World					150			150
Cape Technicon			38		50	90		178
CSIR Pretoria	20							20
MEDUNSA						35		35
North-West Univ.		200	400	400	200	150	300	1,650
Rhodes Univ.	168							168
Univ. of Botswana		200	300			300		800
Univ. of Cape Town	290				100	130	75	595
Univ. of Durban Westville	1,100	200						1,300
Univ. of Port Elizabeth	150			20	45	15		230
Univ. of Stellenbosch	1,210			100	280	360	205	2,155
Univ. of the Western Cape	20				20	30		70
Univ. of the Witwatersrand	400							400
Western Cape College	40	30						70
	3,418	630	338	120	645	960	280	7,841

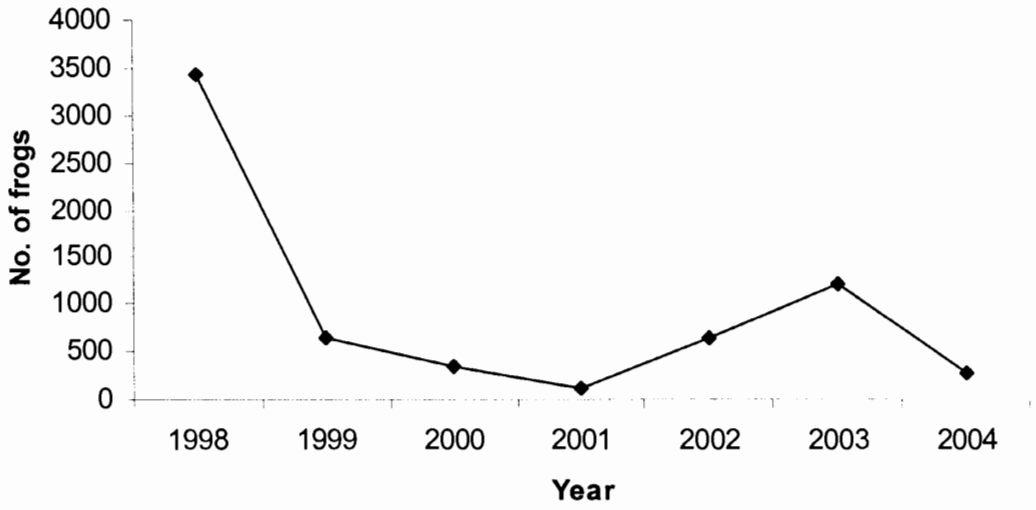


Figure 5-4 Trend in the numbers of *Xenopus laevis* utilised by South African academic and research facilities for the past seven years. These numbers only represent frogs that were purchased from the main suppliers investigated in this study.

5.3.5 Conservation Implications

Depletion of South African Populations

The exploitation of wild *X. laevis* is a potential threat to the integrity of populations. Uncontrolled harvesting practices can lead to the rapid depletion of a population (Weldon 1999). The collection frenzy of *X. laevis* soon after the discovery of the *Xenopus* pregnancy assay, contributed to the disappearance of the species from the Cape Flats (Hey 1986). The efficacy of aquatic traps in the collection of *X. laevis* ensures that large numbers of frogs are removed from a source in a single trapping. Trapping success depends among other factors on the initial population density, number of traps used, type of bait used, duration of trapping and trapping frequency. Such trapping practices have been applied successfully in controlling *X. laevis* numbers at a fish farm in the Eastern Cape, South Africa (Schramm 1986). However, overland migratory behaviour of *X. laevis* enables the species to colonise ponds previously depleted of frogs within two weeks (Schramm 1986). Natural colonisation can occur only at sites where the adjacent populations are situated within migratory distance, which is up to 1,500 m (Lobos and Garin 2002).

When harvesting *X. laevis* from the wild, care has to be taken not to overexploit the source. By following simple precautionary measures the effect of harvesting on population numbers can be minimised. To ensure that members of the reproductively-mature population remain within the population, a portion (e.g. 10%) of the larger frogs should be removed from the batch and released. This is advantageous to both the survival of the population and the continuance of the supplier's business. By taking steps to maintain an active breeding population, suppliers will be guaranteed a reliable source. When collecting specifically for the angling market, selective removal of the right sized frogs should be practiced (35-50 mm snout-vent length). To prevent the depletion of populations, frequent visits to sources should be avoided. Instead, sources should be visited on a rotational basis that allows enough time for the populations to recover (e.g. twice a year).

Feral Populations

Whenever captive animals are moved to other countries, there is a risk that some individuals could escape to the wild. In the case of *X. laevis*, the high numbers that are exported and the many global destinations, increase the risk of escape. The same intrinsic features that rendered this species suitable for laboratory study (e.g. high fecundity, able to tolerate a wide range of environmental conditions) provide escaped individuals with a selective advantage to survive in a novel environment.

Feral populations of *X. laevis* have been detected since 1944 and are known to persist in several countries around the world including the UK, France, Ascension Island, Chile and USA (Tinsley and McCoid1996, Fouquet 2001, Lobos and Jaksic 2005). Three of these countries where large populations of feral *X. laevis* exist, UK (England, Wales) France and the USA (Arizona, California) are also large export destinations for *X. laevis* thereby increasing the opportunity for accidental or intentional escape. It is believed that a population in Ascension Island (South Atlantic) originated from frogs that were imported directly from South Africa (Loveridge 1959). It is not clear from where the feral frogs from Chile were introduced (Lobos and Measey 2002), however the point of entry has been identified as Caren lagoon, near Santiago's international airport (Jaksic 1998). Populations from an additional five states of the USA are now presumed extinct due to single animal introductions, eradication measures and severe weather conditions or combinations of these factors (Tinsley and McCoid 1996).

Xenopus laevis are generalist predators of the aquatic environment where invertebrate prey make up the largest portion of their diet (Measey 1998, McCoid and Fritts 1980a, Schoonbee et al. 1992). The diet can also be supplemented with its own tadpoles, small vertebrates and other terrestrial prey (Inger and Marx 1961, McCoid and Fritts 1980b, Lobos and Measey 2002, Kazadi et al. 1986). As so far as the threat *X. laevis* poses as a predator to native amphibians and fish, the evidence seems inconclusive. Laferty and Page (1997) found that the endangered tidewater goby (*Eucyclogobius newberryi*) that coexist with *X. laevis* in the Santa Clara River estuary, California, made up a part of the diet, but *Xenopus* did not cause a decline in fish numbers. Measey (1998) remarks that a combination of high population densities of *X. laevis* and trophic cascades prompted by the presence of exotic predators may significantly affect on native species.

Transport of Amphibian Pathogens and Parasites

Of particular relevance to the global trade in *X. laevis* is that whenever this animal is translocated, the parasites and pathogens that it might be harbouring are translocated as well. This risk is augmented by the fact that *Xenopus* is host to an exceptional array of metazoan and protozoan parasites, represented by over 25 genera from seven invertebrate groups (Tinsley 1996). Evidence exists for the presence of parasites endemic to Africa in feral *X. laevis* from southern California (Laferty and Page 1997). The likelihood that parasites could switch hosts is dependant on the type of parasite. Host switching is unlikely in the instances of strictly host-specific parasites or where a specific intermediate or final host is required to complete the life-cycle. *Cephaloclamys namaquensis,* an intestinal tapeworm of *X. laevis* has been recorded in *Rana angolensis* in Zimbabwe (Mettrick 1963). This has raised the concern that the tapeworm could spread to other *Rana* species in import countries (Laferty and Page

1997). Interviews with the suppliers have shown that they are often well informed about the role that *X. laevis* plays as a transport host. Much of their knowledge was generated from past experiences with parasite outbreaks at their facilities. Despite this awareness, suppliers seem to be oblivious to the effects of *X. laevis* as a carrier of infectious diseases as a direct consequence of negligent collection and confinement practices. The trade in *X. laevis* has been identified as a major source of the international dissemination of an emerging infectious disease of amphibians, the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Weldon et al. 2004). It is imperative that suppliers and all frog handlers be made aware of practices that promote the spread of amphibian related diseases and of mitigation measures that can decrease this risk.

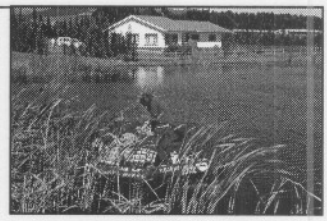
5.4 Conclusion

Very few animals have served scientific and medical research quite like *Xenopus* has. After a long history of utilisation, *X. laevis* is still in high demand outside South Africa. Locally the demand seems to have stabilised at a level below that of pre-1975. Characteristics associated with the trade including suppliers, source of frogs, destinations and uses of frogs have changed over time. The trade is characterised by a consistent demand, but fluctuates between years in terms of number of frogs exported and experiences a combination of regular and changing export destinations. Suppliers and overseas breeding houses maintain an established infrastructure to provide for the demand of the end-users. Wild frogs are continually collected and exported from South Africa to be used directly in research programmes or to provide genetic variation for in-house breeding colonies. Effective control over the export trade by regulatory bodies and rational exploitation by suppliers will ensure that *X. laevis* will remain a viable natural resource. The empirical data generated from this study can aid in establishing the role of *X. laevis* in pathogen or parasite dispersal. By linking the trade route to occurrences of infection, the risks of exporting infected frogs can be determined. With an increased movement of amphibians between countries for research and other purposes (food and pet trade) there is a growing need for quantitative investigations that can support movement and quarantine restrictions that may be enforced.



Chapter 6

RISK ANALYSIS OF CHYTRIDIOMYCOSIS IN THE *Xenopus laevis* TRADE



6.1 Introduction

6.1.1 Risk Identification

There exists in the world today a productive global trade in amphibians for purposes ranging from experimental animals, to cuisine delicacies, to pet animals. Since the mid 1930s, South Africa has been a major supplier of African clawed toads, *Xenopus laevis* for scientific research in many countries across the globe and has in recent years exported almost 10,000 frogs annually to more than 30 countries (Chapter 5 of this thesis). The biggest demand for this species is in the scientific fields of molecular and embryology research (Weldon 1999). It has recently been shown that archived specimens of *X. laevis* from the Cape (the centre of the frog trade) were infected with one of the most virulent pathogens of amphibians, the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Weldon et al. 2004). Chytridiomycosis is widespread in South Africa in *X. laevis*, however, the infection appears to be sub-clinical in this species (Weldon et al. 2004).

The association of *B. dendrobatidis* with amphibian declines has been established through field observation during events of mass mortality (Lips 1999, Young et al. 2001), retrospective analysis of populations that had declined due to previously unknown causes (Carey et al. 1999, Bradley et al. 2002) and through experimental trials (Berger et al. 1998, Nichols et al. 2001). Although the amphibian chytrid is not host-specific, a varying degree of sensitivity exists among metamorphosed amphibians (Anura and Caudata) that can be sub-clinical to lethal infections.

The process of determining how *B. dendrobatidis* becomes established in populations that then suffer sudden declines, is difficult and is not known in most cases that have been studied. One explanation is that the fungus was somehow introduced into the affected populations (Berger et al. 1998, Daszak et al. 1999). Two bodies of evidence support this process of chytridiomycosis establishing in a naïve population. A timeline coupled with a pattern of amphibian deaths in Australia suggests that chytridiomycosis was introduced and spread from one or more point sources. Mounting evidence suggests a similar pattern of deaths in Central

and South America (Lips 2004, Lips et al. 2004). Secondly, amphibian species that do not become diseased when infected with *B. dendrobatidis* and that are transported globally, can assume the role of natural carriers that can transmit the disease to other species from different geographical regions (Daszak et al. 2004, Hanselmann et al. 2004, Weldon et al. 2004).

By dominating the export trade in amphibians from South Africa, *X. laevis* can be recognised as a commodity with a high risk for disseminating chytridiomycosis on a global scale. This puts amphibians both nationally and internationally at risk of acquiring chytridiomycosis through transmission via this species. At an amphibian diseases conference in Cairns, Australia it was recommended that high priority should be given to chytridiomycosis risk assessments in countries where the disease is eminent (Speare 2001). Considering the large movement of *X. laevis*, there is an urgent need to quantify the risks of infected frogs being exported from South Africa. In addition, chytridiomycosis appears on the World Animal Health Organization's (OIE) wildlife list of diseases requiring quarantine restrictions in the movement of amphibians between countries. Because South Africa is a member state of the OIE, it is timely to pre-empt any quarantine or movement restrictions that may be recommended. Within the context of the OIE's import risk analysis the following key definitions can be formulated:

Hazard ..... *Batrachochytrium dendrobatidis* is a biological agent, which may have an adverse effect.

Commodity ..... *Xenopus laevis* is a biological article of trade that is exported from South Africa.

Risk ..... The likelihood that *B. dendrobatidis* may cause disease in amphibians acquired via *X. laevis* in the importing countries.

6.1.2 Description of the Commodity

Xenopus laevis is a member of the Pipidae family. These are strictly aquatic frogs with large fully-webbed feet, which in most species end in keratinised claws (Duellman and Trueb 1994). *Xenopus* possess several morphological characteristics adapted to their aquatic existence such as a dorsoventrally flattened and streamlined body with lateral line organs (Passmore and Carruthers 1995). What makes these animals unique is that they lack a tympanum, tongue, vocal cords and movable eyelids. Because the tadpoles are suspension feeders, they lack keratinised mouthparts. The genus *Xenopus* consists of 17 species, which are endemic to sub-Saharan Africa. *Xenopus laevis* occupies the relatively cooler highlands between the Cape and the plateaux of Cameroon and Nigeria, excluding the Zaire Basin and the hotter lowlands of eastern Africa (Tinsley et al. 1996). In South Africa, *X. laevis* is common and widespread and absent only from northeastern KwaZulu-Natal and parts of the extreme Western Cape.

This species occurs in almost any natural and man-made water bodies including streams, rivers, dams, ponds, roadside pools and even sewage purification works. *Xenopus laevis* does not seem to be threatened in any part of its range and is not listed on the South African Red Data list for threatened species (Measey 2004).

6.1.3 Objectives

The aim of this study was to clarify the uncertainties concerning the health risk that chytridiomycosis poses to amphibians in countries that import *X. laevis*, which may act as a carrier of the disease. If we make the assumption that the rate of transmission is related to prevalence in the source host, then prevalence is an indication of the likelihood of transmission taking place. Although the prevalence of *B. dendrobatidis* in archived *X. laevis* specimens has been determined (Weldon et al. 2004), no information is given on the prevalence in frogs intended for the export market. The objectives of this study were:

- to conduct a risk assessment on the role of the trade in *X. laevis* in the international dissemination of chytridiomycosis by combining quantitative analysis (using prevalence data to support consequences and likelihood) with qualitative analysis (using descriptive scales to describe the magnitude of potential consequences and likelihood),
- to develop and advance mitigation measures that would help prevent, recognise and manage the disease in the trade sector, based on the outcome of the risk assessment.

This risk analysis was conducted on a voluntary basis without any prior consultation with possible stakeholders, which may include suppliers, animal health organizations, customs officials, nature conservation authorities or import facilities. This analysis should therefore be regarded as an initial screening activity to identify risks, which may require more detailed analysis.

6.1.4 Use of Descriptive Scales

In order to classify the likelihood of potential events occurring in the risk being assessed and the consequences of those events, this study made use of qualitative descriptive scales (Tables 6-1 and 6-2) that were tailored from the guidelines provided by the Australia/New Zealand Standards (Australia/New Zealand Standard 1999) to meet the needs of the current riskassessment.

Table 6-1 Qualitative measure of the likelihood of a risk event occurring.

Level	Descriptor	Description
I	Almost certain	Is expected to occur in most circumstances
II	Likely	Will probably occur in most circumstances
III	Possible	Might occur at some time
IV	Unlikely	May occur only in exceptional circumstances

Table 6-2 Qualitative measure of consequences for type of exposure and effect of infection on host species. The two consequences are independent of each other for each level.

Level	Descriptor	Description
1	Minor	Zoospores released, but die; Sub-clinical infection
2	Moderate	*Xenopus* escape, but die; Sporadic deaths
3	Major	Zoospores released and survive; Population declines
4	Catastrophic	Escaped *Xenopus* survive; Species extinction

6.2 Chytridiomycosis Survey

In an attempt to quantify the risk of spreading chytridiomycosis, *X. laevis* were screened to determine the prevalence of *B. dendrobatidis*. The frogs were obtained from three types of sources: seven wild populations in the Western Cape Province (these sites serve as sources of *X. laevis* to commercial suppliers) and one wild population from the Northern Cape Province, four batches from supply houses of the export trade, and five feral populations.

Frogs from wild populations were collected with baited bucket traps that were set overnight in the various ponds. A maximum of 50 frogs were removed from the traps for tissue collection. A pair of scissors was used to remove a piece of interdigital webbing from one hind foot of each frog and the skin individually placed in 70% ethanol filled vials. Dissecting equipment was sterilised after use on each frog by immersion in 70% ethanol. Disinfectant ointment (Bethadine, Adcock Ingram Ltd.) was applied directly to the wound. The bucket traps were hosed off and allowed to dry when working between sites.

Frogs from one supplier (Xenopus Express) were collected and handled at their facility in Somerset-West. Frogs ordered from a second supplier (African Reptile Park) were kept in large concrete containers and handled within three days of arrival. Tissue was collected in the same manner from fixed specimens of feral populations that were collected before 2002 for

purposes other than amphibian disease work by Prof. Richard Tinsley from Bristol University, UK. After 2002 three more feral populations from California, England and France were surveyed for the amphibian chytrid fungus. Histological sections, prepared using described methods (Berger et al. 1999b) were used to identify the amphibian chytrid fungus.

6.3 Chytridiomycosis Prevalence Data

The amphibian chytrid fungus was detected in *X. laevis* from all three of the categories represented by this study: wild populations, frogs from trade facilities and feral populations. The incidence in wild frogs was highly variable, being absent in some of the populations in the Western Cape to being present in 89.7% of animals from the Northern Cape sample. In the six wild populations that the amphibian chytrid was detected, the prevalence was never lower than 8.3% and averaged 38.1% (Table 6-3). If we include the two populations that were chytrid-free, the average prevalence for this category of *X. laevis* is reduced to 28.6% (STD = 27.3). The amphibian chytrid was present in batches from both of the trade facilities, although one of the three batches was chytrid-free. The two facilities differed little in the prevalence of the infection, with an average prevalence of 10.1% (STD = 0.1). From the nine samples of feral populations examined, only two had an incidence of chytridiomycosis, both from California (1983 and 2000). Prevalences were 3% and 14.3%.

A comparison between the percentages of infected samples of the different categories of *X. laevis* sources, indicate that fewer samples from the trade (66.7%) were infected than from the wild (75.0%) and that even fewer feral populations (22.2%) were infected (Figure 6-1). Similarly, the mean prevalence from the categories decreased from wild populations to trade samples, to feral populations.

Table 6-3 Prevalence of *Batrachochytrium dendrobatidis* in 20 samples of *Xenopus laevis*. The first eight samples are from population surveys conducted in the Western Cape (WC) and Northern Cape (NC) provinces of South Africa. Three samples were obtained from suppliers of the export trade situated in the WC and Eastern Cape (EC). The last nine samples are from feral populations from the USA, UK and France.

Sample	Collection date	No. of frogs	Prevalence (%)
Airfield, Stellenbosch, WC	Sep-2001	48	29.2
Simonsvlei farm, Klapmuts, WC	Sep-2001	50	34.0
Welmoed farm, Stellenbosch, WC	Feb-2002	51	0
Bellevue farm, Paarl, WC	Feb-2002	48	0
Welvanpas farm, Wellington, WC	Feb-2002	48	8.3
Botriver, WC	Feb-2002	49	28.6
Strand golf club, WC	Feb-2002	43	39.5
Pedroskloof farm, Kammieskroon, NC	Aug-2004	29	89.7
Xenopus Express, Somerset-West, WC	Sep-2001	50	10.0
African Reptile Park, Knysna, EC	Jun-2003	98	10.2
African Reptile Park, Knysna, EC	Mar-2004	50	0
San Diego, California, USA	1983	33	3.0
Arizona, USA	1986	10	0
Virginia, USA	1987	10	0
Arizona, USA	1988	47	0
California, USA	2000	21	14.3
Wales, UK	Jul-2000	43	0
Scunthorpe, England, UK	Jul-2003	100	0
Deux-Sèvres department, France	2004	40	0
California, USA	Jun-2004	48	0

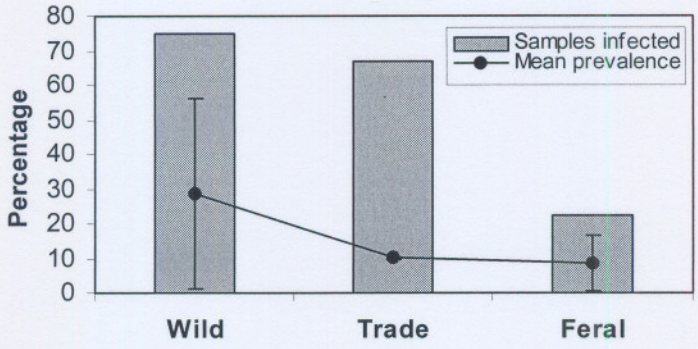


Figure 6-1 Percentage of samples infected and mean prevalence of the infected samples for *Xenopus laevis* from wild populations in South Africa, trade facilities and feral populations.

6.4 Risk Assessment

6.4.1 Release Assessment

The release assessment involves describing the *X. laevis* trade pathway through which *B. dendrobatidis* may be introduced from South Africa to another country, and estimating the likelihood of this process occurring.

Release Pathway

The movement of *B. dendrobatidis* through the *X. laevis* trade from the time of capture in natural water bodies to the final destination, whether it is a science laboratory, the aquarium of a pet keeper, or even a foreign water body of an import country, could potentially involve seven steps (Figure 6-2). When local suppliers visit an infected site they risk spreading the fungus to adjacent sites (Figure 6-2 1a). Simultaneously or alternatively, infected frogs, when collected, can contaminate the holding facilities (Figure 6-2 1b). From here, chytridiomycosis spreads to new sites when other regions are visited on later collection trips (Figure 6-2 2). When ordered, potentially infected frogs are then dispatched by the suppliers to customers locally (Figure 6-2 3a) and abroad (Figure 6-2 3b). In the importing country the frogs go either directly to the end-users or to large breeding facilities. The latter then operate as suppliers, further spreading the potentially infected frogs to end-users nationally and internationally (Figure 6-2 4). Finally, in some cases, chytridiomycosis could spread from these two sources (end-users and breeding facilities) through escaped frogs and be transmitted to native species (Figure 6-2 5).

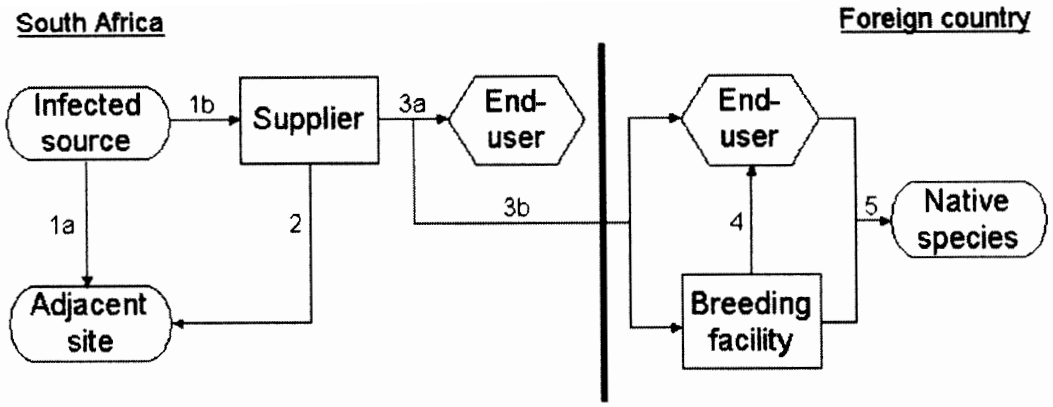


Figure 6-2 Flow chart of the potential movement of the amphibian chytrid fungus through the local and international trade in *Xenopus laevis*.

The trade pathway is an open system that allows for continuous and multiple entry and escape because *B. dendrobatidis* could keep on spreading from point sources in both South Africa and foreign countries through any number of amphibian species and other animate and inanimate vectors. Furthermore, it describes the basic steps in the movement of *B. dendrobatidis* once it enters the *X. laevis* trade pathway until disease incidence can no longer be linked directly to *X. laevis*. The last step (transmission to native species) does not form part of the deliberate movement of the frogs, but occurs as a result of negligence at the final site of captivity.

Likelihood of Chytridiomycosis being Released via the Trade Pathway

When commercial suppliers collect frogs from a contaminated source, it is almost certain that the disease will be transferred to adjacent sites through trapping equipment or be transported to the holding facilities via infected frogs. Before a single frog is collected and exported however, there are various opportunities that facilitate the spread of the *B. dendrobatidis* between sites within the country.

- The opportunity exists in South Africa for human induced spread of chytridiomycosis either through accidental or intentional means. Possible transmission could be facilitated by a number of carriers including field biologists working in wetlands or river habitat and people that are dependent on natural or artificially created water bodies for consumer purposes and in agriculture. In a developed country, transmission through

the visiting of water bodies for human consumption does not contribute to the spread of chytridiomycosis to the same extent.

- It is also possible that a number of animate vectors could spread the fungus between sites of which frog-to-frog transmission is the most obvious. Transmission is expected to be higher during the breeding season when amphibian activity reaches a peak and individuals congregate at breeding sites. *Xenopus laevis* is one of the few frog species in South Africa (and in the world) that is known for mass migration (Hewitt and Power 1913, Lobos and Measey 2002), thus increasing the likelihood of introducing chytridiomycosis to adjacent sites.

Except for the collection of wild *X. laevis*, none of these situations are unique to South Africa, unless we consider the collection of *X. laevis* from feral populations. In the event of one of the above-mentioned carriers coming in contact with a source that is frequented by suppliers, the involved carrier then indirectly links an outside source with the trade pathway.

When suppliers visit multiple sources of wild *X. laevis* they are likely to encounter an infected site, because of the wide distribution of chytridiomycosis in the area that suppliers operate in. The indication from the disease survey is that not all animals from a contaminated source are necessarily infected and that prevalence differs among sites. This implies that the animals that are selected (sub sample - usually large females) from the sample that has been caught from contaminated sites may contain any combination of infected and uninfected individuals. The possibility, although unlikely, exists that the entire sub sample may be uninfected. At the holding facilities of suppliers the frogs from different sites are often sorted and mixed. Generally the mixing of samples will increase the likelihood of *B. dendrobatidis* being present in the final batch. It can be predicted that when frogs from clean sites (*B. dendrobatidis* not present) are grouped with those caught in contaminated sites the probability of finding an infected individual decreases. Over time, regardless of the initial prevalence, all the animals that are kept in the same enclosure may acquire chytridiomycosis because of an accelerated transmission of the disease in crowded conditions. Disease and parasites can become a threat to frog breeding when frogs are confined in high concentration, under-fed or are kept in unhygienic conditions (Hey 1949, Negroni 1996). This rate of transmission is dependent on susceptibility to infection/disease which is known to differ between populations (McDonald and Alford 1999, Bell et al. 2004) behavioural characteristics of the host (Bradley et al. 2002), and external factors including: husbandry conditions (time intervals between cleaning of aquaria, crowding etc.) and the length of captivity. Because chytridiomycosis prevalence is impossible to predict at sources and likely to change depending on a number of factors when hosts are

kept in captivity, a more applicable measure of risk should simply be the presence or absence of the disease in a given sample intended for export.

Ultimately, the likelihood of infected *X. laevis* leaving the country depends on the animal health certification policy that resides with the Veterinary Services Directorate of the Department of Agriculture. Veterinary Services policy stipulates that quarantine and veterinary diagnostic testing of animals intended for export depends on the requirements of the import country. According to a spokesperson of Veterinary Services in the Western Cape Province, not a single batch of *X. laevis* has been quarantined in recent years. Because none of the import countries of *X. laevis* require prior diagnostic testing of chytridiomycosis, it is almost certain that infected frogs have free passage to the respective destinations.

6.4.2 Exposure Assessment

The exposure assessment involves describing the pathway through which amphibians in the importing countries become exposed to chytridiomycosis through the *X. laevis* trade, and estimating the likelihood of this exposure occurring.

Exposure Route

In the importing country, amphibians can become exposed to chytridiomycosis through contact with the infective stage of *B. dendrobatidis*, the zoospores. The zoospores are waterborne and can therefore transmit to captive amphibians when enclosures share the same water or equipment, or transmit to wild amphibians that visit a contaminated source. A water body can become contaminated through one of two processes, or both. Firstly, water that was in contact with infected *X. laevis*, such as aquarium water, could be disposed of without treatment and end up in water bodies that harbour amphibians, thus exposing them to zoospores that were released into the aquarium water before it was discarded. Secondly, infected frogs that escaped from captivity or that were deliberately released into the environment, may act as a constant source of *B. dendrobatidis* zoospores as long as they remain infected. Survival of escaped *X. laevis* will depend on the availability of prey, the presence and effect of novel predators, whether or not environmental conditions are favourable and whether eradication is attempted. In only a few cases, were escaped frogs able to overcome adverse conditions and/or invasive species control measures resulting in successful breeding populations as shown by the history of the persistence of feral *X. laevis* populations (Tinsley and McCoid 1996).

Except for the UK, a parallel seems to exist between the presence/absence of chytridiomycosis in those countries that are known to harbour feral *X. laevis* and the incidence

of chytridiomycosis in the respective *X. laevis* populations. Amphibian species from the state of California that have been diagnosed with chytridiomycosis have suffered population declines, such as the Yosemite toad, *Bufo canorus* and the mountain yellow-legged frog, *Rana muscosa* (Green and Kagarise Sherman 2001, Green et al. 2002), others such as the western toad, bullfrog, pacific tree-frog and foothills yellow-legged frog only experienced morbidity events and do not seem to be adversely affected by the infection (Green et al. 2002). Chytridiomycosis was very recently detected in feral *Rana catesbeiana* in the UK (Pasmans et al. 2005), but has not yet been detected in France, a country that harbours chytrid-free feral *X. laevis* populations. The infection status of *X. laevis* from the remaining two countries outside Africa, Chile and Ascension Island, remains to be determined. No occurrences of chytridiomycosis from any amphibian have yet been reported from these two countries.

Likelihood of Exposure Taking Place

Customs practices in the import country could determine whether the risk of exposure in the country in question is eliminated, reduced or unaffected. Countries that do not have animal health legislation equipped to deal with chytridiomycosis upon import of amphibians, are likely to experience exposure of the environment to the disease. The probability of exposure to amphibians is dependent on a number of factors that can occur independently or together.

- The intended distribution of *X. laevis* within a country will determine the geographical area that can potentially become exposed. This likelihood of exposure increases with the number of destinations and with an increase in the number of animals that are distributed.
- Exposure risk is related to the intended use of *X. laevis* (laboratory animal or exotic pet). Exposure by pet owners is highly likely, because they do not have the same level of training in disposal practices for used material as laboratory workers. It is not uncommon for owners of exotic pets to simply discard the animals in the environment when they lose interest in them. However, this does not exclude the possibility of contamination by laboratory workers that do not comply with proper disposal practices.
- Another factor that influences likelihood of exposure with regards to geographical distribution is human and amphibian demographics that determine the availability of potential vectors. Although chytridiomycosis is not zoonotic, humans may act as vectors that spread the disease when contaminated equipment or footwear come in contact with aquatic habitat of amphibians. Exposure risk will be highest in regions known for a rich amphibian biodiversity and abundance or amphibian habitat that is regularly visited by people.

- Environmental characteristics also influence exposure risk. Both *X. laevis* and *B. dendrobatidis* cannot survive in regions that experience extreme temperatures. In Africa, *X. laevis* occupies mainly temperate Savannah habitat, whereas *B. dendrobatidis* remains viable at a temperature range of between 4 and 30°C (Longcore et al. 1999, Berger 2001). It is therefore unlikely that amphibians will be exposed to chytridiomycosis in parts of the tropics or arid regions where the average temperature often exceeds 30°C. As for sub-4°C averages; these temperature zones are usually unsuitable for amphibians.

6.4.3 Consequence Assessment

The consequence assessment involves describing the potential consequences of amphibians becoming exposed to chytridiomycosis and estimating the likelihood of them occurring. Exposure of amphibians to *B. dendrobatidis* zoospores will almost certainly lead to transmission because it has not been demonstrated in clinical trials that there are species that resist infection when exposed to zoospores In addition, the wide range of amphibian taxa that have been diagnosed with chytridiomycosis suggests that the pathogen has no host specificity (Speare and Core Working Group 2001). The release and exposure pathways may have consequences regarding the type of exposure, whereas chytridiomycosis transmission to native amphibians may have consequences for species and population integrity, and from an economical perspective.

Consequences of Exposure Type for Infection Potential

The consequences related to the type of exposure are influenced by two factors; 1) the form in which *B. dendrobatidis* is exposed (active infection on escaped frogs vs. released zoospores from captive frogs) and 2) the environment into which *B. dendrobatidis* is released (favourable vs. hazardous for survival). In a hazardous environment zoospores from a source of captive frogs are expected to have minor consequences (least chance of transmission). On the other hand, infected *X. laevis* are expected to have moderate consequences, because *B. dendrobatidis* might survive longer on its host and therefore have more opportunities to transmit to native amphibians. When *B. dendrobatidis* is released into an environment with favourable conditions the consequences become more severe. Infected *X. laevis* may have catastrophic consequences over contaminated water that might have major consequences as so far infection potential goes.

Consequences of Infection for Amphibian Populations

It is difficult to predict what effect chytridiomycosis will have on a particular species. Numerous case reports describing the effect of chytridiomycosis on amphibian assemblages from various

locations on the globe, as well as monographs on the subject have been published or are available online. These available works can be studied to estimate the consequences of *B. dendrobatidis* infection in particular species and areas of concern by import countries of *X. laevis*. Various negative consequences have been observed among different species and populations when exposed to chytridiomycosis ranging from minor (sub-clinical) to catastrophic (species extinction). Evidence from wild populations has shown that *B. dendrobatidis* often has major consequences (population declines) for species assemblages, such as at the Fortuna Forest Reserve, Panama (Berger et al. 1998), Rocky Mountain National Park, Colorado, USA (Muths et al. 2003) and Pen`alara Natural Park, Spain (Bosch et al. 2001). In a few cases species are believed to have disappeared completely due to chytridiomycosis. These species include among others *Atelopus cruciger* from Palmichal, Estado Carabobo, Venezuela in 1986 (Bonaccorso et al. 2003), *Taudactylus acutirostris* from Big Tableland, Queensland Australia in 1996 (Berger 2001), and *Nectophrynoides asperginis* from Kihansi Gorge, Tanzania (Chapter 9 of this thesis). Amphibian populations that do not appear to be declining, despite the prevalence of chytridiomycosis, may experience moderate consequences in the form of sporadic deaths. Amphibian losses due to chytridiomycosis are not limited to wild amphibians, but have been reported from frog farms in Uruguay (Mazzoni et al. 2003) and from laboratories and zoos in the USA (Nichols et al. 1996, 1998, Parker et al. 2002).

The international demand for *X. laevis* may have provided the opportunity for chytridiomycosis to be disseminated around the world. Over the past seven years exports of *X. laevis* have included destinations in North and South America, Western and Eastern Europe, the Middle East, South and East Asia, Australia and New Zealand. Of the 31 countries that have imported *X. laevis* since 1998, chytridiomycosis has only been detected in wild amphibian populations from eight countries (Table 6-4). It is not possible at this stage to make deductions regarding the association of the occurrence of chytridiomycosis with countries that have recently imported *X. laevis*, because chytridiomycosis surveys have only been conducted in nine of these countries.

Table 6-4 Association of chytridiomycosis in wild amphibians with countries that have imported *Xenopus laevis* from South Africa since 1998.

Import country	Chytridiomycosis survey conducted	Chytridiomycosis detected	Reference
Argentina	Yes	Yes	Herrera et al. (2005)
Australia	Yes	Yes	e.g. Berger et al. (1998)
Austria	No	—	
Belgium	No	—	
Brazil	Yes	Yes	Carolina et al. (2005)
Canada	Yes	Yes	Ouellet et al. (2003)
China	Yes	No	Fu and Zheng (2005)
Denmark	No	—	
England	Yes	Yes	Pasmans et al. (2005)
Finland	No	—	
France	Yes	No	Weldon unpubl. data
Germany	No	—	
Hong Kong	No	—	
Hungary	No	—	
Israel	No	—	
Italy	No	—	
Japan	No	—	
Kuwait	No	—	
New Zealand	Yes	Yes	e.g. Waldman et al. (2001)
Nigeria	Yes	No	Weldon unpubl. data
Republic of China	No	—	
Republic of Korea	No	—	
Russia	No	—	
Scotland	No	—	
Singapore	No	—	
Spain	Yes	Yes	Bosch et al. (2001)
Sweden	No	—	
Switzerland	No	—	
Thailand	No	—	
The Netherlands	No	—	
United States	Yes	Yes	e.g. Carey et al. (1999)

Economic Consequences

Chytridiomycosis will have some economic effects on the *X. laevis* trade sector in South Africa should it be detected in stock animals. No immediate loss of stock animals should be encountered because *X. laevis* do not show any apparent clinical symptoms usually associated with chytridiomycosis, nor do the frogs die from the infection. All enclosures where frogs are kept would have to be disinfected and additional quarantine areas would have to be constructed (see section on recommendations for captive frog hygiene). During this period, there could be restrictions on the movement of frogs, both to and from facilities that could lead to reductions in income.

The economic consequences of chytridiomycosis to the importing countries could be applicable to three sectors, namely the customs division, scientific laboratories and fieldwork and monitoring.

- Customs practices that may involve costs include quarantine and isolation costs as well as diagnostic testing and treatment costs of chytridiomycosis.
- Scientific laboratories that stock in-house colonies of *X. laevis* may need to provide for costs involving diagnostic testing of all amphibian species that are kept at their facilities and control, disinfection or treatment costs.
- Costs involved with fieldwork as a result of chytridiomycosis may include disinfection and other costs required for preventing anthropogenic spread of the disease, costs for monitoring species at contaminated and adjacent sites, or eradication costs of feral *X. laevis* populations.

6.4.4 Risk Estimation

The risk estimation integrates the release assessment, exposure assessment and consequence assessment to produce a summation of the findings used to estimate risk.

General Considerations

Due to the nature of the hazard under investigation (a transmissible disease) the risk of an amphibian from an import country acquiring chytridiomycosis as a result of the risk process (*X. laevis* trade) can be traced back to the initial incidence of the disease at the start of the risk process (the collection of wild *X. laevis* in South Africa). It can therefore be said that the potential risk at the start of the pathway (wild *X. laevis* from contaminated sources, intended for export) is only realised during the event of an amphibian in the import country becoming exposed to chytridiomycosis. At the same time, the level of risk, once the hazard has reached this point (when transmission is almost certain to occur), is not dependant on the variance of

any preceding step (e.g. whether amphibians were exposed to feral *X. laevis* or to zoospores flushed with used aquarium water), but is a factor of the intrinsic characteristics of the amphibian species. It is therefore difficult to predict the level of risk unless data regarding host-pathogen relationship is available for the species in question. Thus the consequences of exposure will occur irrespective of the variation in steps preceding exposure. Therefore, a more informative measure of risk is the likelihood of transmission taking place, which is influenced by variation in the preceding steps of the release and exposure processes. A number of variances in model inputs or activity steps of the release and exposure routes exist that, when combined in a chronological fashion, serve as an estimate of the risk of transmission.

Dichotomous variation in release inputs that influence exposure risk:

Step 1

Likelihood of encountering *B. dendrobatidis* in the wild
- Collect from single source — **Possible**
- Collect from multiple sources — **Likely**

Step 2

Likelihood of *B. dendrobatidis* present in export batch
- Samples separate, select one for export — **Possible**
- Samples mixed, select export sub sample — **Likely**

Dichotomous variation in exposure inputs that influence exposure risk:

Step 3

Likelihood of *B. dendrobatidis* entering a country
- Disease dealt with through customs practices — **Unlikely**
- Disease not dealt with through customs — **Almost certain**

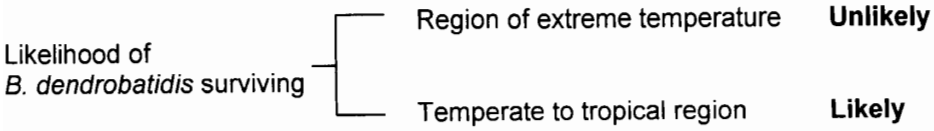
Step 4

Likelihood of *B. dendrobatidis* ending up in the environment
- *X. laevis* intended for laboratory animal — **Possible**
- *X. laevis* intended for pet animal — **Likely**

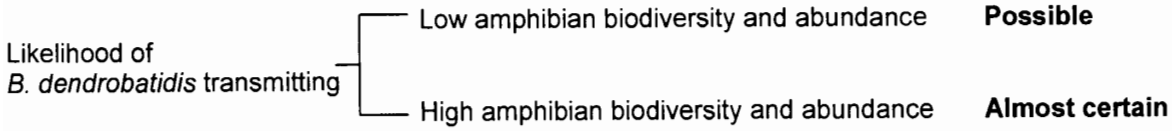
Step 5

Likelihood of *B. dendrobatidis* persisting until transmission
- Zoospores released — **Unlikely**
- Frogs released — **Almost certain**

Step 6



Step 7



Risk Allocation

A weight was assigned to each step of the risk process based on the likelihood of the step occurring. The following standard weight classes were assigned:

Likelihood	Weight
Unlikely	1
Possible	2
Likely	3
Almost certain	4

Risk levels are derived at by adding the assigned weights of each step that makes up the risk process. Using this scoring system, risks were assigned to one of three levels:

Risk level	Score range
Low risk	7-13
Moderate risk	14-20
High risk	21-28

Upper and lower levels of the risk score were derived at by calculating the maximum and minimum possible score. Thus the maximum score is *number of steps* x *maximum weight* or 7 x 4 = 28. Similarly the minimum score is *number of steps* x *minimum weight* or 7 x 1 = 7. This configuration of weighted steps gives 49 possible combinations or scenarios that all yield a risk score of between 11 and 24. A few exposure scenarios have been proposed, that when they occur, will pose risks of one of the three levels.

High risk

Scenario 1

Suppliers collect frogs from multiple sources and select frogs for export from a mixture of these and other frogs at the holding facilities. The frogs enter a country without any customs practices aimed at reducing the risk of importing chytridiomycosis. Frogs are destined for the pet trade and eventually end up in an environment with favourable conditions for both host and pathogen and that is rich in amphibians (risk score = 24).

Scenario 2

Suppliers collect frogs from a single source, but select frogs for export from a mixture of these and other frogs at the holding facilities. The frogs enter a country without any customs practices aimed at reducing the risk of importing chytridiomycosis. Frogs are destined for the pet trade and released zoospores end up in an environment with favourable conditions for both host and pathogen and that is rich in amphibians (risk score = 21).

Moderate risk

Scenario 3

Frogs are collected from a single source and batches are kept separate at the holding facilities of which one batch is selected for export. At the port of entry the frogs are subjected to customs practices aimed at reducing the risk of importing chytridiomycosis. Frogs are destined for a scientific laboratory and eventually end up in an environment with favourable conditions for both host and pathogen and that is rich in amphibians (risk score = 18)

Scenario 4

Suppliers collect frogs from multiple sources. At the holding facilities these frogs are not mixed with other frogs from previous collections, but are exported as a single batch. The frogs enter a country without any customs practices aimed at reducing the risk of importing chytridiomycosis. Frogs are destined for the pet trade and released zoospores end up in an unfavourable environment with low amphibians biodiversity and abundance (risk score = 15).

Low risk

Scenario 5

Suppliers collect frogs from multiple sources and select frogs for export from a mixture of these and other frogs at the holding facilities. At the port of entry the frogs are subjected to customs practices aimed at reducing the risk of importing chytridiomycosis. Frogs are destined for a scientific laboratory and released zoospores eventually end up in an unfavourable environment with low amphibians biodiversity and abundance (risk score = 13).

Scenario 6

Frogs are collected from a single source and batches are kept separate at the holding facilities of which one batch is selected for export. At the port of entry the frogs are subjected to customs practices aimed at reducing the risk of importing chytridiomycosis. Frogs are destined for a scientific laboratory and released zoospores eventually end up in an unfavourable environment with low amphibians biodiversity and abundance (risk score = 11).

6.5 Risk Evaluation

This risk assessment has shown that *X. laevis* can potentially spread chytridiomycosis through the scientific trade. Activities related to the trade are not solely responsible for local and international dissemination of the chytrid fungus but play a major part, especially via frogs being exported. The *X. laevis* trade does not pose a direct threat to other amphibians, but once the pathogen is transmitted, it becomes a threatening process that could severely affect on population and species survival. The likelihood of transmission to novel amphibian species is increased when *X. laevis* escape and establish feral populations. Although there is no evidence to link any specific case of chytridiomycosis associated population declines to feral *X. laevis*, transmission via this species remains a high risk. Because the trade in *X. laevis* plays such an important role in the development of scientific technologies, avoiding the risk of potentially introducing chytridiomycosis by banning the trade is simply not an option. Careful consideration of the likelihood of occurrences in the trade process and the consequences of the occurrences can generate actions to reduce the risks from occurring.

6.6 Recommendations to Reduce Risks

Because chytridiomycosis is a recently-emerged disease of amphibians of which aspects of the pathogenicity and epidemiology are not fully understood yet, any attempt at drafting disease management protocols should be based on the latest knowledge and be open to future improvements. A basic requirement is that the protocol should focus on taking precaution to disrupt the normal mode of transmission among amphibians and between amphibians and other vectors, including humans. The protocol should be adapted to any unique conditions of the involved institutions, regions or countries, while considering this requirement.

6.6.1 International Activities

Despite the wide occurrence of chytridiomycosis very few countries have developed official management protocols to control the disease. Australia, that has suffered many species and

population declines due to the chytridiomycosis pandemic, is at the forefront of developing amphibian diseases prevention strategies. The New South Wales National Parks and Wildlife Service has developed a hygiene protocol for the control of diseases in frogs (NSW NPWS 2000) and policy measures to control amphibian movements have been established by the Parks and Wildlife Commission of the Northern Territory (see Mazzoni et al. 2003). Australia has recently drafted a national Threat Abatement Plan aimed at preventing the spread of chytridiomycosis to chytrid-free regions and to decrease the effects on currently infected populations (Speare 2004). On a global level, the International Union for Conservation of Nature has published guidelines for amphibian translocations that include specific quarantine and testing recommendations for chytridiomycosis (Cunningham et al. 2001). A code of practice to prevent accidental transmission during fieldwork has been drafted by the Declining Amphibian Population Task Force (DAPTF) and is available from URL: http//ventura.fws.gov/es/protocols/dafta.pdf. In recent years available disease protocols, such as that of the DAPTF, have been tailored and implemented as standard procedure during amphibian monitoring studies (Waldman et al. 2001, Bell 2002, Dodd 2003). Mitigation protocols can only be successful when not only field workers comply with it, but also regulating bodies implement the strategies at policy level. Numerous authors have urged for more stringent veterinary surveillance and quarantine guidelines and for an improvement in the efforts to curtail the legal and illegal trade in amphibians (Mazzoni et al. 2003, Bell et al. 2004, Daszak et al. 2004, Hanselmann et al. 2004). Besides obvious advantages for conservation, Leung et al. (2002) determined that an approach at preventing biodiversity loss could be less costly and more effective than future control and eradication measures.

6.6.2 Treatment Options for the *Xenopus laevis* Trade

Due to the wide occurrence of the amphibian chytrid fungus in wild *X. laevis* populations, a strict pathogen hygiene protocol is essential to controlling the spread of the disease through the frog trade. The movement of *B. dendrobatidis* through the pathway can be interrupted if it is somehow eradicated during a specific step through deliberate human intervention or a natural occurrence, such as a change in ambient temperature outside the tolerance range of the fungus. It has been demonstrated in captive Australian red-eyed tree frogs, *Litoria chloris* that an elevated temperature of 37°C is effective against *B. dendrobatidis* (Woodhams et al. 2003). Although the risk of spread is possible throughout all the steps that are involved in the *X. laevis* trade, the same likelihood of spread is not shared among the different steps. Because the initial steps in the pathway generate the risk - the collection from wild populations and husbandry practices at supplier facilities - successful control of the disease within the country will greatly reduce the risk of infected frogs being exported. To improve managing this process the protocol should function around controllable units based on the steps that structure the

trade pathway. These control units include the handling of frogs in the field, husbandry practices at the supplier facilities and import/export regulations. A further consideration in the selection of risk reduction options is that the cost of implementing each option should justify the extent of risk reduction gained from it.

Field Hygiene

Many of these basic procedures are contained in the already existing management protocols mentioned in section 6.6.1 and have been tailored to meet the requirements of the *X. laevis* trade. When first visiting a site or when moving between sites, it is important to prevent the transfer of the disease organism from footwear and equipment. An effective chemical disinfectant that kills the chytrid fungus quickly is sodium hypochlorite, the active ingredient in household bleach. Footwear should first be rinsed in water and scraped clean of all mud and debris before being transferred to a disinfection bath containing the sodium hypochlorite. Rinsing with water first will prolong the life of the disinfection solution because organic matter accelerates breakdown of the solution (Johnson et al. 2003). Soak footwear for 2 minutes in a 2% sodium hypochlorite solution while scrubbing with a coarse brush. Other equipment such as nets and traps should be disinfected by either emersion or scrubbing with sodium hypochlorite. Complete the routine by rinsing in running water. When no disinfection solution is available or when only one site is visited at a time, equipment can be dried for a long enough period to allow for complete evaporation of water because the chytrid fungus does not survive desiccation (Johnson et al. 2003). Never handle live, sick or dead frogs with bare hands. Disposable gloves should be worn when handling the frogs, to prevent the risk of spreading the disease through direct contact. Following these basic field hygiene procedures will prevent human transfer of chytridiomycosis between sites.

Captive Frog Hygiene

Suppliers should follow a four-step approach to ensure that the frogs are rid of a potential infection and that they remain uninfected.

- Step 1: Prior to housing frogs, all tanks, aquaria and equipment should be disinfected with sodium hypochlorite. Alternatively equipment, etc. can be immersed in hot water (at least 50°C), because *B. dendrobatidis* growth in broth cultures is arrested at 28°C and prolonged temperatures of 30°C can be lethal (Longcore et al. 1999, Johnson et al. 2003, Piotrowski et al. 2004).
- Step 2: Upon arrival at captive facilities, frogs should be kept in a quarantine room and prophylactic treatment for chytridiomycosis administered. Disposable gloves should be worn when servicing quarantine animals. A formalin/malachite green solution has

effectively been used to cure infected *Xenopus tropicalis* (Parker et al. 2002). The frogs are treated for 24 hours every other day for four treatments in baths made up of 25 ppm formalin and 0.10 mg/L malachite green (Parker et al. 2002). In between baths, frogs should be kept in treated enclosures with clean water. Up to 1,000 frogs can be treated for ZAR 0.50, an almost negligible cost to ensure that disease-free animals leave the facility. The breakdown of the costs involved for treating *X. laevis* was calculated, assuming that you can treat two frogs per litre of solution, as follows:

FORMALIN	
Concentration	**Cost (ZAR)**
100% (1L) or 1,000,000 ppm	8.60
0.0025% (1L) or 25 ppm	0.000215
Cost per 1,000 frogs	0.1075

MALACHITE GREEN	
Concentration	**Cost (ZAR)**
100 g	37.00
0.1 mg (makes 1L of solution)	0.000037
Cost per 1,000 frogs	0.0185

TOTAL COST	
One treatment of 1,000 frogs	**0.126**
Four treatments of 1,000 frogs	**0.504**

- Step 3: Treated frogs can now be transferred to disinfected enclosures.
- Step 4: To prevent wastewater from possibly exposing the environment to chytridiomycosis, water drained from all enclosures should be collected and either treated with sodium hypochlorite (100 mg/L for 30 minutes) or heated above 50°C before disposal (Johnson et al. 2003).

The following general precaution measures should also be taken: place footbaths with disinfectant at all entrances where frogs are kept, use and keep dedicated equipment inside the different enclosure rooms, and prevent aquaria and tanks from sharing water or the same filtration system. Following the four-step approach and complying with the general precautions will increase the pathogen hygiene efficiency that will minimise the risk of infected frogs from reaching the end-user.

Trade Regulations

As part of the function of the Veterinary Services of the Provincial Government of South Africa involves monitoring animal disease risks and preventing the spread of diseases, their diagnostic services should be expanded to recognise chytridiomycosis in *X. laevis* intended for the trade. A monitoring program should be adopted whereby a sample from the export batch (e.g. 10%) is examined for the presence of *B. dendrobatidis* before export using the sensitive PCR technique. If the suspect organism is detected, the involved supplier should share the responsibility for treating the entire batch for the condition.

Each importing country has to evaluate their system of veterinary services and surveillance programmes to recognise *B. dendrobatidis* at customs checkpoints to prevent it from infecting native amphibian populations. Customs houses that require that amphibians be kept in quarantine after arrival should be informed that observation of hosts alone can not be applied to *X. laevis* with regards to chytridiomycosis, because of the sub-clinical infection in this species, but should be supplemented with diagnostic testing of the disease. Frogs that die during transport should be submitted for necropsy and examined for evidence of diseases before healthy frogs can be released from quarantine. Importers need to be fully informed not only about the consequences of the invasion of foreign amphibian species, but of the risks that introduced pathogens could pose to the local amphibian fauna.

6.7 Conclusion

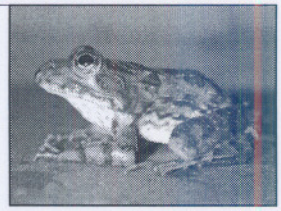
Through this risk assessment, a risk to amphibian populations has been identified and analysed in terms of the range of potential consequences and the likelihood of these consequences occurring. The likelihood was further analysed to produce an estimated level of risk and options have been proposed to reduce these risks. A risk management system for the export of *X. laevis* needs to be established that will implement and maintain the system while considering the recommendations provided in this risk analysis. Effective functioning of the risk management system would involve communication through the interactive exchange of information on risk among risk assessors, risk managers and other interested parties. Conveying the findings of this risk analysis to the Western Cape Nature Conservation Board (WCNCB) has set this process in motion. The WCNCB, which is responsible for the issuing of collection permits for *X. laevis* in the Western Cape Province and thus regulate the export of this commodity from South Africa, have agreed to further the process of implementing a management strategy at policy level.

Given the context that the amphibian chytrid is already widespread in South Africa especially through infected *X. laevis*, a concern that may arise is what effect mitigation, if implemented now, will have on preventing, as yet, unaffected species and populations from transmitting the disease. We have determined that this country plays a key role in the international spread of *B. dendrobatidis*. Consequently we, as a country, have an obligation to protect amphibians outside of our borders from acquiring chytridiomycosis by intervening in this risk process. Association with the OIE denotes that South Africa must comply with the requirements of this organization regarding chytridiomycosis by adopting measures that control the movement of amphibians. With mitigation actions in place it is possible to prevent future epidemics of as yet unidentified diseases.



Chapter 7

QUANTITATIVE MEASUREMENT OF *Batrachochytrium dendrobatidis* INFECTION IN LIVE FROGS



7.1 Introduction

The amphibian chytrid fungus, *Batrachochytrium dendrobatidis* grows and completes its development in the keratinised layer of the epidermis, the *stratum corneum* of metamorphosed frogs and in the keratinised mouthparts of tadpoles. On the anuran body, the fungus is concentrated in the region of the feet, hind legs and pelvic patch. These areas are the least susceptible to dehydration and in direct contact with the substrate or water and therefore maximally exposed to potential transmission of the disease organism. The infective stage is a motile zoospore that can either re-infect the same individual or transmit to other hosts when released into water. After locating a susceptible animal, zoospores penetrate the skin where sporangia develop in the *stratum granulosum*, the layer of the epidermis just below the top layer, the *stratum corneum*, and release their zoospores through discharge papillae when they reach the surface through the natural process of skin regeneration. A short lifecycle of 4-5 days when grown in pure culture (Longcore et al. 1999) ensures that the infection spreads rapidly on and between amphibians as long as moist or wet conditions prevail.

Various microscopic techniques are used to identify chytridiomycosis (Berger et al. 1999b, Longcore 2001). Diagnostic tests specific for *B. dendrobatidis* that rely on antigen function and molecular properties have also been developed (Berger et al. 2002, Annis et al. 2004). However, except for the real-time PCR Taqman assay (Boyle et al. 2004), none of the methods serve as a tool to quantify infection. The ability to quantify an infection has application in comparative experimental and basic research in the fields of pathology, immunology and epidemiology. The real-time PCR assay has many advantages because it operates with a high degree of sensitivity for diagnostic purposes in skin samples with the ability to quantify the infection down to a single zoospore (Boyle et al. 2004). It does not, however, provide quantitative information on the pattern of spread on the host and is not readily available due to the level of technology it involves. Various authors have made incidental comments on the intensity of infection while describing the histologically observed microscopic lesions of infections from case reports of mortality events and the outcome of experimental transmission. In these references, infection intensity has been described in terms of degree of chytrid

numbers (Nichols et al. 2001), percentage of skin surface infected on histological sections (Daszak et al. 2004, Hanselmann et al. 2004) and layers of infected keratinaceous cells (Burrowes et al. 2004, Daszak et al. 2004, Hanselmann et al. 2004). It becomes complicated to draw comparisons between species or experimental groups when various methods are in use of which the paired relationships have not been determined. Furthermore, the variability of *B. dendrobatidis* infection among toe clips, where not all toes of the same individual are infected, can often lead to false negatives in the data set (Boyle et al. 2004, Retallick et al. 2004) resulting in an under-representation of prevalence in populations. This variability becomes more apparent in animals with low levels of infection, thereby increasing the chances of overlooking the suspect organism. In these cases a need therefore exists for a standardised technique by which *B. dendrobatidis* infection can be quantified not just for comparative research, but also for providing accurate information that will increase the confidence of detecting infection through histological surveys. Such a technique should be accessible to most workers in the field and should preferably not require advanced skills beyond accurately diagnosing chytridiomycosis.

In this chapter the candidate presents a quantification technique for *B. dendrobatidis* in apparently healthy and moribund frogs that makes use of one of the most commonly noted clinical symptoms of chytridiomycosis, namely the tendency to slough skin more frequently. Excessive sloughing may be a physiological response to the irritation caused by the infection. Sloughed skin contains clusters of chytrid thalli, the abundance and distribution of which correspond to the section of skin on the donor frog. By utilising this sloughed skin the infection can be quantified by determining sporangium density across the foot. An imprint of the foot is left in skin sloughs from which the different components of the foot (toes, webbing etc.) can be distinguished from each other. The procedures for quantification are demonstrated on the Cape river frog, *Afrana fuscigula*, which according to Channing (2004) is a common inhabitant of rivers, streams and dams in the Western Cape, Eastern Cape and parts of the Northern Cape provinces as well as the central plateau of South Africa . In addition, the morphology of the hind feet was used to characterise the micro-spatial distribution of the infection. The feet of *A. fuscigula* are extensively webbed, pointed toes contain subarticular tubercles, and an inner metatarsal tubercle is present on the base of the foot (Figure 7-1). This chapter yields three sets of information: 1) a step-by-step approach to quantify infection with *B. dendrobatidis*, 2) a characterisation of *B. dendrobatidis* infection in *A. fuscigula* and 3) application of the quantification technique in the selection of tissue types for histological diagnosis of chytridiomycosis.

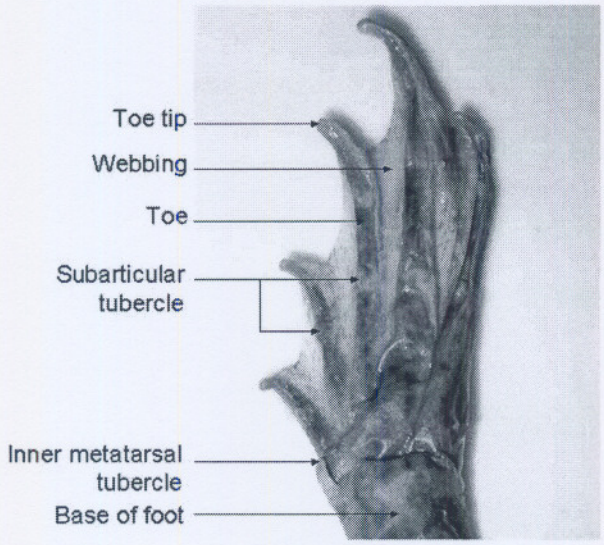


Figure 7-1 Ventral view of the external morphology of the hind foot of *Afrana fuscigula*.

7.2 Material and Methods

7.2.1 Source and Collection of Specimens

During May 2004 a disease survey of frogs was carried out in the semi-desert Namaqualand region in the Northern Cape Province that forms part of the Succulent Karoo biome. A large breeding population of *A. fuscigula* were found inhabiting a shallow stream on private land on the plateau of the Kammiesberg (Alt. 1,100 m). The perennial stream is fed by seepage from a pond, built in a riverbed that only receives water during a short rain season in winter. Water from the stream collects in a second smaller pond where more frogs were found along its well-vegetated banks. No moribund or sick frogs were observed and apparently healthy adults were collected and placed in separate containers that were transported in large cooler boxes. Frogs were individually housed in an air-conditioned room (20°C) in rectangular transparent plastic containers (220 mm long, 120 mm wide, 170 mm high) with a lid in which air holes were drilled. The containers were fitted with a tailored base that divided the substrate into a wet and an elevated dry zone.

7.2.2 Technique for Quantifying Sporangia in Sloughs of Feet

The technique used to quantify sporangia in sloughs of feet was developed by the candidate and is described in detail below. The candidate has presented the methodology for the technique in a manner that will allow easy replication by others.

Equipment Required

(1) Plastic containers (600 ml bottles work well)

(2) Dropper bottle
(3) Distilled water
(4) Microscope slides and cover slips (22 X 40 mm)
(5) Sharp needles (X2)
(6) Dissecting microscope
(7) Compound microscope with eyepiece graticule
(8) Stage micrometer
(9) Mechanical counter

Preparation of Skin Samples

Each frog is placed in a separate container with 100 ml of distilled water and left for 24 hours in a temperature controlled room (20°C) to allow sufficient time for pieces of skin to slough. Slides for microscopic examination are prepared as follows:

(1) Remove skin sloughs of the feet from the water (identified by their shape and the presence of dense impressions from the tubercles) and transfer to a drop of water in the centre of a microscope slide.
(2) While observing with a dissecting microscope (20 X), use two sharp needles to spread out the skin evenly over the slide with the tubercles facing upward.
(3) Add a cover slip and observe with a compound microscope (100 and 400 X).

Counting of Zoosporangia

(1) Select the 40 X objective of the compound microscope and place the stage micrometer in position on the mechanical stage.
(2) Measure the diameter of the microscopic field of view.
(3) Calculate the area of the microscopic field by using the equation: πr^2.
(4) Align the prepared slide so that the longest section of skin moves along the x-axis of the microscope stage (Figure 7-2).
(5) Starting at one end and working your way along the designated axis, count the sporangia within each field with the aid of a mechanical counter.
(6) Note down the number of sporangia in each field. Note that because the fields do not overlap, but touches at the edges, two roughly triangular areas (with concave sides) are omitted between adjacent fields.
(7) Align the slide so that the widest section of skin moves along the Y-axis of the microscope stage (Figure 7-2) and repeat steps 5-6.
(8) Calculate the mean number of sporangia observed per field of view. Knowing the field area, the "per field" number can now be converted to sporangia/mm^2.

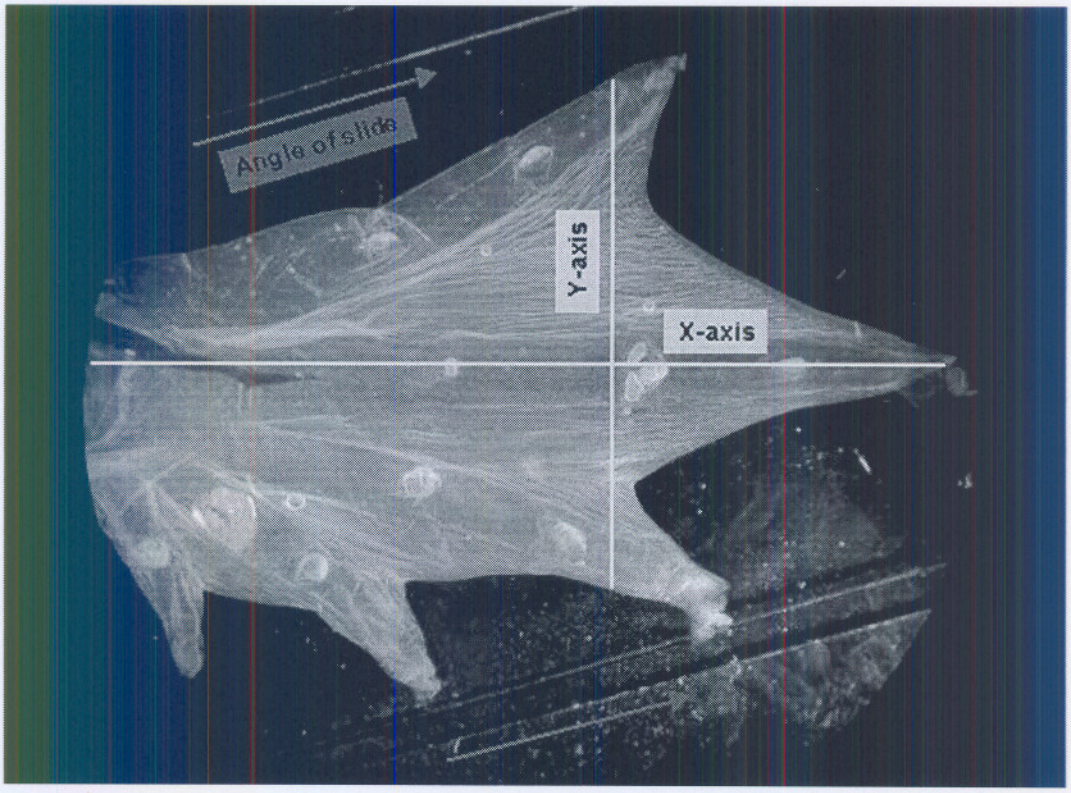


Figure 7-2 Wet mount of sloughed skin from the foot of *Afrana fuscigula*. The perpendicular lines indicate the longest (X-axis) and widest (Y-axis) sections of the skin along which sporangia are counted within microscopic fields.

7.2.3 Additional Count Data and Measurements

Sporangium density was also calculated for different sites on the feet namely toes, toe tips, tubercles, webbing and base of the foot. The sites are easily distinguished from one another in sloughed skin by their shape, texture and relative position to one another that make out the shape of the foot (Figure 7-3). Because of the size limit of most of these components it is not possible to count the fields in an X/Y configuration, more often fields were counted in a single straight line.

Dimensions of sporangia and cluster size were measured with an eyepiece graticule and expressed as mean ± standard deviation (SD). A grouping of sporangia was defined as a cluster when the area of uninfected skin between such groupings was wider than the diameter of the larger of two adjacent groupings. Only groupings of 10 sporangia and more were measured to determine cluster size. In addition, cluster density (mean no. of clusters per square millimetre) was also determined.

Specimens were screened for a second time after a week in captivity to confirm infection status and again after four weeks, but no quantification data was taken.

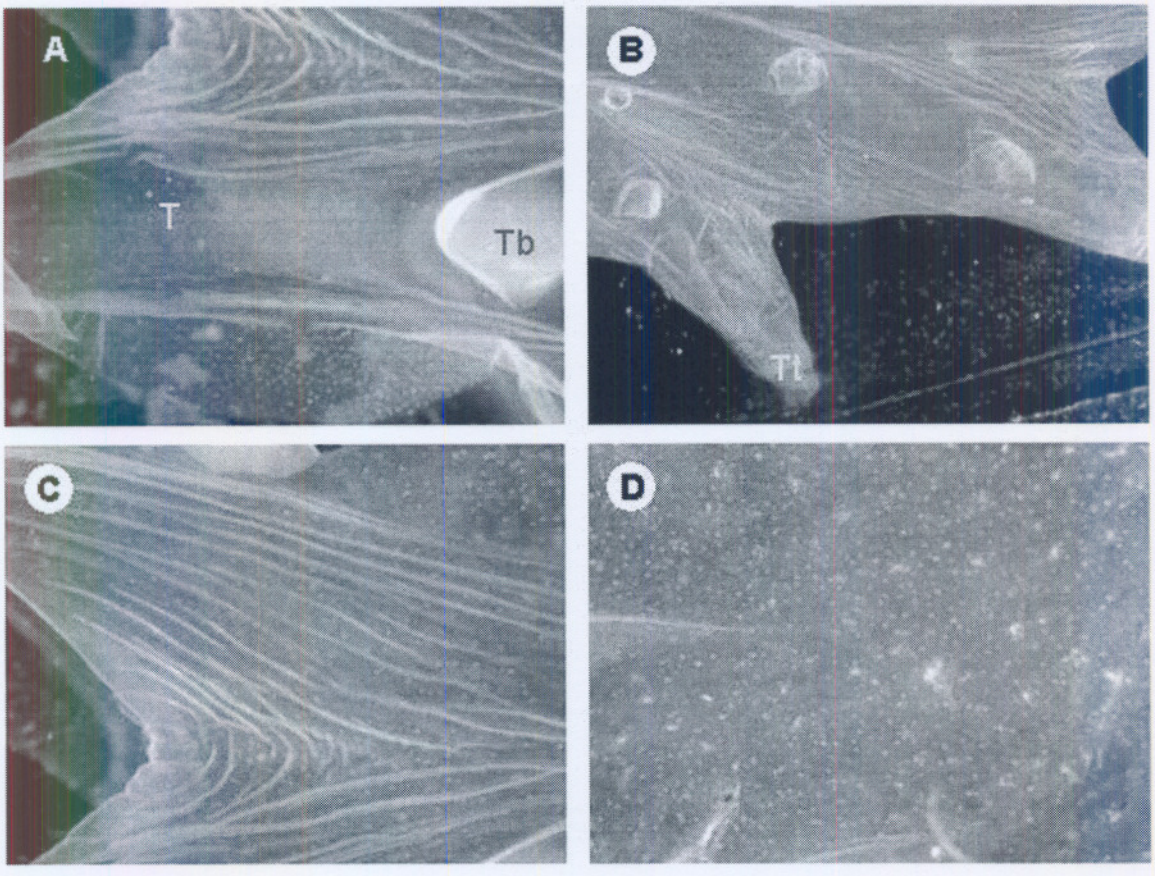


Figure 7-3 Light micrographs of imprints on sloughed skin left by the components of the anuran foot. These components are distinguished from one another by their shape, texture and position. A) Toes have a smooth appearance and are interrupted with one or more oval-shaped, densely-tissued tubercles, T = toe, Tb = tubercle, B) Toe tips are situated at the distal end of toes and consist of coarser tissue Tt = toe tip, C) webbing is found in between toes and has numerous small folds, D) the base of foot consists of smooth, thin tissue.

7.2.4 Statistical Analysis

All statistical analyses were computed using the Statistica 7 software package. The Shapiro Wilk test was used to test for normality of the count data (sporangia per field). Transformations were performed on the count data by using log, square root and square transformations. Kruskal-Wallace tests were used to analyse bivariate associations between the density of *B. dendrobatidis* on the toes, toe tips, tubercles, webbing and foot base.

The Poisson frequency function was used to approximate binomial probabilities of detecting *B. dendrobatidis* on a 3 x 3 mm piece of skin when using histology as diagnostic tool. The calculations make use of the principle that sporangia are grouped together in clusters, that all sections are of equal size (6 µm), and are based on the assumption that the data follows a

Poisson distribution. The Poisson frequency function is given by the following formula taken from Rice (1995):

$$N \geq \frac{-1}{\lambda} \ln(e)$$

where N = number of sections

λ = no. of colonies / total no. of sections

$e = 1 - \%$ probability

7.3 Results

7.3.1 Identification and Infection Rate

Infected skin is distinguished from healthy skin by the presence of clusters of sporangia that disrupt the mosaic pattern of squamous epithelium cells (100 X). Upon closer examination (400 X) a crust of embedded and exposed sporangia within undefined epidermal tissue becomes visible (Figure 7-4). The undefined appearance of the epidermis (coarse, lacking cell wall) in the vicinity of the infection is the result of a hyperkeratotic reaction. Larger clusters often have an area near the centre that lack sporangia, but retains the hyperkeratotic tissue. In smaller clusters especially, infected epithelial cells have a flaky appearance because of partial or complete detachment. Small clusters from the base of the foot are often associated with modified epidermis cells that function to increase the grip, almost like warts. Sloughed skin contained sporangia of *B. dendrobatidis* in various stages of development within the same cluster especially young sporangia, and mature sporangia with colonial morphology, zoospores and discharge papilla (Figure 7-5). Fewer empty sporangia were observed. Zoosporangium size (mean ± SD) was measured at 6.9 ± 1.6 µm (n = 113) and reached a maximum size of 11.7 µm.

Prevalence was high at 61% (n = 25/41) at the time of capture. Apart from excessive sloughing of the skin, no other clinical symptoms usually associated with chytridiomycosis were observed at the time. After one month in captivity all experimental animals were still alive and *B. dendrobatidis* could only be detected in 9.8% (n = 4/41) of frogs. All accept one of the 16 frogs that initially tested negative for chytridiomycosis, remained infection-free.

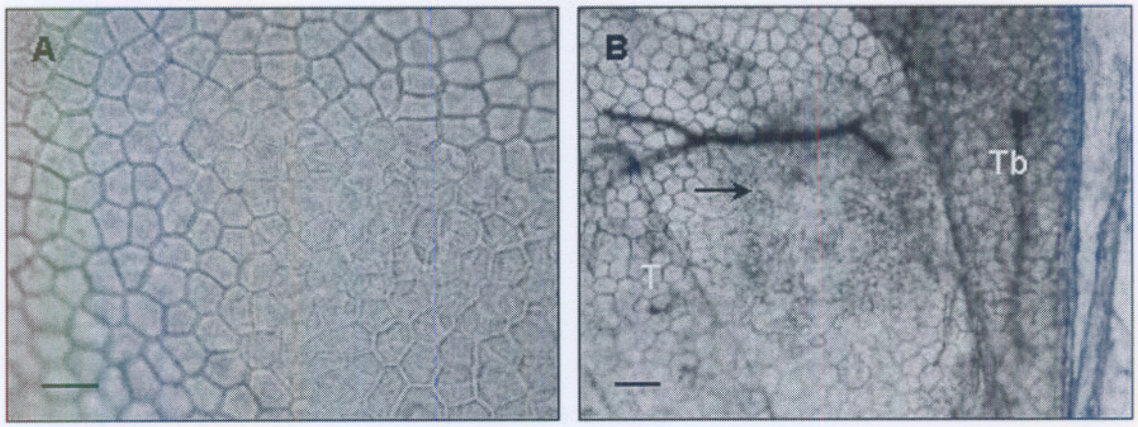


Figure 7-4 Light micrographs of toe skin. A) On the surface of healthy skin the squamous epithelium forms a continuous layer of cells (400 X, scale bar = 20 µm). B) When infected with chytridiomycosis, the squamous pattern of the epithelium is disrupted by clusters of sporangia that appear as darker patches with circular thalli, such as this donut-shaped cluster indicated by the arrow. T = toe, Tb = tubercle (100 X, scale bar = 30 µm).

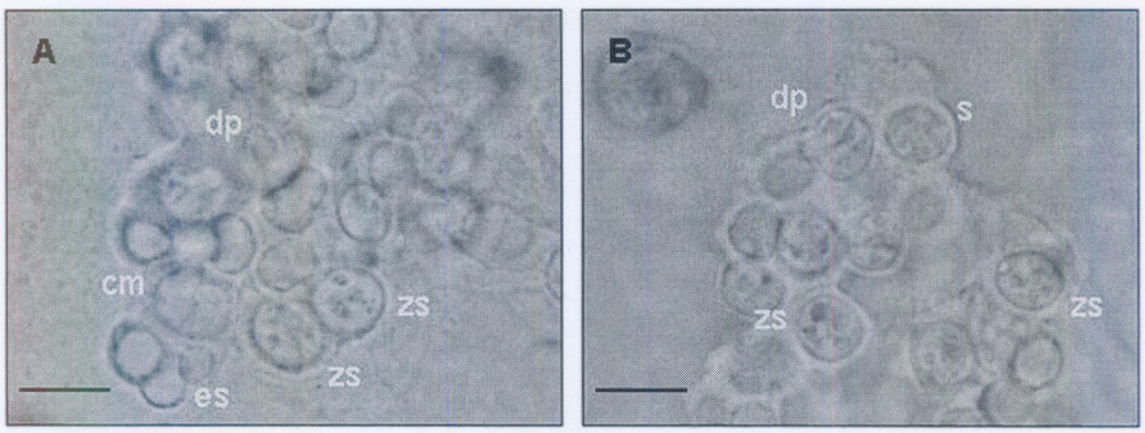


Figure 7-5 Light micrographs indicate that various developmental stages of *Batrachochytrium dendrobatidis* are visible in sloughed skin. A, B) cm = colonial morphology, dp = discharge papilla, es = empty sporangia, s = young sporangium, zs = sporangium with zoospores (600 X, scale bar = 10 µm).

7.3.2 Zoosporangium Density

Only skin sloughs that had intact toes, webbing and base of the foot were considered for the counting of sporangia. Adequate skin sloughs for quantification were attained from 13 infected specimens. The sporangium density between specimens ranged from 1.0 – 533.4/mm^2 with a mean of 55.2 /mm^2. Even larger differences in sporangia per field were counted, from zero to a

minimum of 3.71/mm^2 to a maximum of 2,228/mm^2. These values were calculated from 575 fields of which 18.8% had sporangia, totalling 8,536 sporangia. The majority of fields with sporangia (74.1%) were adjoined on either one or both sides by fields in which sporangia were detected, resulting in a series of mostly empty fields interrupted by clumped fields with sporangia and less frequently by only single fields with sporangia. Cluster size is highly variable and is not only determined by the number of sporangia but also by the density of the sporangia. As an indication of this variation, the mean size for clusters of 10 sporangia was 572.4 ± 203.4 µm^2. A cluster of 135 sporangia covered an area of 0.043 mm^2 and a cluster of 177 measured just 0.009 mm^2. The shape of small clusters usually conforms to a circular or elliptical shape but clusters become more variable with increased cluster size.

7.3.3 Zoosporangium Density per Site

The normality curve was skewed to the right (Figure 7-6), primarily because of two exceptionally high observations (dense sporangia) on the tubercles and a large component of observations without any sporangia. None of the transformations produced a normal distribution and hence the count data were analysed with non-parametric tests. Overall, sporangium density across the different sites on the foot differed significantly from each other ($p = 0.001$), indicating that sporangia are not evenly spread over the ventral surface of the foot. Multiple paired comparisons of the mean ranks of sporangium density from all sites indicated that a significant difference ($p = 0.0091$) exists between the density on toe tips and on webbing (Table 7-1). The pattern of sporangium density that emerged from the comparison of the density variables of the sites indicates that density decreases from the distal end of the foot (toe tip) to the proximal end (foot base) and from the elevated region (tubercles) to the lower region of the webbing (Figure 7-7). Infection of the foot can be divided into three categories according to sporangia density (Table 7-2), namely a high-density site (toe tips, 160.8/mm^2) moderate-density sites that include tubercles (58.4/mm^2) and toes (56.4/mm^2), and low-density sites that include the webbing and foot base (7.4 and 5.9/mm^2 respectively). Toe tips and tubercles have the smallest surface area and are often completely covered in sporangia, when infected. In contrast, sites that have large areas such as the foot base and webbing (relatively extensive in *A. fuscigula*) have only isolated clusters of sporangia. In only 16.7% of infected specimens were all five sites infected. Most often (41.7%) the infection was restricted to two sites only. Differences also occurred in terms of the frequency with which sites were infected (Figure 7-8). The most frequently infected site was toes (62.1% of specimens); twice as often than what the tubercles and the base of the foot were infected and more than three times as often as webbing.

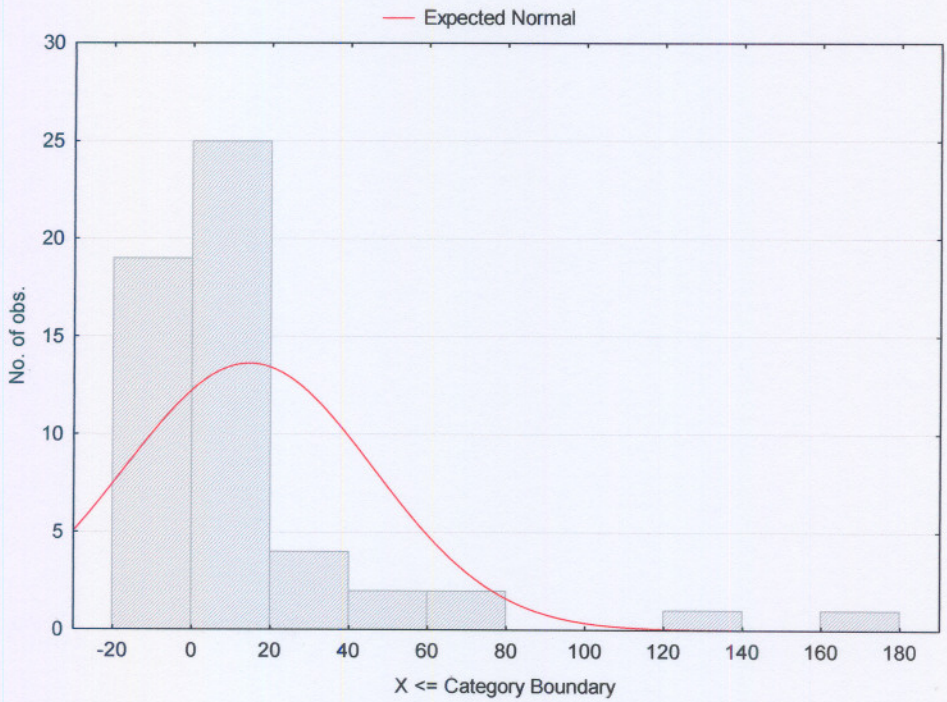


Figure 7-6 Normality curve of the "sporangia per field data" used to determine sporangium density on the foot of *Afrana fuscigula*. The curve is skewed to the right and therefore the data set was treated as non-parametric data (Shapiro Wilk, $p < 0.05$).

Table 7-1 Two-sided significant levels associated with comparisons of *Batrachochytrium dendrobatidis* intensity between each of the sites on the foot of *Afrana fuscigula*. A significant difference ($p = 0.0387$) exists between the density on toe tips and on webbing (indicated in red).

Kruskal-Wallis test: H (4, N= 54) =13.49051 p =.0091

Multiple Comparisons p values (2-tailed)

	Toe tip R:32.778	Tubercle R:31.083	Toe R:36.333	Web R:17.364	Base R:19.000
Toe tip		1.000000	1.000000	0.292657	0.566427
Tubercle	1.000000		1.000000	0.366897	0.728422
Toe	1.000000	1.000000		0.038689	0.100763
Web	0.292657	0.366897	0.038689		1.000000
Base	0.566427	0.728422	0.100763	1.000000	

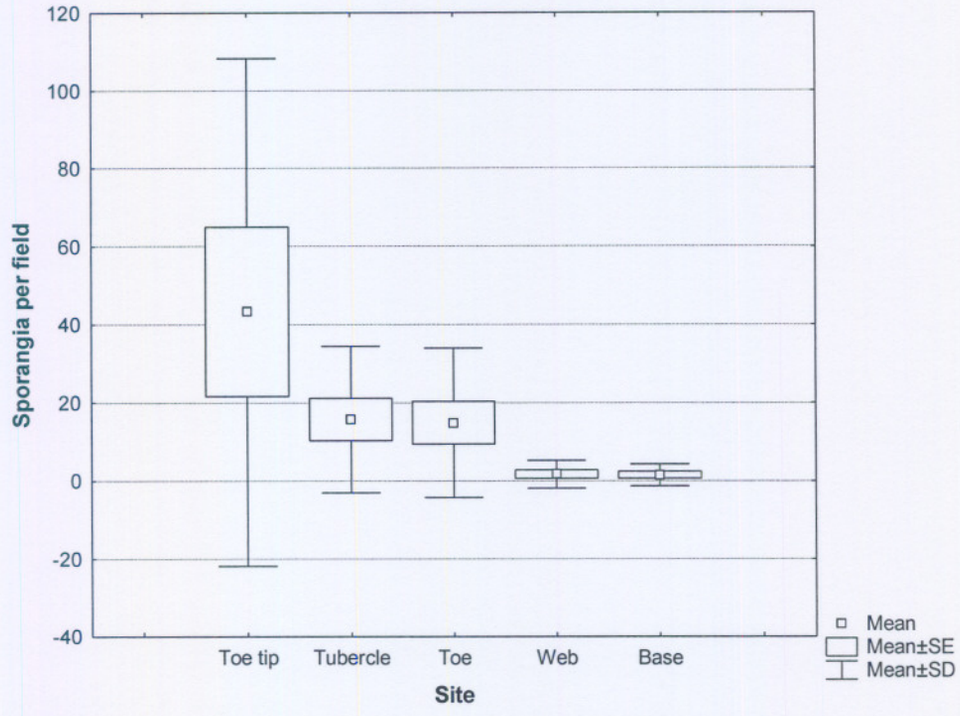


Figure 7-7 Mean, standard error and standard deviation graph of the distributions of *Batrachochytrium dendrobatidis* sporangium density data from different sites of the foot.

Table 7-2 Comparison of *Batrachochytrium dendrobatidis* sporangium density on the different sites of the frog foot and the number of fields from which sporangia/mm^2 were calculated.

Site	No. examined	No. fields (average per site)	Sporangium density (mean ± SD)
Toe tips	26	39 (1.5)	160.8 ± 241.8
Tubercles	63	102 (1.6)	58.4 ± 69.5
Toes	29	353 (12.2)	56.4 ± 70.7
Webbing	28	199 (7.2)	7.4 ± 13.3
Foot base	22	206 (9.4)	5.9 ± 10.4

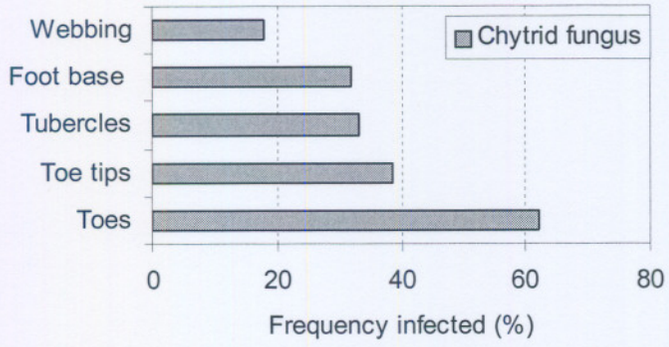


Figure 7-8 Frequency with which the different sites on the frog foot are infected with *Batrachochytrium dendrobatidis*.

7.3.4 Probability of Detecting Infection through Histology

The occurrence of chytrid clusters on the skin fit the Poisson distribution when the following two assumptions are made. Firstly, although a variation in cluster size exists that may influence detection probability, the total surface area covered by clusters in relation to the surface area of the skin being sectioned is negligibly small. The mean total surface area covered by clusters varies between 0.2 and 0.6% of the skin being sectioned (Table 7-3); an equivalent of 1–3 sections out of 500 possible sections. Secondly, that the clusters are independent and that there is no reason for clusters to group together, but that they will be randomly distributed on the skin. Mean cluster density for a 9 mm^2 skin sample increased in the same order between sites as did sporangium density, namely foot base (1.57 clusters), webbing (2.22 clusters), toe (8.61 clusters), tubercle (10.88 clusters) and toe tip (12.0 clusters). Because cluster density varies between sites on the foot, the random distribution of clusters applies to distribution within the individual sites rather than to the whole foot. Because the Poisson frequency function is dependant on cluster density, the number of sections required for each level of probability increases proportionately between sites of decreasing cluster density (Table 7-4). To detect *B. dendrobatidis* with 95% probability in an *A. fuscigula* from this study would require 125 sections through a toe tip sample, 138 sections through a tubercle sample and 174 sections when sectioning toe tissue. It is important to realise that these numbers of sections do not imply the number needed to detect *B. dendrobatidis* and should be interpreted as follows: there is a 95% probability that *B. dendrobatidis* will be detected when 125 sections are made through a toe tip sample from this study etc. Therefore *B. dendrobatidis* could be detected in the first, the 125th or on any section in between. When sectioning webbing of *A. fuscigula*, the detection of a 90% probability or more would require

more than 500 sections and therefore a larger tissue sample. The cut-off probability on a 9 mm^2 foot base sample is closer to 80%. A line graph of these values clearly illustrates the differences in work effort required between skin samples of varying cluster density (Figure 7-9). The graph also facilitates determining the number of sections required for any given probability level of detection.

Table 7-3 Values and formulas used to calculate the surface area on a 3 x 3 mm piece of skin that is infected with *Batrachochytrium dendrobatidis*.

Site	Density (sporangia/mm^2)	Area (μm^2)	Area (9 mm^2)	% Cover
	x	$y = \pi r^2 (x)$	$z = 9y(10^{-6})$	$z/9\%$
Foot	55.2	2065	0.019	0.21
Toe tip	160.8	6015	0.054	0.60
Tubercle	85.4	3195	0.029	0.32
Toe	56.4	2110	0.019	0.21
Webbing	7.4	277	0.002	0.03
Foot base	5.9	221	0.002	0.02

Table 7-4 Probability matrix for detecting *Batrachochytrium dendrobatidis* through a given number of histological sections on a 3 x 3 mm piece of skin derived from the Poisson frequency function. Differences in the required number of sections for a given probability level are accounted for by the mean number of colonies. Values indicated in red exceed the maximum number of sections that can be made on a 9 mm^2 skin sample (N_{max} = 500).

Probability	No. of sections				
	Toe tip	Tubercle	Toe	Webbing	Foot base
0.1	4	5	6	24	34
0.2	9	10	13	50	71
0.3	15	16	21	80	113
0.4	21	23	30	115	163
0.5	29	32	40	156	221
0.6	38	42	53	206	292
0.7	50	55	70	271	383
0.8	67	74	93	362	512
0.9	96	106	134	519	733
0.95	125	138	174	675	954
0.99	192	212	267	1037	1467

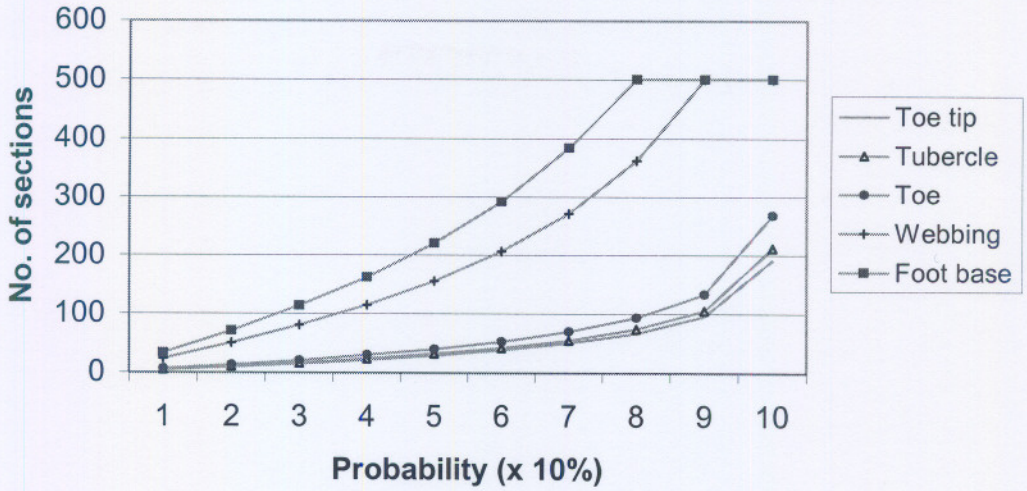


Figure 7-9 Line graph illustrating the relationship between probability level of detection and number of sections required for different cluster densities of *Batrachochytrium dendrobatidis*.

7.4 Discussion

7.4.1 Direct Microscopic Count as Quantification Technique

The case study of *A. fuscigula* has demonstrated that it is possible to reliably quantify *B. dendrobatidis* infection in apparently healthy frogs, a technique that can be used also on moribund frogs. Quantification data from within population differences can be applied to comparative susceptibility, transmission and pathogenicity studies between species. The method for the collection of skin limits the application of the technique, because it has to be used with caution for terrestrial frogs that are known to drown easily (e.g. members of the Microhylidae family). Although the method has not been tested on urodelids, it is expected to be applicable to this group due to the morphological similarities of the feet to anurans and the ability to maintain the animals in water that will allow the collection of skin sloughs. In fully aquatic amphibians the infection is not necessarily limited to the hind legs and ventrum, but may include the dorsal surfaces such as with *Xenopus* (clawed frogs) (Parker et al. 2002). Infections of 100% have been detected in *X. laevis* populations from examining only interdigital webbing (Weldon unpublished data) suggesting that the infection is evenly distributed across the foot or that the infection is very high, or both. If there exists no significant difference in distribution across the foot in aquatic frogs, site selection is not important when collecting tissue for histological diagnostic techniques such as conventional histology (e.g. haematoxylin and eosin stain) and the chytrid specific immunoperoxidase test (Berger et al. 2002).

As a diagnostic tool, using whole pieces of skin slough instead of skin scrapings has advantages regarding the ease of the screening, in that skin scrapings often contain pigmented cells that make it more difficult to distinguish *B. dendrobatidis* sporangia. Furthermore, sloughed skin is usually only 1-2 cell layers thick, which makes focusing through the tissue layer easier, whereas skin scrapings can consist of multiple tissue layers. The advantage of maintaining the anatomical relationships when examining sloughed skin is that the observer can easily establish which layer is being observed by orientation from the surface. This is important for locating sporangia that are often restricted to the top two layers. In skin scrapings this orientation is lost and deciding whether suspicious structures are at the surface or within deeper layers, is often difficult, owing to the disruption of the relationship of the layers. On the other hand, skin scrapings are favoured when attempts are made at isolating *B. dendrobatidis* because sloughed skin is usually more contaminated with bacteria and other fungi.

7.4.2 Infection in *Afrana fuscigula*

Despite high prevalence and relatively heavy infections in some of the *A. fuscigula*, the infected frogs did not appear to be adversely affected by the infection and 84% were able to clear the infection after a month in captivity. The change from the colder winter temperatures to room temperature may have been a causing factor in the observed clearance of infection because prevalence and mortality rate due to chytridiomycosis are known to be higher at lower temperatures (Berger et al. 2004, Retallick et al. 2004). The presence of large numbers of developing and mature sporangia in skin sloughs as opposed to empty sporangia, suggests that the majority of zoospores were released directly into the water and did not immediately re-infect the skin. Frogs that spent a lot of time on the elevated sections of the enclosures experienced a further reduction in exposure time to zoospores in the water. A combination of these two factors may also have contributed to chytridiomycosis being cleared. This observation is contrary to previous reports of chytridiomycosis in *A. fuscigula* where mortality events and sporadic deaths of juvenile and adult frogs occurred in populations from Namaqualand and the Eastern Cape Province (Hopkins and Channing 2003, Lane et al. 2003a). It seems that the outcome of infection in this species is unpredictable and that the advancement to mortality may depend on the presence of co-factors. In 1996 at a site less than 200 km from the source of frogs for this study, a population of nearly 50 *A. fuscigula* died at the start of the wet season after a prolonged period of drought (Chapter 4 of this thesis). A retrospective examination of dead specimens collected at the site revealed a 100% prevalence of *B. dendrobatidis*. The susceptibility of amphibians to develop disease due to chytrid fungus is known to vary between species. Whereas some species display morbidity and some experience mortality, others do not develop any clinical symptoms and appear to be resistant

to *B. dendrobatidis* (Green et al. 2002, Daszak et al. 2004). Variation in susceptibility to chytridiomycosis within a species has been demonstrated in experimentally infected *Rana yavapaiensis* and *Rana boylii* and the tiger salamander, *Ambystoma tigrinum* (Davidson et al. 2003). It would therefore appear that *A. fuscigula* exhibits a degree of resistance to *B. dendrobatidis* as long as optimum environmental conditions prevail.

7.4.3 Micro-spatial Occurrence on Skin

The wide range of differences in sporangium density between individuals of the population could be due to the length of infection. It can be expected that recently infected individuals will not be as heavily infected as those that have carried the infection for an extended period of time. The rate of sloughing could also be partly responsible for measured differences in sporangium density. Within specimen variation in sporangium density per field is symptomatic of an over-dispersed infection. The frequent occurrence of sporangia in adjoining fields indicates that *B. dendrobatidis* often occurs in clusters in the skin. The formation of clusters is a direct result of the colonisation strategy of the organism whereby colonies have a tendency to spread concentrically from the original loci of infection resulting in a core of empty, hyperkeratotic tissue. Cluster growth is also promoted by the limited time (< 24 h) and short distance (< 20 mm) that zoospores are able to swim before they encyst (Piotrowski et al. 2004). It appears that after the core or older sporangia have been shed, the hyperkeratic tissue does not become recolonised, resulting in a donut-shaped cluster. Two possible explanations why zoospores are not likely to infect hyperkeratotic skin are: 1) zoospores might require skin tissue with normal anatomical arrangements to be able to penetrate, or 2) the amphibian host could develop local resistance to *B. dendrobatidis* in areas previously infected. A similar growth pattern is also noticeable when *B. dendrobatidis* is grown in culture on nutrient agar. In culture, swimming zoospores tend to encyst at the edges of the existing colony that gradually expands as a consequence of this behaviour. Therefore low sporangium counts per field may reflect an area of recent infection or it may be the periphery of an expanding cluster. On the other hand, high sporangium densities are counted in the active growing regions of older clusters.

Differences in sporangium density between different areas of the foot as well as site infection frequency indicate that although any area of the foot is likely to become infected, certain areas are more likely to remain heavily infected. The most heavily infected sites on the foot (toe tips, tubercles and toes) are also the areas that are particularly in close contact with the substrate due to the protruding surfaces of the tubercles and toe tips and the clasping ability of the toes and thus the most likely areas to encounter zoospores on wet substrates. Of further significance is that the toes and protruding areas are most likely subjected to abrasion during

contact with the substrate or other frogs during amplexus (the mating ritual) and therefore greatly contributes to the spread and direct transmission of the fungus due to the concentration of the infection in areas that are exposed to severe abrasion.

7.4.4 Use of Density Data in Histology

Examining a test sample of skin sloughs prior to conducting a histological survey can greatly enhance the chances of eliminating false negatives from the population data. When selecting tissue types for histological diagnosis, quantification data can provide information about 1) the part of the foot that must be selected for histology 2) the minimum number of the identified part that must be collected from each specimen if the particular part occurs in multiples (all parts except base of foot) and 3) the number of sections that need to be made in order to detect the infection at a certain level of probability. The probability of detecting *B. dendrobatidis* is not only a factor of the sporangia density in a site, but also the frequency with which the site is infected in a population sample. The probability matrix produced by the Poisson frequency function illustrates that site selection for histology samples can have a marked influence on the number of sections that would be required to detect the amphibian chytrid at a specific level of probability.

7.5 Conclusion

The technique described in this chapter for quantifying *B. dendrobatidis* infection in live frogs does not require high level technology and yields reliable data that can be summarised as follows:

- The direct sporangium count makes it possible to express infection in terms of sporangia density, thus quantifying the infection.
- Comparing sporangium density between different sites provides a method to determine which part the foot must be used in histological surveys to provide the best chance of detecting *B. dendrobatidis*.
- Applying sporangium density data to the histological technique allows the calculation of the probability of detecting chytridiomycosis through set section repetitions.

The application of this technique to quarantine regulations that involve daily examinations is encouraged because it not only provides non-invasive presence/absence data, but also allows the investigator to quantify the response of chytridiomycosis to treatment regimes. Regular surveillance of amphibians has also been instigated at scientific laboratories and on frog farms following chytridiomycosis epidemics (Parker et al. 2002, Mazzoni et al. 2003). Further work is

needed to determine what the minimum requirement for skin slough size should be to be useful in detecting chytridiomycosis because frogs with early or light infections often do not shed as much skin or as frequently as frogs with major infections.



Chapter 8

MOLECULAR ANALYSIS OF *Batrachochytrium dendrobatidis* FROM SOUTH AFRICA



8.1 Introduction

The amphibian chytrid fungus *Batrachochytrium dendrobatidis* is considered to be an emerging pathogen based on the quick rate at which populations decline where chytridiomycosis is involved and the distinct geographic and temporal progression of the disease (Laurance et al. 1996, Daszak et al. 1999, Lips 1999). The recent emergence and introduction of chytridiomycosis is supported by molecular evidence (Morehouse et al. 2003), however, the origin of *B. dendrobatidis* has not yet been resolved through molecular analysis. Support for Africa as the origin of the amphibian chytrid has been demonstrated by epidemiological data that sprout from a histochemical analysis of wild and archived specimens of the African clawed frog, *Xenopus laevis* (Weldon et al. 2004).

In the first attempt to investigate the relationship of isolates the rDNA internal transcribed spacers (ITS gene) in strains from Australia, Central America and North America were used during which seven strains were identified (Morgan 2000). It was later discovered that *B. dendrobatidis* had multiple copies of the ITS region and that this gene was therefore not suitable for taxonomic work ([7]R. Speare pers. com. 2004). Early comparison of the 18S rDNA of chytrid strains from wild-caught Australian and captive American frogs did not show significant differences (see Berger et al. 1999a). In the most recent effort to help resolve the population genetics of *B. dendrobatidis* Morehouse et al. (2003) sequenced four variable loci of 35 strains of the organism and analysed the data with statistical parsimony to illustrate relationships between genotypes. Some of the important findings of the multi locus sequence typing (MLST) study were that a low level of genetic variation existed among strains (five variable nucleotide positions were detected among 10 loci) and that the population genetic structure resembled that of a newly emerged pathogen from a single, founder strain. It was also shown that the North American population formed a distinct gene pool. Multilocus sequence types from Panama and Australia were similar indicating a recent introduction of the disease into these two regions.

[7] Prof. Rick Speare, James Cook University, Townsville, Queensland, Australia

This study is an attempt to determine the level of genetic variation among isolates of *B. dendrobatidis* from South Africa and to compare the relationship of these strains with those tested by Morehouse et al. (2003). Three additional polymorphic loci were genotyped to the four used in the Morehouse paper (James et al. in prep.), thus strengthening the dataset upon which genetic relationships can be constructed. The possibility of extracting DNA from archived specimens and using it in the MLST was also investigated.

8.2 Material and Methods

8.2.1 Isolation and Cultivation of *B. dendrobatidis*

During August 2003 Prof. Rick Speare (James Cook University, Townsville, Australia) and Dr Joyce Longcore visited our laboratory to provide training and help with the isolation and cultivation of *B. dendrobatidis*. Several isolation attempts were made from live, apparently healthy frogs during the stay of our collaborators and during the next few months thereafter. Frogs from sources described in Chapter 4 were used for this purpose. Infection status was determined by light microscopic examination of wet mounts, followed by isolation. Details on isolation procedure are available from Longcore (2001). Six to ten pieces of infected skin (<1 mm) were placed on 100 mm petri dishes with TGh agar and antibiotics (modified by J. Longcore from her original recipe). The cultures were incubated at 21°C and their progress checked daily by examination under a compound microscope (100 X magnification). During this time contaminants (filamentous fungi, yeasts and bacteria) were cut out from the plates with a curved scalpel within a flow-through laminar – a procedure termed "weeding" by Longcore (2001). After about a month, each isolate (colony on piece of skin) that was actively growing were split in two. The one half remained on the TGh agar plate and the other half was transferred to a 1% tryptone agar plate. When colonies reached a size of ± 3x3 mm they were transferred to 30 ml glass culture tubes with screw caps containing 10 ml of tryptone broth medium. After initial incubation at 21°C for 1-2 weeks, the cultures were made up to a 50 ml volume (5 ml culture with 45 ml media). The 50 ml cultures were stored at 6°C and passaged once every two months into fresh media.

Media Recipes

TGh agar

Tryptone (Conda)	8 g
Gelatin hydrolosate (Sigma)	2 g
Agar bacteriological (Biolab)	10 g

1) Mix with distilled water to 1,000 ml.

2) Autoclave at 121°C for 15 min. and allow media to cool to 50°C.
3) Add the following antibiotics:

Penicillin-G, sodium salt (Sigma)	200 mg/l
Streptomycin sulphate (ICN)	300 mg/l
Tetracycline (Sigma)	20 mg/l

4) Pour media into petri dishes (makes approximately 40 plates)

1% Tryptone agar

Tryptone	10 g
Agar bacteriological	10 g

1) Mix with distilled water to 1,000 ml.
2) Autoclave at 121°C for 15 min. and allow media to cool to 50°C before pouring plates.

1% Tryptone broth

Tryptone	10 g
Distilled water	1,000 ml

1) Autoclave at 121°C for 15 min. and allow media to cool to room temperature.
2) Media can be stored at 4°C until needed.

8.2.2 Source and Collection of Frog Samples

It was decided to attempt DNA extraction from archived specimens that had been diagnosed with chytridiomycosis through conventional histochemical analysis (Chapter 4 of this thesis). These specimens were part of the herpetological collections of Bayworld (PEMA), Natal Museum (NM), National Museum (NMB) and Transvaal Museum (TM) and had been collected on different dates spanning from 1943-2000 for various reasons other than a systematic survey of diseases (Table 8-1). Formal requests for specimen loans were sent to the respective museums informing them of the intended use of the specimens for molecular research that required the removal of tissue samples. Specimen loans were granted by all of the involved museums. Because of anatomical differences between species and the preservation condition of specimens skin samples were not collected from the same region on all specimens, however, these were all regions that are most frequently infected by *B. dendrobatidis* namely toes, webbing, and skin from the thighs and abdominal pouch. A clean scalpel was used on each specimen. Skin samples were placed in plastic vials containing 70% ethanol and sent to Dr Alex Hyatt at the Australian Animal Health Laboratory (AAHL), Geelong for DNA analysis.

Table 8-1 Origin of skin samples used in the real-time Taqman PCR assay.

Museum number	Species	Collection year	Skin sample
NMB/A960612C11	*Afrana fuscigula*	1996	digit, thigh skin
NMB/A960612C12	*Afrana fuscigula*	1996	digit, thigh skin
PEMA/DT5	*Amietia vertebralis*	2000	webbing, thigh skin
PEMA/JR13	*Amietia vertebralis*	2000	webbing
PEMA/JR15	*Amietia vertebralis*	2000	webbing
PEMA1471	*Heleophryne regis*	1981	thigh skin, abdominal pouch
PEMA1488	*Heleophryne regis*	1982	thigh skin, abdominal pouch
NMB112	*Xenopus gilli*	1943	digit and webbing
PEMA1093	*Xenopus laevis*	1972	webbing
TM44180	*Xenopus laevis*	1973	digit and webbing
NM7301	*Xenopus laevis*	1985	digit and webbing
NMB5208	*Xenopus laevis*	1991	webbing
NM7737	*Xenopus laevis*	1995	webbing
NMB6322	*Xenopus laevis*	1996	webbing
NMB/AL961112A13	*Xenopus laevis*	1996	webbing
TM80486	*Xenopus laevis*	1996	webbing
TM70695	*Xenopus muelleri*	1991	webbing
TM83100	*Xenopus muelleri*	1998	webbing
TM30801	*Xenopus petersii*	1965	digit and webbing

Abbreviations: (NM) Natal Museum Pietermaritzburg, (NMB) National Museum Bloemfontein, (PEMBA) Bayworld Port Elizabeth, (TM) Transvaal Museum Pretoria.

8.2.3 Real-time Taqman PCR Assay

When received at AAHL the samples were processed as follows in a dedicated PCR suite for detection and quantification of *B. dendrobatidis*.

1) Using a clean blade for each sample, the tissue was cut in half. Approximately 10 mg was selected for subsequent processing.
2) The tissue was rehydrated in phosphate buffered saline overnight at 4°C.
3) Tissue was then transferred to magnalyser tubes with 250 µl lysis buffer.
4) The sample was then bead-beated (2 x 40 sec.) and allowed to stand at room temperature for 30 min. prior to being microfuged (2 min.).

5) 50 µl proteinase K was then added, vortexed and spun briefly followed by overnight incubation at 55°C.
6) DNA was extracted on MagnaPure using total nucleic acid kit and eluted in 50 µl.
7) 2 µl was used in the real-time Taqman PCR assay using described criteria (Boyle et al. 2004).
8) The assay was repeated using 8 µl.
9) In addition, 3 µl of sample was also run on a 1% agarose gel for detection of DNA.

8.2.4 Multilocus Sequence Typing

Five actively growing isolates were selected for molecular analysis (CW26, CW27, CW29, CW34, CW36). One ml of broth culture was transferred to fresh 1% Tryptone agar plates and allowed to air dry in a laminar flow hood, sealed and incubated at 21°C for 10 days. These cultures (hereon referred to as the South African isolates) were sent to chytrid fungus specialist, Dr Joyce Longcore from the University of Maine, Orono USA. No import permit was required by the US Department of Agriculture because *B. dendrobatidis* is a non-pathogenic microorganism to livestock, poultry or humans. Dr Longcore transferred the cultures onto new plates of agar with antibiotics, incubated them, and made stock cultures in 1% tryptone broth in glass, screw-capped culture tubes. Dr Longcore currently maintains these isolates as part of her already extensive collection of *B. dendrobatidis* isolates from across the globe. Two sets of the South African isolates, one on 1% tryptone plates and one in 50 ml plastic, screw-capped centrifuge tubes, were sent to Dr Tim James and colleague Dr Rytas Vilgalys from Duke University, Durham USA for molecular analysis.

When received at Duke University the samples were processed as follows:

1) The cultures were inoculated onto a piece of cellophane overlaying a Petri dish containing 1% tryptone and 1% agar.
2) The actively growing culture was harvested by scraping from the cellophane surface and the tissue was lyophilized.
3) DNA was extracted from ~50 mg of tissue using the method of Zolan and Pukkila (1986).
4) The South African strains were genotyped from seven polymorphic loci using the methods described in Morehouse et al. (2003). Four of these loci (CTSYN1, APRT13, LSU35, R6046) were described in Morehouse et al. (2003), and three additional loci (BdC5, BdC18, and BdC24) will be described later (James et al. in prep).
5) A UPGMA phenogram was calculated for the MLST data of the 5 South African isolates and 35 global isolates (Morehouse et al. 2003) by using the program PAUP* v. 4 (Swofford 2002).

The 35 global *B. dendrobatidis* strains were isolated from 19 amphibians from North America, Panama, Africa and Australia (Morehouse et al. 2003) (Table 8-2).

Table 8-2 Source of origin and host species of *Batrachochytrium dendrobatidis* strains used for multilocus sequence typing. The table has been reconstructed from Morehouse et al. (2003) and the South African strains (CW26, 27, 29, 34, 36) have been added to the table.

Strain	Origin	Host
JEL197	National Zoological Park, DC, USA	*Dendrobates azureus*
JEL198	National Zoological Park, DC, USA	*Dendrobates azureus*
JEL213	Mono Co., California, USA	*Rana muscosa*
JEL214	Mono Co., California, USA	*Rana muscosa*
JEL215	Mono Co., California, USA	*Rana muscosa*
JEL226	Yavapai Co., Arizona, USA	*Rana yavapaiensis*
JEL229	Montrose Canyon, Arizona, USA	*Hyla arenicolor*
JEL230	Montrose Canyon, Arizona, USA	*Rana yavapaiensis*
JEL260	Quebec, Canada	*Rana catesbeiana*
JEL262	Quebec, Canada	*Rana catesbeiana*
JEL264	Quebec, Canada	*Rana catesbeiana*
JEL277	Arizona, USA	*Ambystoma tigrinum*
JEL284	Wisconsin, USA (captive)	*Rana pipiens*
JEL289	Milford, Maine, USA	*Rana pipiens*
JEL231	Mesquite Wash, Arizona, USA	*Rana yavapaiensis*
JEL254	Orono, Maine, USA	*Rana pipiens*
JEL258	Orono, Maine, USA	*Rana sylvatica*
JEL203	Bronx Zoo, Ohio, USA	*Dyscophus guineti*
JEL270	Point Reyes, California, USA	*Rana catesbeiana*
JEL271	Point Reyes, California, USA	*Rana catesbeiana*
JEL282	Toledo Zoo, Ohio, USA	*Bufo americana*
545	Melbourne, Victoria, Australia	*Litoria lesueuri*
98 1810 3	Tully, Queensland, Australia	*Nyctimystes dayi*
99 1385 12	Rockhampton, Queensland, Australia	*Litoria caerulea*
PM5	Panama	*Smilisca phaeota*
PM7	Panama	*Smilisca phaeota*
JEL253	Melbourne, Victoria, Australia	*Limnodynastes dumerilii*
JEL225	Wisconsin, USA (captive)	*Xenopus tropicalis*

Table 8-2 continued

Strain	Origin	Host
JEL239	Imported from Ghana	*Xenopus tropicalis*
JEL240	Imported from Ghana	*Xenopus tropicalis*
JEL245	Imported from Ghana	*Xenopus tropicalis*
JEL273	Clear Creek Co., Colorado, USA	*Bufo boreas*
JEL275	Clear Creek Co., Colorado, USA	*Bufo boreas*
PM1	Panama	*Eleuthodactylus caryophyllaceum*
JEL274	Clear Creek Co., Colorado, USA	*Bufo boreas*
CW26	Springbok, Northern Cape, SA	*Bufo robinsoni*
CW27	Kammiesberg, Northern Cape, SA	*Afrana fuscigula*
CW29	Kammiesberg, Northern Cape, SA	*Xenopus laevis*
CW34	Kammiesberg, Northern Cape, SA	*Xenopus laevis*
CW36	Port Elizabeth, Eastern Cape, SA	*Afrana fuscigula*

8.3 Results

8.3.1 *B. dendrobatidis* Cultures

Isolation of *B. dendrobatidis* was attempted from 43 individuals including frogs, toads and clawed toads, and from both tadpoles and post-metamorphic frogs (Table 8-3). Almost two thirds of the isolation attempts produced viable cultures. An isolate was determined as being viable by the presence of zoospores swimming in the film of water surrounding the host tissue and fungus colony. In most of the viable isolates zoospores were released within two days of isolation. However, in some of the isolates sporangia remained dormant for up to two weeks before releasing their zoospores. The number of days an isolate survived was calculated as the last day that free-swimming zoospores were observed. Eight cultures were lost during the first week of growth in culture due to unknown causes, and nine cultures died within one or two days of each after about three weeks in culture. It is suspected that the incubator that was used at the time may have caused the death of these nine cultures because volatile organic chemicals were used in the incubator prior to it housing the cultures and the incubator was without ventilation holes. New isolates were henceforth incubated in a temperature-controlled room at 21°C. A few more cultures were lost due to bacterial and filamentous fungal contaminants that overgrew *B. dendrobatidis*. Five isolates from two species (*Afrana fuscigula* and *Xenopus laevis*) and two geographical regions (Namaqualand and the Eastern Cape) are currently actively growing in stock cultures.

Table 8-3 Origin of *Batrachochytrium dendrobatidis* isolates from South Africa and their progress in culture.

Isolate number	Host species	Locality	Days survived	Suspect cause of death
CW1	*Afrana fuscigula*	Springbok	5	Unknown
CW2	*Bufo robinsoni*	Springbok	5	Unknown
CW3	*Bufo robinsoni*	Springbok	5	Unknown
CW4	*Afrana fuscigula*	Kammiesberg	5	Unknown
CW5	*Afrana fuscigula*	Kammiesberg	0	
CW6	*Afrana fuscigula*	Kammiesberg	0	
CW7	*Afrana fuscigula*	Kammiesberg	20	Faulty incubator
CW8	*Afrana fuscigula*	Kammiesberg	0	
CW9	*Xenopus laevis*	Kammiesberg	18	Faulty incubator
CW10	*Xenopus laevis*	Kammiesberg	1	Unknown
CW11	*Xenopus laevis*	Kammiesberg	20	Faulty incubator
CW12	*Xenopus laevis*	Kammiesberg	19	Faulty incubator
CW13	*Xenopus laevis*	Kammiesberg	0	
CW14	*Afrana fuscigula*	Goegap NR	20	Faulty incubator
CW15	*Afrana fuscigula*	Goegap NR	19	Faulty incubator
CW16	*Afrana fuscigula*	Goegap NR	20	Faulty incubator
CW17	*Afrana fuscigula*	Kammiesberg	20	Faulty incubator
CW18	*Afrana fuscigula*	Kammiesberg	0	
CW19	*Afrana fuscigula*	Kammiesberg	5	Unknown
CW20	*Afrana fuscigula*	Goegap NR	19	Faulty incubator
CW21	*Afrana fuscigula*	Kammiesberg	0	
CW22	*Bufo robinsoni*	Springbok	5	Unknown
CW23	*Bufo robinsoni*	Springbok	0	
CW24	*Afrana fuscigula*	Kammiesberg	5	Unknown
CW25	*Afrana fuscigula*	Springbok	10	Unknown
CW26	*Bufo robinsoni*	Springbok	Remains viable	
CW27	*Afrana fuscigula*	Kammiesberg	Remains viable	
CW28	*Afrana fuscigula*	Kammiesberg	104	Bacterial contamination
CW29	*Xenopus laevis*	Kammiesberg	Remains viable	
CW30	*Cacosternum boettgeri*	Kenton on Sea	0	
CW31	*Cacosternum boettgeri*	Kenton on Sea	0	
CW32	*Cacosternum boettgeri*	Kenton on Sea	0	
CW33	*Xenopus laevis*	Kammiesberg	0	
CW34	*Xenopus laevis*	Kammiesberg	Remains viable	
CW35	*Afrana fuscigula*	Port Elizabeth	21	Fungal contamination
CW36	*Afrana fuscigula*	Port Elizabeth	Remains viable	
CW37	*Afrana fuscigula*	Port Elizabeth	148	
CW38	*Tomopterna natalensis*	Bella-Bella	0	
CW39	*Tomopterna natalensis*	Bella-Bella	0	
CW40	*Afrana dracomontana*	Lesotho	1	Bacterial contamination
CW41	*Afrana dracomontana*	Lesotho	2	Bacterial contamination
CW42	*Afrana dracomontana*	Lesotho	0	

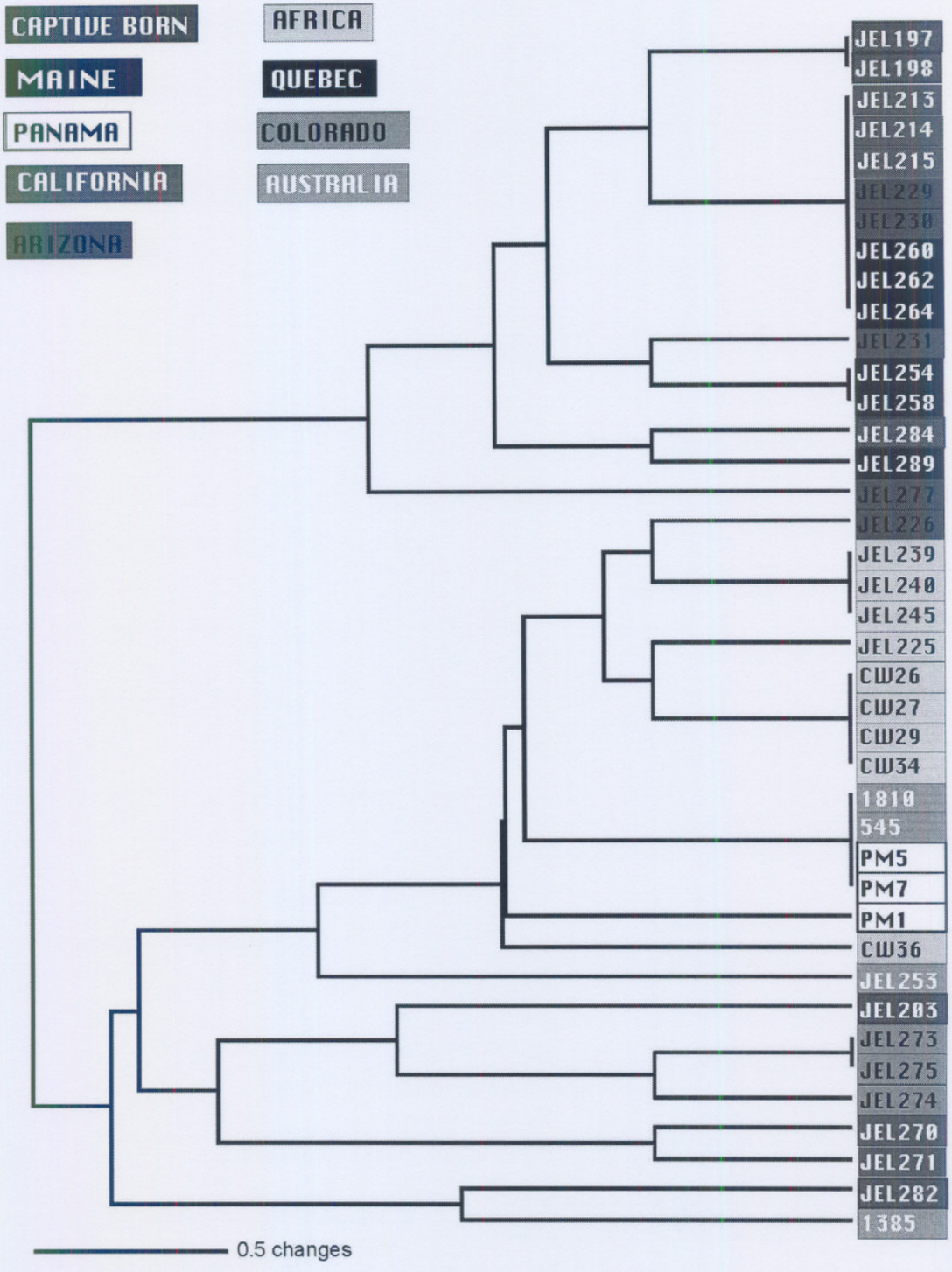
8.3.2 Molecular Analysis

All Taqman assays were negative for *B. dendrobatidis.* In addition, no DNA was detected on the 1% agarose gel. The inability to detect DNA in either assay (Taqman and gel) indicates that it was not possible to extract DNA from the skin samples. An explanation for this may be attributable to the possible prolonged use of formalin in the preservation of the samples. The use of 10% neutrally buffered formalin as fixative, followed by preservation in 70% ethanol is widely practiced by herpetologists. Certain PCR inhibitors have recently been discovered in some samples submitted for Taqman analysis and is currently being investigated further ([8]A. Hyatt pers. com. 2005). However, this is unlikely to be the cause of negative test results for the South African samples because DNA was not detected on the gel.

MLST of the 40 *B. dendrobatidis* strains revealed 22 genotypes. The relationships of the 40 strains can be viewed graphically by the construction of a phenogram (Figure 8-1). Each branch represents a change in genotype at one or more loci. Groups that are connected by shorter branches imply closer similarity between groups. Two major clusters are identified: JEL197-JEL277 represents an all North American group (n = 16), and the second, larger cluster contains the African, Australian, Panamanian and remaining North American strains (n = 24). Two multilocus genotypes exist among the South African strains that relate to their geographical distribution in the country. Four strains (all from Northern Cape) were identical in genotype regardless of their host. The strain from the Eastern Cape (CW36) had a unique genotype and is the only strain of the 40 samples to be homozygous at the APRT13 locus. Strong geographical relatedness is demonstrated by the African strains. The Northern Cape genotype shows up closely on the tree to the strains from *Xenopus tropicalis* that were imported to the USA from Ghana and from a distribution facility in the USA.

[8] Dr Alex Hyatt, Australian Animal Health Laboratory, Geelong, Victoria, Australia

Figure 8-1 UPGMA phenogram indicating the relationship between *Batrachochytrium dendrobatidis* isolates from Canada, USA, Panama, Africa and Australia.



8.4 Discussion

The inability to extract DNA from the archived samples is unfortunate, because the samples represented strains from a wide geographical range in South Africa. In addition, if such a procedure is successful and the DNA is used for MLST a temporal pattern of the genetic data would emerge that could prove to be useful in interpreting the history of divergence of *B. dendrobatidis* in the sub-continent.

It is clear from the MLST data that differences in genotype do exist among the global *B. dendrobatidis* population and that some of the MLST genotypes are more closely related than others. The pattern of relatedness is not dependent on host and only roughly correlates with geographical distribution. The strongest support for a geographical pattern is demonstrated by the smaller of the two major clusters (JEL197-JEL227). It can be deduced that divergence of this cluster took place in North America, because the cluster solely comprises of strains from this continent. However, a second minor cluster containing North American strains (Colorado, California and captive born) groups more closely to the Panama and Old World strains.

An analysis of genetic material may reveal clues to better understand the origins of organisms. Generally, one would expect to find greater genetic diversity among individuals from the source population than the sink population. It has recently been hypothesised that *B. dendrobatidis* originated in southern Africa based on epidemiological evidence (Weldon et al. 2004). The initial genetic analyses presented here does not support an "out of Africa" hypothesis. This point is argued by a low genetic diversity and distinct genotypes of the South African strains. On the other hand, the relationships indicated by the tree are supported by very few differences. It is too early at this stage of the investigation to reject the “out of Africa” hypothesis for a number of reasons.

- Samples from only two regions in South Africa were included in the analysis although *B. dendrobatidis* is known to be widespread in the sub-continent and in Africa (Chapter 4 of this thesis). Moreover, both these geographically separate regions harbour *B. dendrobatidis* with distinct MLST genotypes.
- The dataset that has been analysed is biased towards North American strains with 60% originating from the USA and Canada.
- The population structure remains incomplete because the sampling does not cover the known range of chytridiomycosis for example New Zealand, Tanzania, Spain and Ecuador (Bell et al. 2004, Bosch et al. 2001, Ron et al. 2003, Weldon and Du Preez 2004b).
- Furthermore, the global occurrence of the disease might be underestimated because new localities are being discovered due to a spreading epidemic as is currently being witnessed

in Central America (Lips 1998, 1999, Bell et al. 2004, Lips et al. 2004) or discoveries from random surveys. Ecological niche modelling has shwon that the distribution of suitable regions for *B. dendrobatidis* is extensive in the New World and the application of New World-based models on the Old World shows equally high predictability for the disease (Ron 2005).

It is apparent from the current study that more samples are needed to help elucidate the population structure of *B. dendrobatidis*. Not only should samples include a diversity of regions and host species, but sample sizes should also be considered. A meaningful sample size may include up to 50 independently cultured isolates from every region ([9]T. James pers. com. 2005). These sample sizes would allow quantitative measures of genotypic or genetic diversity to be statistically compared between geographic regions. In a southern African context the extreme Western Cape is an area that should receive high priority for attempted *B. dendrobatidis* isolation. The reason being that the known carrier host, *X. laevis* from this region, is exported globally for scientific research (Chapter 5 of this thesis, Weldon et al. 2004). Strains from the Western Cape are expected to bear the closest similarities with those from certain other countries in support of the dissemination pathway of *B. dendrobatidis* via the *X. laevis* trade. It would be most informative to compare the MLST of the known African strains to that of the virulent strain believed to be responsible for the population crash of *Nectophrynoides asperginis* from Kihansi Gorge, Tanzania (Chapter 9 of this thesis, Weldon and du Preez 2004b). The population genetic study of *B. dendrobatidis* will also benefit from developing more variable markers, such as microsatellite loci or amplified length polymorphisms (Morehouse et al. 2003). The team from Duke University is currently investigating this aspect.



[9] Dr Tim James, Duke University, Durham, USA

Chapter 9

CASE STUDY: DECLINE OF THE KIHANSI SPRAY TOAD, *Nectophrynoides asperginis*, FROM TANZANIA



9.1 Introduction

The Kihansi spray toad, *Nectophrynoides asperginis*, was discovered in 1996 in the Kihansi Gorge of south-western Tanzania (Poynton et al. 1998) and is distinctive in Africa in terms of the habitat it occupies (Figure 9-1). Its entire known habitat is restricted to less than 0.15 km^2 of a unique vegetation type within a narrow strip of moist forest bordering the gorge. Continuous spray generated by the Kihansi River as it flows over the Udzunqwa scarp, showers patches of herbaceous vegetation and moss-covered rocks to create a series of spray wetlands. Using the IUCN risk criteria, *N. asperginis* is considered to be critically endangered and the entire genus is listed under Appendix 1 of the Convention on International Trade on Endangered Species. The species' extremely restricted distribution makes it highly vulnerable to extinction due to a catastrophic event such as severe habitat alteration or introduction of a virulent disease. Spray toads are live bearing, which makes them even more vulnerable because of low fecundity and prolonged gestation. The toads are active during the day and at night although there is a slight shift in position from the dense vegetation mat to the more exposed rock surfaces after dark (Poynton et al. 1998). The species occurs in extremely high densities of up to 4.7 toads/m^2 of wetland vegetation (Channing et al. in prep.).

Concurrent with the description of the Kihansi spray toad, concerns were felt about the chances of survival of this Tanzanian endemic (Poynton et al. 1998). The spray wetlands were in danger of undergoing severe alteration because of a ten-fold reduction in water flow due to the requirements of a hydroelectric plant that was implemented in May 2000. The bypass flow released from the newly constructed dam above the main Kihansi falls was not enough to generate the spray that characterised the gorge and sustained the unique ecosystem within it. Without the spray conditions a range of plant and animal species were threatened. Ecological monitoring of various species and communities, as well as microclimate data collected since the diversion, allowed the progress of the Kihansi spray toad, among others, to be closely followed. A Joint Donor Supervision Mission initially carried out monitoring in October/November 2000 and reported on recommendations for conservation including the Kihansi spray toad. A short-term project, the Immediate Rescue and Emergency Measures

(IREM) that followed the mission (June 2001 – March 2002) included studies of *N. asperginis* and the spray wetland habitat and the implementation of mitigation measures (NORPLAN 2002a). During 2001/2002 a long-term project was established, the Lower Kihansi Environmental Management Project (LKEMP), that is currently still in operation. One of the main objectives of LKEMP is realising a series of medium-term measures to ensure the long-term conservation of the Kihansi Gorge ecosystem.

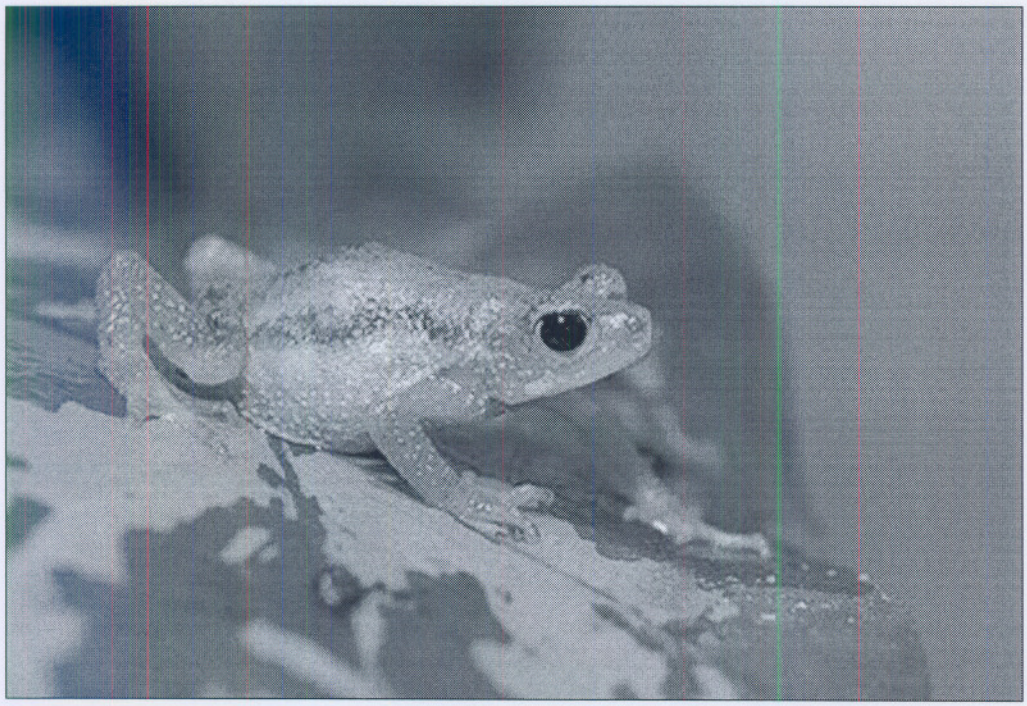


Figure 9-1 The Kihansi spray toad, *Nectophrynoides asperginis*. Photo, courtesy of Jule Larsen Maher, WCS-Bronx Zoo, Bronx, NY, USA.

The spray toad population experienced an initial decline during the dry season following the diversion (October 2000 – March 2001) and faced almost certain extinction in the gorge. The Tanzania Electrical Supply Company Ltd. (TANESCO) contracted a Norwegian engineering company, NORPLAN, to implement mitigation measures that would simulate the spray zone conditions of high relative humidity and low, constant temperatures. Elaborate sprinkler systems were installed in the Upper, Lower and Mid-gorge spray wetlands (NORPLAN 2001b) (Figure 9-2). The sprinklers, which receive water from tributaries of the Kihansi River, have been irrigating the Upper spray wetland since March 2001. The mitigation measures appeared to be highly successful because the spray toad population soon began to recover in the affected wetlands. Population census data of the Upper Spray wetland from Channing et al. (in prep.) indicate that between March 2001 and June 2003 the population estimates substantially increased from 1,752 to 17,978. During July/August 2003 population numbers of *N. asperginis*

unexpectedly plummeted. Less than a month after the last census on 4 July 2003, the foreman at Kihansi reported that no toads could be detected at the Upper spray wetland. The non-sightings of spray toads in some of the wetlands and a dramatic reduction in numbers at others continued during the month of July. The Mhalala population appeared to be unchanged at the time. During 3-5 August, LKEMP's Technical Advisor systematically quantified the extent of the apparent population decline in all of the wetlands. The attempted census confirmed that *N. asperginis* had experienced a dramatic population crash because only 61 toads in total were encountered from only three of the five wetlands. From the time that the census was conducted in June 2003 only six dead toads were recovered (5 on 28 July, 1 on 4 August), all from the Mid-gorge spray wetland (LKEMP 2004a). No obvious explanation could be given for the population crash at the time.

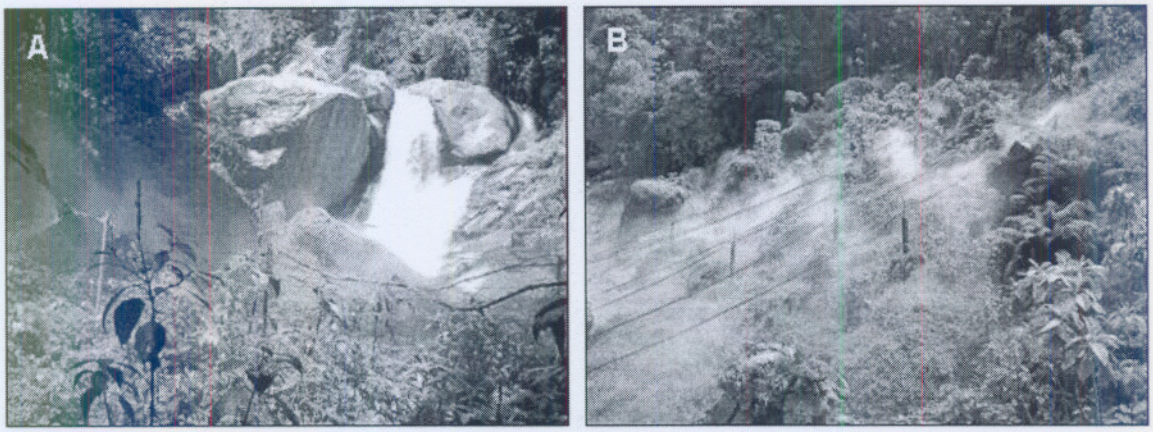


Figure 9-2 Examples of the spray wetlands, core habitat of the Kihansi spray toad, A) Close up view of the Mid-gorge spray wetland with much reduced waterfall in background. B) Lower spray wetland. Note network of sprinklers constructed to simulate the conditions previously created by waterfall spray from the Kihansi River.

Here the candidate presents the results from an investigation into the possible causes of the population decline of *N. asperginis.* It appears as if a combination of factors related to the construction of the Kihansi Dam may have been involved through their synergistic action. The strongest evidence, however, points towards a disease-mediated decline caused by the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*. The objectives of this study were to assess the status of the spray toad population and other amphibians of Kihansi Gorge and adjacent Udagaji Gorge through a systematic survey, to investigate possible causes of the decline, and to advise LKEMP on mitigation options that would aid in the recovery of the spay toads and to minimise the risks of the same event recurring in Kihansi Gorge. The study was performed under the auspices of LKEMP.

9.2 Material and Methods

9.2.1 Description of Study Site

As the Kihansi River passes over the Udzungwa escarp it races down a 100 m high waterfall that generates a spray zone as water hits rocks within and surrounding the plunge pool. The average water flow used to measure 16 m^3/s, but, since the diversion, has been reduced to a bypass flow of 1.5 – 1.9 m^3/s (NORPLAN 2001a). It is within this spray zone on the banks of the gorge that the Main Falls spray wetland is situated. The 50-300 mm thick wetland vegetation consists of a variety of moss, fern, grasses and eastern African forbs (Poynton et al. 1998). Consistent silt-filled spray turns this pasture of herbaceous vegetation into a boggy wetland. More waterfalls farther down the gorge used to generate similar spray zones that sustained three more wetlands in descending altitude, the Upper spray wetland, Lower spray wetland and Mid-gorge spray wetland (Figure 9-3). To the west of the Kihansi River the Mhalala spray wetland is situated on the steep banks of a tributary that joins the Kihansi just below the Upper spray wetland. The adjacent Udagaji Gorge was selected as a reference site because it does not share the same catchment as Kihansi River and has a similar amphibian community structure as Kihansi Gorge, apart from *N. asperginis*. James Camp, situated near the Lower spray wetland, was used as base camp and field laboratory.

9.2.2 Frog Survey

During November 2003 a field trip to Kihansi and adjacent Udagaji Gorges was undertaken to investigate the decline of the spray toads. The candidate was accompanied by Dr Jeremy Thompson (Technical Advisor, LKEMP) and Prof. James P. Gibbs (Conservation Biologist, State University of the New York College of Environmental Science and Forestry). The search effort was divided into surveying the different spray wetlands and nearby forest streams of Kihansi Gorge on three consecutive nights, followed by one night of surveying near the main falls of Udagaji Gorge. During the day reconnaissance trips were made to the spray wetlands where the vegetation and rock surfaces were systematically scanned with binoculars from the periphery of the wetlands for the presence of spray toads. Collections in the spray wetlands and surrounding forest were conducted after dark by making use of both visual encounters and audio cues. Installed pathways (consisting of concrete blocks) that dissect the spray wetlands as well as two sprinkler lines that run perpendicular with the river, were used as transects. A 500 mm band of vegetation was systematically searched along the transects. Before entering and upon exiting the wetlands, footwear were scrubbed and disinfected with a 2% sodium hypochlorite solution. Precaution was taken to reduce the risks of possibly introducing diseases to the Mhalala spray wetland by limiting the number of observers to two and by not entering the wetland at night. Surgical gloves were worn at all times when frogs were handled and changed between species and between sites. Frogs were individually placed in plastic

bags and returned to James Camp for processing. The identification keys and species descriptions in Schiøtz (1999), Channing (2001) and NORPLAN (2002b) were used to identify frogs in the field.

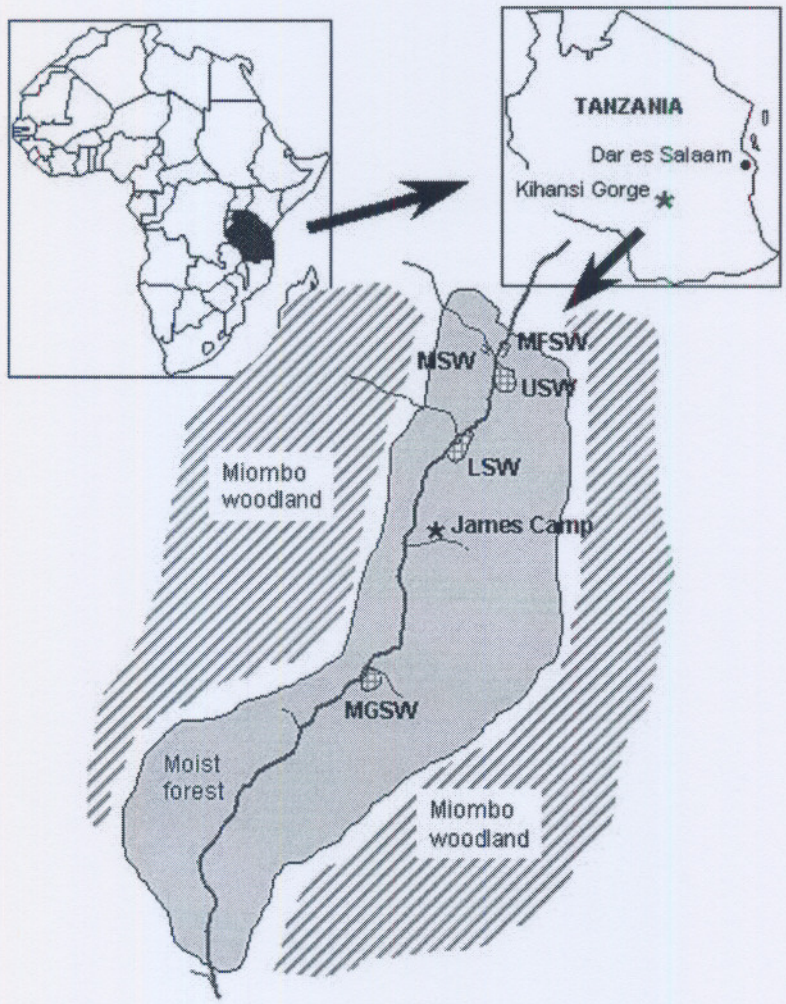


Figure 9-3 Diagrammatic representation of Kihansi Gorge indicating the course of the Kihansi River and location of spray wetlands. MFSW = Main Falls spray wetland, MSW = Mhalala spray wetland, UPS = Upper spray wetland, LSW = Lower spray wetland, MGSW = Mid-gorge spray wetland. The asterisk shows the location of the base camp (James Camp).

9.2.3 Tissue Collection and Processing

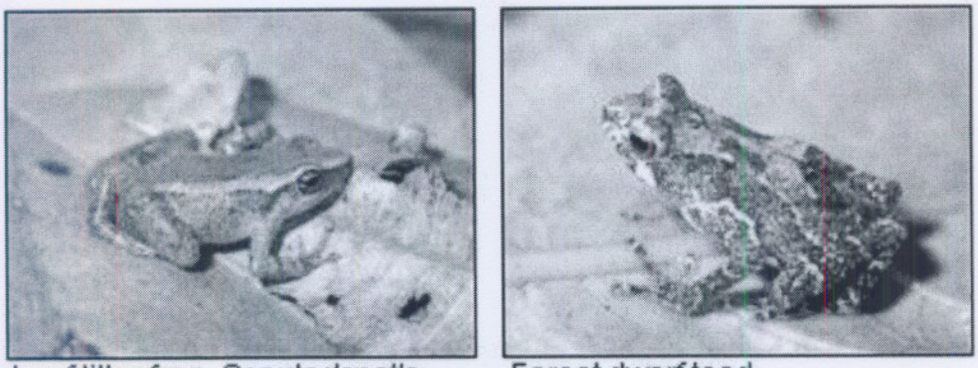
Apart from the specimens that were collected on the November 2003 survey, archived specimens of *N. asperginis* and *N. torneirri* were examined histologically for chytridiomycosis using techniques described in Chapter 2. Archived specimens were from the private collections of Prof. Alan Channing (University of the Western Cape) and of LKEMP that were collected during monitoring visits to Kihansi Gorge between 1997 and 2003. The fifth toe from the left hind foot was removed from live frogs and fixed in 70% alcohol in separate vials. After the

procedure, feet were treated with the antiseptic ointment, Bethadine (Adcock Ingram Ltd.) and frogs were released at the site of collection. Tissue processing and interpretation was conducted at the North-West University, South Africa. Tissue samples were dehydrated in an alcohol series, embedded in paraffin wax and sectioned at 6 µm. Sections were stained with haematoxylin and eosin and examined with a compound microscope for the presence of *B. dendrobatidis* thalli. The left hind hind of the fixed spray toad carcases collected during the mass mortality event and a single toe from the rest of the archived specimens were removed and prepared for histological analysis in the same way.

9.3 Results

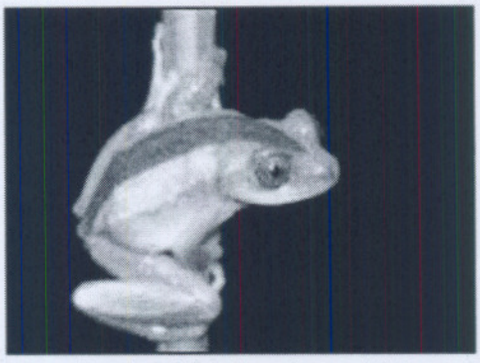
9.3.1 Species Diversity and Distribution

More than three months had passed since the last sighting of a live *N. asperginis* when the investigation into the species decline was conducted by the candidate in November 2003. Despite intensive searching during the day and at night, no spray toads could be found in any of the spray wetlands where it is known to have occurred. Five species, representing four families, were found during simultaneous searches around the wetlands and along streams in Kihansi and Udagaji Gorge (Table 9-1). Images of these frogs demonstrate the diversity of species found in the gorge (Figure 9-4). Torrent frogs (*Arthroleptidis yakusini*) used to be common in the spray wetlands, but were only found at a forest stream near James Camp and at Udagaji Gorge. On the fringes of the Upper and Mid-gorge spray wetlands, a few forest dwarf toads (*Nectophrynoides tornieri*) and Fornasini's spiny reed frogs (*Afrixalus fornasinii*) were found on top of herbaceous vegetation. One of the species, the plain grass frog (*Ptychadena anchietae*), was not listed in the field guide to frogs of the Kihansi Gorge (NORPLAN 2002b) and can be considered a new record for the gorge. New localities for *A. fornasinii* in Kihansi Gorge were recorded at the Mid-gorge and Upper spray wetlands (previously only known from the Lower spray wetland).

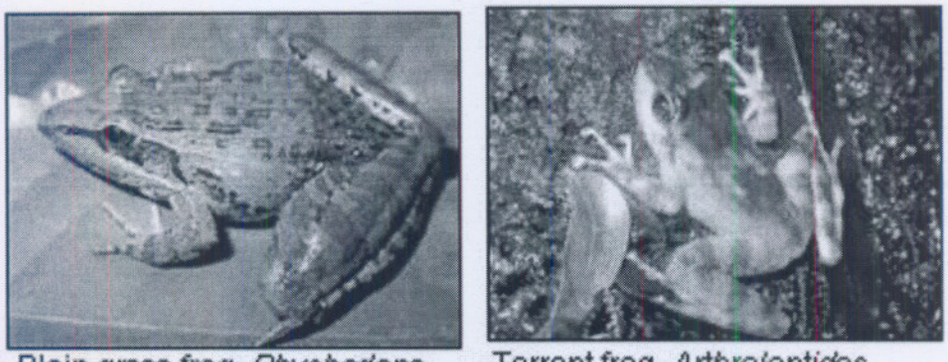


Leaf litter frog, *Scoutedenella xenodactylus*

Forest dwarf toad, *Nectophrynoides tornieri*



Greater leaf-folding frog, *Afrixalus fornasinii*



Plain grass frog, *Phychadena anchietae*

Torrent frog, *Arthroleptides yakusini*

Figure 9-4 Examples of frogs collected at Kihansi and Udagaji gorges, Udzungwa Mountains, Tanzania during the November 2003 survey.

Table 9-1 Anuran species found in and near the spray wetlands of Kihansi Gorge and the main falls of Udagaji Gorge.

Species	Family	Locality				
		MGSW	LSW	USW	JC	UG
Schoutedenella xenodactylus	Arthroleptidae	X				X
Nectophrynoides tornieri	Bufonidae	X		X		X
Afrixalus fornasinii	Hyperoliidae	X		X		
Arthroleptidis yakusini	Ranidae				X	X
Ptychadena anchietae	Ranidae		X			X
No. of species		3	1	2	1	4

Abbreviations: (MGSW) Mid-gorge spray wetland, (LSW) Lower spray wetland, (USW) Upper spray wetland, (JC) James Camp, (UG) Udagaji Gorge.

9.3.2 *B. dendrobatidis* Infection Rate

Tissue samples for processing were collected from 32 specimens from Kihansi Gorge and 13 specimens from Udagaji Gorge during the survey in November 2003 (Table 9-2). The only records for which no tissue samples were collected are *A. fornasinii* from the Upper spray wetland (call identified only), the leaf litter frog (*Schoutedenella xenodactylus*) and *P. anchietae* from Udagaji Gorge (both visual encounters). Chytridiomycosis was subsequently detected in *P. anchietae* from the Lower spray wetland in Kihansi Gorge and in *A. yakusini* from Udagaji Gorge.

Four *N. asperginis* specimens were severely infected with *B. dendrobatidis*. Except for one toad collected in June 2003, the rest of the specimens were collected dead on site at the time of the population crash. Together with two more specimens these six toads represent the last individuals of *N. asperginis* to have been collected. This evidence suggests that the prevalence of *B. dendrobatidis* must have been very high in the spray toad population at the time of the population crash. Histological sections revealed that the infections were concentrated in the anterior half of the feet (toes and toe tips) where almost 50% of the surface contained focal lesions with chytrid thalli (Figure 9-5). Clusters of sporangia were up to 70 µm thick and embedded within hyperkeratotic cells. Autolysis in one of the specimens collected in July 2003 had advanced beyond the point where a positive diagnosis of a cutaneous infection could be made using the current diagnostic technique. Contrary to the high prevalence of *B. dendrobatidis* during the population crash, no evidence of the pathogen was detected in any of the 81 spray toads collected during the six years preceding 2003.

Table 9-2 Prevalence and distribution of chytridiomycosis in frogs from Kihansi Gorge and Udagaji Gorge.

Species	Site	Collection date	Specimens examined (no. infected)
Schoutedenella xenodactylus	Kihansi	Nov. 2003	17 (0)
Nectophrynoides asperginis	Kihansi	Oct. 1997	1 (0)
		Oct. 1999	17 (0)
		May 2000	1 (0)
		Oct. 2000	54 (0)
		Nov. 2000	1 (0)
		Feb. 2001	2 (0)
		Aug. 2001	1 (0)
		Oct. 2001	1 (0)
		Sep. 2002	3 (0)
		Jun. 2003	2 (1)
		Jul. 2003	3 (2)
		Aug. 2003	1 (1)
Nectophrynoides tornieri	Kihansi	May 2000	3 (0)
		Aug. 2001	3 (0)
		Sep. 2002	2 (0)
		Nov. 2003	4 (0)
	Udagaji	Nov. 2003	2 (0)
Afrixalus fornasinii	Kihansi	Nov. 2003	1 (0)
Arthroleptidis yakusini	Kihansi	Nov. 2003	9 (0)
	Udagaji	Nov. 2003	11 (3)
Ptychadena anchietae	Kihansi	Nov. 2003	1 (1)

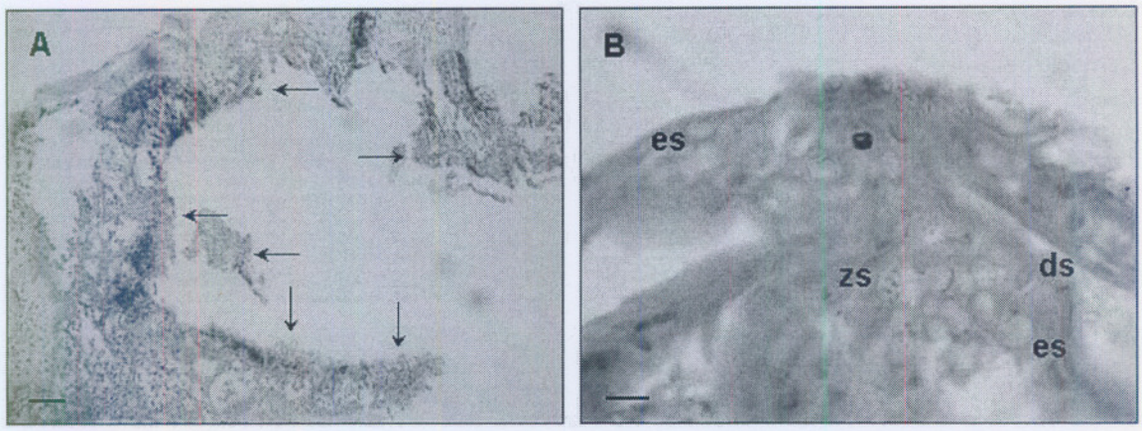


Figure 9-5 Micrographs of haematoxylin and eosin stained sections of *Nectophrynoides asperginis* skin infected with *Batrachochytrium dendrobatidis*. A) Arrows indicate the position of *B. dendrobatidis* clusters in superficial and sloughed epidermis (scale bar = 60 µm). B) The concentration of sporangia in various stages of development is symptomatic of an active infection; ds = developing sporangium, es = empty sporangium, zs = sporangium with zoospores (scale bar = 10 µm).

9.4 Discussion

9.4.1 Evidence for Chytridiomycosis as Cause of Decline

The rapidity and severity with which the Kihansi spray toad population crashed is typical of an initial epidemic experience in a naive population from a virulent pathogen. The decline at Kihansi Gorge occurred more rapidly than declines attributable to chytridiomycosis in Latin America and Australia, which happened over a few months to three years (Laurance et al. 1996, Alford and Richards 1997, Young et al. 2001, Lips et al. 2003). Evidence suggests that the amphibian chytrid fungus played a major part in the decline of the Kihansi spray toad. Chytridiomycosis was positively identified in dead specimens collected at the time of the population crash. Histopathological analysis demonstrated a severe infection of the epidermis and a particularly high prevalence among the animals that died. The timing of the mortality event coincides with the first appearance of *B. dendrobatidis* in *N. asperginis* and the related *N. tornieri* because chytridiomycosis was neither detected in six years of archived specimens pre-dating the population crash nor in any of the translocated toads that died in USA zoos and had been submitted for necropsy (Lee 2003). It can therefore be deduced that chytridiomycosis was introduced shortly before the epidemic occurred.

Although *B. dendrobatidis* is likely to infect any amphibian, those species that have been most severely affected share certain characteristics. These include low fecundity, aquatic larvae

associated with streams, restricted geographical distribution and high elevation (Berger et al. 1998, Lips 1999, Young et al. 2001, Bosch et al. 2001). Except for aquatic larvae, *N. asperginis* from Kihansi Gorge displays all of these characteristics. The exceptionally high densities in which spray toads occur implies that contact between individuals must be high, thus providing ample opportunity for frog-to-frog transmission of chytridiomycosis. During the intermittent high-flow tests in September 2002, when the sprinklers were turned off, the spray toads responded to the change in conditions by congregating in the part of the wetland closest to the waterfall that still received small amounts of natural spray (Channing et al. 2003). If the spray toads displayed the same behaviour during the June 2003 high-flow test when *B. dendrobatidis* was already present, the rate of disease transmission would have been elevated considerably. The constant 100% relative humidity and low temperatures that characterise the spray wetland habitat create the perfect conditions for *B. dendrobatidis* survival and for indirect transmission of zoospores. Isolates of *B. dendrobatidis* grow best at low temperatures (4-25°C) and experience mortality when temperatures rise above 30°C (Longcore et al. 1999, Piotrowski et al. 2004), and the zoospores require water or a moist environment for transmission.

9.4.2 Other Possible Reasons for Decline

Collecting and Monitoring

Apart from the type specimens collected in December 1996 and October 1997, 500 toads that were transferred to the USA for captive breeding in December 2000 and a few hundred voucher specimens collected on population surveys during 2000 – 2003, spray toads were not removed from Kihansi Gorge. It does not appear that the early scientific collections adversely affected the medium-term population trend, because the spray toad population was on an upward curve until the population crash. However, any future collections will have to be subjected to strict scrutiny until census data indicate that the population has recovered to its previous size. It is unlikely that illegal collecting can persist due to the inaccessibility of Kihansi Gorge and the strict control of human traffic to the gorge by TANESCO and LKEMP officials.

A bigger threat may be related to the method used when searching for spray toads in vegetation plots. The method can be destructive to wetland habitat because vegetation is removed from the plot during the search and replaced afterwards. Furthermore, spray toads that take cover in the thick carpet of wetland vegetation can easily be trampled on especially because they occur in such high densities. It is highly unlikely that the population crash was directly related to census activities because numerous censuses were carried out in the past without any apparent harm to the spray toad population.

Predation and Foraging Associations

There exists a delicate balance between host plant communities and insects in the spray wetlands that constitute the main diet of the Kihansi spray toad. The spray wetland vegetation underwent marked changes following diversion; marsh and streamside species died back and weedy species entered the wetland (Quinn et al. 2003). The long-term implications of the dramatic change in the abundance of certain plant species and in the vegetation structure on the insect prey of *N. asperginis* has not been determined. Shifts in insect community structure have also been observed especially among species that are specialist feeders and those that are particularly susceptible to desiccation (Channing et al. 2003). However, preliminary examination of gut contents of *N. asperginis* suggests that this toad feeds on a wide range of insect families (Poynton et al. 1998) and is therefore not dependant on the abundance of one particular species.

Very little is known about the natural enemies of the Kihansi spray toad. During past ecological monitoring only three cases of predation on spray toads were observed (Channing et al. in prep.). The predators included crabs on two occasions and army ants on a single occasion. Retreat and significant changes in vegetation structure may render the wetlands more accessible to conventional, as well as opportunistic predators. Although overall predation on the spray toads may have increased, it can be expected that the effect thereof on population size would be a gradual process and insignificant in terms of total population size, especially because no introduced predators have been observed.

Alteration of Flow Regime

The bypass flow from Kihansi Dam is subject to manipulation, because it forms part of the Lower Kihansi Hydroelectric Plant. The regulation of water flow allows for a relatively constant volume (1.5-1.9 m^3/s) for most of the year, except for a short period during summer when the Kihansi River is in flood. Intermittent high flow manipulations of varying regime (4-8 m^3/s) were tested during 2002 as part of mitigation measure investigations (Channing et al. 2003). During the high flow tests, the sprinkler systems were turned off to measure changes that might occur in the generation of natural spray and to test the effect thereof on the wetland ecosystem. Although the study concluded that a peak flow of 8 m^3/s was not sufficient to generate substantial amounts of spray to cover the entire wetland, the spray toad population was found to be unchanged. In June 2003 intermittent high flows of 12 and 16 were released (Channing et al. 2003). This time, however, the volume of water was sufficient to generate enough spray to cover the entire wetland. Witness accounts testified that the colour of the waterfall turned visibly darker during testing. It appears that the sediment that had accumulated at the bottom of the dam since its construction was flushed when the sluices were opened to allow the larger

bypass flow of 16 m^3/s and was sprayed onto the wetlands. Ever since the diversion, the spray toads were exposed to either rainwater or filtered sprinkler water from one of the tributaries of the Kihansi River. The water quality from spray generated from dam water would have been quite different from what the toads had become accustomed to. It was during the census conducted in the days following these high flow tests that the highest population estimates since the diversion were recorded, and just under one month before the population was reduced to less than 100 individuals. The sprinkler team at Kihansi indicated that spray toad numbers began to decline within two weeks after the high flow study. Sudden and significant increases in flow and velocity can cause changes in water temperature, which has been shown to inhibit growth and development of juvenile spotted tree frogs in eastern Australia (Gillespie 1997). It is possible that the altered conditions in water quality that the spray toads experienced during the last intermittent high flows could have caused a stress reaction.

Agro-chemical Pollution

The undulating landscape of the Kihansi River catchment has fertile soil with large stretches of former Miombo Woodland now converted into agricultural land. According to agricultural monitoring agencies based in Iringa the chlorine-organic insecticide endosulfan (thiodan) is widely applied in these areas ([10]J. Thompson pers. com. 2003). Exposure to endosulfan has been known to impair normal development and reproduction of amphibians. Harris et al. (2000) reported deformities in American toads (*Bufo americanus*) and northern leopard frogs (*Rana pipiens*) that had been exposed to 0.1 ppm endosulfan. In the red-spotted newt (*Notophthalmus viridescens*) endosulfan exposure impaired the pheromonal system that led to disrupted mate choice and lowered mating success (Park et al. 2001). It has also been demonstrated that endosulfan can cause mortality in tadpoles of *Bufo melanostictus* and *Rana tigrina* (LC50 of 0.123 mg/L and 1.8 mg/L respectively) (Gopal et al. 1981). Runoff from crops containing pesticides and fertilisers could have settled in the dam basin and accumulated over time. Sediment particles that were released with the high flows may have transferred bound chemical residues onto the spray wetlands. Five soil samples at Kihansi have been analised, but endosulfan was not detected. It is believed that the samples were not taken from strategically important locations and that more extensive analysis is warrented (J. Thomson 2005). Considering the reports of endosulfan induced adversities, this insecticide could be potentially hazardous to *N. asperginis*. It can be expected that the release of pesticides would affect a range of biota in the wetlands and not only the spray toads, however, plant and invertebrate communities appeared to be healthy shortly after the high flow studies (Channing

[10] Dr Jeremy Thompson, LKEMP, Dar es Salaam, Tanzania

et al. in prep.). Sensitivity to environmental change due to the permeable nature of amphibian skin, could account for differences in reaction to pesticide exposure.

Multiple Factors

The combination of *N. asperginis* behaviour (high-density population, tendency to congregate) and spray wetland conditions could explain the rapid rate at which the species decline occurred. It seems that the construction of the hydroelectric dam and the reduction in water flow of the Kihansi River created a series of conditions and events that predisposed *N. asperginis* to lethal *B. dendrobatidis* infection despite elaborate mitigation measures to rescue the spray toads and maintain spray wetland integrity. The drying of the wetlands caused a gradual change in invertebrate prey availability and a possible increase in predation that are linked to shifts in vegetation structure. During the intermittent high-flow tests the toads were instantly exposed to silt laden water of poorer quality than what the toads had been used to. Pesticide residues bound to sediment particles could have compromised the immune system of the spray toads even further. However, water and sediment samples from the dam and spray wetlands were not tested for the presence of the widely used insecticide endosulfan and hence the involvement of this insecticide in the population crash remains speculative. Environmental changes acting synergistically with other factors such as increased concentration of pollutants or a clumped distribution, could stress amphibians sufficiently to compromise their immune systems and increase disease transmission between susceptible hosts (Carey and Bryant 1995, Collins and Strofer 2003). The testing of a mitigation measure (intermittent high-flows) may have provided the final trigger that caused the spray toads to succumb to chytridiomycosis and would have coincided with an accelerated rate of transmission if the toads behaved by moving to the wettest part of the wetland.

9.4.3 Introduction and Dissemination Pathways of Chytridiomycosis

The recent introduction of chytridiomycosis into Kihansi Gorge raises questions as to how the pathogen was introduced. Knowledge of possible entry pathways is necessary to effectively control a further spread of the disease. A number of aspects must be considered when estimating the likelihood of chytridiomycosis dissemination within the study area. The already existing stock of chytridiomycosis within the gorges can disseminate between sites through a number of vectors or the stock can be amplified by recurring inoculation doses through a number of pathways.

Without effective pathogen prevention mechanisms, Kihansi Gorge is vulnerable to transmission of chytridiomycosis through a range of pathways. These pathways have been ranked from highest perceived risk to lowest perceived risk (Table 9-3). In terms of *B.*

dendrobatidis viability, accidental introduction of frogs seems the most likely pathway for introduction into Kihansi Gorge. It has been remarked that three of the species found at Kihansi Gorge, *A. fornasinii*, *Hyperolius puncticulatus* and *Chiromantis xerampelina* could have been accidentally introduced, because Kihansi Gorge forms a disjunctive locality from their known range in Tanzania (NORPLAN 2002b). It is possible that these frogs could have been transported from lowland sites in for example, fruit and vegetable supplies and landscaping equipment such as pipes, especially during the construction phase of the sprinkler system. The displacement of Australian tree frogs (family Hylidae) in fresh produce is regarded as a major source of chytridiomycosis dissemination in the country (NSW National Parks and Wildlife Service 2000). *Hyperolius puncticulatus* and *C. xerampelina*, also tree frogs, are ecologically similar species to the Hylidae and therefore share similar behavioural patterns. If these frogs were infected, contact with local anuran species could have resulted in subsequent transmission of the disease.

Kihansi Gorge is often visited by scientists from various disciplines such as botany, entomology, herpetology, hydrology and others for ecological monitoring purposes. The possibility exists that they could have come into contact with an infected source during fieldwork, especially those working in wetland habitats. Failure to sterilise their equipment and protective clothing could have resulted in direct transmission or indirect transmission in spray wetland habitat. It was not until June 2003, when chytridiomycosis was already present, that LKEMP commissioned the construction of sterilisation baths at Kihansi Gorge.

Mammals associated with riverine habitat, such as the Cape clawless otter as well as a number of wetland birds were observed in Kihansi Gorge and might be able to transport the amphibian chytrid fungus (i.e. contaminated water droplets), however, there is no evidence that this occurred. Although their numbers are low, these animate vectors are capable of crossing large distances in relatively short time. The reduction in water flow and subsequent disappearance of the natural spray zone have rendered parts of the river that were previously showered with spray, more accessible for potential animate vectors of chytridiomycosis. Besides the cold and wet conditions, the spray generated a draft of 7 knots, thus rendering it even more inaccessible to potential vectors.

Another possible method of introduction is that the network of tributaries and Kihansi River transport zoospores from contaminated upland sources. The amphibian chytrid has been shown to remain viable in sterilised lake water for up to seven weeks (Johnson and Speare 2003) and could therefore potentially survive this method of spread. Although it is possible that infective zoospores could be fed into the gorges directly through the rivers and into the

sprinkler system, the risk remains very low, because it would require enormous quantities of zoospores given the dilution effect created by the large volume of water. This seems unlikely, because a continuous supply of the organisms would have been detected in collections made before June 2003.

Table 9-3 Entry pathways of chytridiomycosis to Kihansi and Udagaji Gorges. Pathways are ranked from the highest to the lowest perceived risk.

Rank	Pathway
1	Introduced anuran vectors.
2	Protective clothing and equipment of scientists (nets, trapping equipment, waders etc.).
3	Other animate vectors (wetland birds, otters etc.).
4	Water from the Kihansi River and other tributaries.

The opportunity exists for a continuous dissemination of the disease within Kihansi Gorge by the maintenance crew that service the sprinkler system on a daily basis. A single contaminated medium could spread the disease between sites. Mechanical transmission could occur if for instance the organism was transported in mud or water from a contaminated source on the carrier's footwear or equipment and the carrier wore the same footwear to a second wetland or used the same equipment. The transmission would be completed if susceptible frogs came in direct contact with the contaminated mud or equipment.

Although *A. yakusini* seems to have retracted from the spray wetlands (at least temporary), the forest stream populations appear to be healthy despite being infected. No baseline data is available for *P. anchietae* so it is not possible to make any deductions regarding the population status of this species, however the specimens that were encountered appeared to be healthy. Both these species can therefore act as reservoir hosts for chytridiomycosis. Considering this gradual change in vegetation composition in the gorge and the highly mobile nature of *P. anchietae*, this frog is a high-risk species for disseminating chytridiomycosis along the river within and beyond the gorge. The risk of *A. yakusini* disseminating chytridiomycosis is limited to streams and surrounding moist forest including the spray wetlands.

9.4.4 Ability of *N. asperginis* to Recover

The observed trend in the Kihansi spray toad population size is not regarded as a natural fluctuation or part of seasonal changes in abundance because of the magnitude (>99% of individuals) and rapidity (3-4 weeks) of the decline and the lack of any subsequent recovery 22

months post-decline. These criteria have been used to document observed negative trends in amphibian populations from Latin America as genuine declines (Lips et al. 2003). The possibility of species extinction is highly likely because not a single spray toad was found during the November 2003 survey. The latest quantitative surveys conducted by LKEMP in April/May 2004 have again failed to locate any spray toads (LKEMP 2004b). The last confirmed record of a Kihansi spray toad was a single individual heard calling at night in the Upper spray wetland in late January 2004. Rarity of species and low reproductive rate, as with *N. asperginis*, have previously been correlated with increased extinction risk (Rabinowitz et al. 1986, McKinney 1997, Puvis et al. 2000, Cardillo 2003). In addition, high endemism and restricted ranges have been used as criteria to predict species extinction of many Latin American amphibian species (Lips et al. 2004). Given the uncertainties about the survival of the Kihansi spray toad throughout most of its known geographical range, the ability of the species to recover to its previously known population size in the wild is doubtful. Low fecundity due to their breeding biology might imply that *N. asperginis* are not very resilient following an epidemic that reduced the population to below detection level. The Eungella day frog (*Taudactylus eungellensis*) disappeared from Eungella National Park, Queensland, Australia after an initial chytridiomycosis epidemic, but was subsequently found in a few remnant populations with low prevalence (Retallick et al. 2004). The event of this happening at Kihansi Gorge is, however, remote. The niche specifications of the spray toads is restricted and their range has been systematically surveyed on two occasions post-decline without detecting the species.

Captive breeding might be the last refuge for the Kihansi spray toad. After an initial population increase in the captive populations, numbers started to fall and the original 500 animals have since dropped to fewer than 100 individuals (Lee 2003). This can mainly be ascribed to a range of illnesses associated with organ inflammatory processes, renal problems, metabolic bone diseases and *Mycobacterium* infections. Because of the declining numbers, the captive populations have now been consolidated from six to two zoos (the Bronx and Toledo zoos). Budget restraints from the zoos and national budget obligations from the Government of Tanzania meant that the captive breeding programme faced serious financial problems. This has prompted the Government of Tanzania to request financial support from the international community for the captive spray toads by establishing a Basket Fund in aid of the programme (LKEMP 2004b).

9.4.5 Previous Conservation Actions

The initial decline of spray toad numbers following the diversion of the Kihansi River resulted in a number of ecological surveys aimed at conserving the population and the greater Kihansi

Gorge ecosystem (NORPLAN 2002a). These short-term mitigation measures can be summarised as follows:

- Aerial surveys of the Udzungwa Escarpment were conducted in an effort to search for other large waterfalls that could potentially create similar habitat conditions to Kihansi Gorge. Other sites were identified and amphibian surveys conducted, but no spray toads were found.
- NORPLAN has, in association with Tanzanian and other international scientists, carried out detailed studies on aspects of the Kihansi Gorge ecosystem. Some of these studies involved the disciplines of herpetology, entomology, botany and microclimate analysis.
- The artificial sprinkler system was installed at three of the spray wetlands as part of the IREM project. The aim was that the sprinkler system would recreate the spray zone conditions that were no longer present due to the reduction in water flow from requirements of the hydroelectric plant.
- In late November 2000, 500 toads were removed from Kihansi Gorge and translocated to the United States for captive breeding purposes. This group of toads was initially split between the Bronx Zoo and Detroit Zoo to increase their chances of survival and to share the husbandry responsibilities (Lee 2003). In the following years toads from the original group and their captive-raised offspring were distributed to an additional four zoos, namely Oklahoma City -, Baltimore -, Toledo - and Buffalo Zoo.
- A sterilisation station for footwear of the sprinkler team and visitors was set up at the top of Kihansi Gorge during June 2003. With LKEMP unaware that chytrid fungus was already in the gorge, the primary objective of the sterilisation programme was to prevent anthropogenic introduction of disease agents.

9.4.6 Proposed Mitigation Actions

The overall objective of the proposed recovery actions is to prevent the extinction of the Kihansi spray toad by identifying the cause of the population decline, monitoring and experimental design, ameliorating known risks and maintaining the captive breeding population. The following recovery actions have not been ordered according to priority, because many of the actions need to be implemented simultaneously. Other actions have already been implemented to some extent and are in need of revision.

Action 1 Further Investigate Causes of the Population Crash

- *Experimental Exposures of the Kihansi Spray Toad to B. dendrobatidis*

Although histopathology analysis identified the amphibian chytrid fungus in dead *N. asperginis*, the clinical course of the disease in this toad is unknown. Susceptibility

experiments should only be conducted when the captive breeding programme has produced sufficient progeny that can be exposed to *B. dendrobatidis* isolates, preferably from the same region.

- *Test for Pesticides in Sediment*

The pesticide hypothesis should be investigated further by quantifying the application of agro-chemicals in the Kihansi River catchment and by analysing sediment samples from the dam and soil samples from the spray wetlands. It is also recommended that the bioaccumulation of pesticides be tested in frogs that visit the spray wetlands such as *N. tornieri* and *A. yakusini*.

- *Examine Climatological Data for Kihansi Gorge*

No documented evidence exists to suggest that ozone depletion and associated increased exposure to UV-B radiation may or may not adversely affect amphibian communities at Kihansi Gorge. Data on the environmental parameters (temperature, rainfall, humidity etc.) at Kihansi Gorge in the months surrounding the mass mortality event should therefore be examined for any atypical events.

Action 2 Ameliorate Known and Perceived Threats

- *Threat: Dissemination of Amphibian Chytrid Fungus*
  Action: Implement a Rigorous Field Hygiene Protocol

A strict pathogen hygiene protocol is essential to control chytridiomycosis within Kihansi Gorge. The protocol functions around critical control points that include traffic control to and from important conservation sites, on site handling of frogs and sprinkler maintenance regulations. The following guidelines are aimed at preventing the entering and spread of diseases by anthropogenic factors.

- When first visiting a site or when moving between sites, it is important to prevent the transfer of the disease organism from footwear and equipment. Footwear should first be rinsed in water and scraped clean of all mud and debris before treating with disinfection solution. For disinfection, the soles should be bathed in a 2% sodium hypochlorite solution for 2 minutes and the rest of the footwear scrubbed for at least 1 minute with the same solution. To prevent wastewater from exposing the environment to chytridiomycosis, the water should be collected and treated with sodium hypochlorite (100 mg/L for 30 minutes).
- Equipment such as nets and traps should be disinfected by either immersion in sodium hypochlorite solution for 2 minutes or allowed to dry.

- To lessen the risk of spreading the disease between frogs as a result of handling, one pair of gloves or one plastic bag per sample (for collecting techniques that crowd frogs) or per frog (for individual frog collecting techniques) should be used.
- Never handle live, sick or dead frogs with bare hands.
- During monitoring or other field surveys that require toe clipping or other surgical procedures, care must be taken to use disposable sterile instruments. Alternatively, instruments should be sterilised between uses on frogs by dipping in 70% ethanol for at least 30 seconds and wiped off with a clean paper towel.
- Continuous maintenance of the sprinkler system is a necessity, however, care must be taken to restrict the number of visits to the wetlands and the number of personnel to the minimum. This will ensure the least disturbance in the wetlands and lower the risk of introducing diseases. Water temperature above 50°C is highly effective for killing chytrid fungus (Johnson et al. 2003) and could be used to disinfect maintenance equipment by immersion.

Threat: Deterioration of Spray Wetland Habitat

Action: Maintain Hydrological Integrity of Spray Wetlands

The continued functioning of the sprinkler system at remaining populated sites as well as extinction sites is vital for the continued survival of the species. Natural and induced re-colonisation of spray wetlands can only occur if the habitat is maintained in a pristine condition.

Threat: Negligent Alteration of Flow Regime

Action: Coordinate Release of Water into Kihansi Gorge

Together TANESCO and LKEMP should coordinate the implementation of high bypass flows to prevent any adverse effects that it might have on the spray toads and spray wetland ecosystem. Channing et al. (2003) recommended that only flows of more than 10 m^3/s should be considered for maintaining conditions in the spray wetlands (e.g. controlling the height of encroaching taller vegetation species).

Action 3 Increase the Total Population to a Viable Size

Maintain the Captive Breeding Programme

The captive breeding colonies at the USA zoos should be maintained for at least the time necessary to rear progeny that can be used for experimental trials aimed at increasing its chances of survival and for possible re-introduction into Kihansi Gorge.

- *Re-establish Breeding Colony at Kihansi Gorge*

If successful, animals produced from the captive breeding programme can be re-introduced into Kihansi Gorge. Because of the eminent risk of re-introducing chytridiomycosis along with the captive-bred toads, all animals should be screened with non-invasive diagnostic PCR techniques for chytridiomycosis prior to their release into the wild. Should chytridiomycosis be detected in any of these toads, the entire batch should be treated prophylactically for the infection followed by at least a month of observation in quarantine before attempting re-introduction. The survival and progress of the re-introduced toads should be carefully monitored to determine whether the population has resumed breeding activity and established a viable progeny *in situ*.

Action 4 Prepare and Implement a Rigorous Monitoring Programme

Monitoring is required to determine whether population declines are continuing, or whether they are part of a long-term fluctuation cycle in population size. Such monitoring is also essential when planning a re-introduction programme and for future experimental trials that might involve habitat manipulation or translocation. The following considerations should form part of the monitoring programme:

- Monitoring should be designed to cause the minimum disturbance to spray wetland habitat and to prevent injury to spray toads.
- It is important that not only the remaining populations be monitored but also all extinction sites in the event that natural re-colonisation occurs.
- Any dead or diseased frogs that are encountered during monitoring and other surveys should be collected and submitted for post-mortem examination.
- Personnel should strictly follow the disease hygiene protocol during fieldwork to prevent inadvertent transmission of disease organisms.

The monitoring methods for spray toads employed during the IREM and LKEMP projects have been evaluated and revised by Gibbs (2003) to form part of a monitoring protocol for the lower Kihansi Gorge ecosystem that will be sustainable over the long-term. The programme focuses on a key set of indicators of the ecological condition of the gorge that were selected on the basis of their information content and ecological relevance, measurement costs, technical feasibility and quality of baseline data. The method for spray toad monitoring involves direct counts on permanent rock plots in the Upper spray wetland and vegetation transects in all spray wetlands.

9.5 Conclusion

The failed efforts to detect *N. asperginis* during our survey and subsequently by LKEMP staff could predict the extinction of this species in the wild. Only extensive searching over time will confirm this. At the time of writing, no live spray toads had been found since August 2003 ([11]J. Thompson pers. com. 2005). The next survey of the amphibians of Kihansi Gorge and the spray wetlands in particular is scheduled for early June 2005 ([12]W. Sarunday pers. com. 2005). The reasons for the Kihansi decline are not completely understood. Multiple factors that might have acted in combination with chytridiomycosis may have been involved. It is suspected that the incidence of chytridiomycosis in *N. asperginis* played a leading role in the species decline. This hypothesis is supported by 1) the rapid rate at which the decline took place, 2) the association of *B. dendrobatidis* with dead toads and 3) the absence of *B. dendrobatidis* in the years leading up to the decline. The case of the Kihansi spray toad marks the first report of a rapid population decline and possible species extinction in the wild of an amphibian species in Africa and the first that is linked to chytridiomycosis. Part of the earlier mitigation measures for this species was the establishment of a captive population in the United States. These animals are the last remaining individuals and final refuge of the species. Recovery of the species is possible only provided that successful breeding commences, sufficient funds be generated to cover the expenses and successful re-introduction strategies are developed and implemented.

An eminent danger is the likelihood that the amphibian chytrid fungus could spread to other amphibians in the region, which could lead to similar results in species that are susceptible to disease. The Udzungwa Mountains form part of the Eastern Arc Mountains, one of the top 25 biodiversity hotspots (Myers et al. 2000), and is well known for its diverse and endemic amphibian species (Jenkins 1992, Burgess et al. 1998). A document containing amphibian disease management for Kihansi Gorge, as discussed in this chapter, was presented by the candidate at the 2nd Expert Panel Meeting of LKEMP in Washington D.C. in January 2004. Some of the recommendations were accepted and have since been implemented at Kihansi Gorge. LKEMP have agreed to further investigate the role of pesticides in the decline event, to implement the field hygiene protocol and to continue to monitor the spray wetlands in search of the spray toads. Maintenance of the sprinkler system and monitoring of spray wetlands, among other threats, imply that the opportunity for human induced transmission of the amphibian chytrid fungus remains high. Apart from the advantages of staff and researchers practicing sterilisation procedures for the well being of amphibians at Kihansi Gorge, it can restrict the spread of diseases to other areas.

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[11] Dr Jeremy Thompson, Technical Advisor LKEMP, Dar es Salaam, Tanzania
[12] Dr Wilfred Sarunday, Project Manager LKEMP, Dar es Salaam, Tanzania

Chapter 10

FINAL DISCUSSION



10.1 Background

Through retrospective disease surveys of archived amphibians that died during events of mass mortality and from sites that experienced population declines or extirpation of amphibian species, we know that chytridiomycosis has threatened amphibians at least for the past four decades. Very little evidence of the pathogen is available from before 1960. This sudden emergence of chytridiomycosis raises the question of why have amphibians only recently experienced the rapid adverse effects that the fungus is capable of causing. One explanation holds that *B. dendrobatidis* is a widespread organism that has emerged as a pathogen of frogs because of either an increase in virulence or increased host susceptibility caused by other factors (Daszak et al. 1999). Contrary to the first explanation, *B. dendrobatidis* may be an introduced pathogen spreading through naive populations (Berger et al. 1998, Daszak et al. 1999). If the amphibian chytrid was introduced into the affected areas, then where was the original source or area of origin? These were the questions that lead to the study of investigating the occurrence chytridiomycosis in South Africa; an area from which nothing was known about the disease apart from a single incidence in *Xenopus laevis* from the Cape (Speare and Berger 2000). The aim was set to determine whether chytridiomycosis had been introduced into the country in recent years and what effect the disease has had on wild frog populations. Because there were no published reports of rapid population declines of South African frogs and associated mass mortality events to guide the investigation it was decided to focus the initial efforts on infection in *X. laevis*. Not only was it known that *X. laevis* was a host of *B. dendrobatidis*, but the species' area of occurrence included almost the entire country and could thus yield data on the geographical distribution of the amphibian chytrid throughout the country. In addition, *X. laevis* was a commodity being utilised as a laboratory and pet animal and was therefore regarded as a high-risk species for disseminating the fungus internationally.

To address the research problem, the main objective of the study was to determine the geographical and temporal distribution of *B. dendrobatidis* in South Africa, as well as the rest of Africa by surveying both extant frogs and archived specimens. Of equal importance was to assess the role that the *X. laevis* trade has played in spreading chytridiomycosis to other countries because this related to the "introduced pathogen" hypothesis. The amphibian chytrid

Histological evidence of chytridiomycete fungal infection in a free-ranging amphibian, *Afrana fuscigula* (Anura: Ranidae), in South Africa

E P Lane[a*], C Weldon[b] and J Bingham[c]

ABSTRACT

The 1st recorded histological evidence of chytridiomycete fungal infection in a free-ranging ranid amphibian in South Africa is presented. Literature on causes of a worldwide decline in amphibian populations is briefly reviewed.

Key words: amphibian, Chytridiomycosis, dermatitis, fungus.

Lane E P, Weldon C, Bingham J **Histological evidence of chytridiomycete fungal infection in a free-ranging amphibian, *Afrana fuscigula* (Anura: Ranidae), in South Africa**. *Journal of the South African Veterinary Association* (2003) 74(1): 20–21 (En.). Department of Pathology, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa.

Declining amphibian populations are a worldwide concern. Proposed aetiological factors, either singly or in combination, include habitat destruction; changes in weather patterns; pollution of water supplies with urea, nitrates, carbaryl, paraquat and petroleum; increased road traffic; increased exposure to ultraviolet radiation; and infectious diseases such as chytridiomycosis, saprolegniasis and infection with *Aeromonas hydrophila* or teratogenic trematodes[1,3,7,9–11,13,14,17,18,21–23].

One of the most commonly identified infectious causes of mortality is cutaneous or oral chytridiomycosis caused by the chytridiomycete fungus, *Batrachochytrium dendrobatidis*[3,8]. Dermatitis associated with a chytridiomycete was described from a number of captive anurans before the original discovery of the amphibian chytrid[15]. Clinical symptoms associated with chytridiomycosis include abnormal behaviour and body posture, as well as excessive sloughing of the skin[4].

Chytridiomycosis has been associated with population declines in Australia, New Zealand, North, Central and South America and, most recently Spain[4,6,16,19]. Amphibian cutaneous chytridiomycosis has been reported in wild *Xenopus laevis* frogs from the Western Cape Province[24]. Retrospective studies of archived material in South African museums have also identified the amphibian chytrid in South African frogs (Weldon, unpubl. data). *B. dendrobatidis* may either have been recently introduced into South Africa or it might have evolved within an African host species such as *Xenopus* spp., and been disseminated around the world in *Xenopus* used as laboratory animals[19]. This communication reports the presence of *B. dendrobatidis* zoosporangia in the superficial epidermis of a Cape river frog (*Afrana fuscigula*) collected in the Eastern Cape province of South Africa; and is the 1st confirmation of the disease in a ranid frog from South Africa.

As part of a small initial survey of free-ranging amphibians in South Africa, a freshly dead amphibian, identified as *Afrana fuscigula*, was collected from Kologha Forest, near Stutterheim, in the Eastern Cape (32°32'29"S, 27°21'57"E). No macroscopic lesions were noted, and the whole frog was preserved in 10 % formalin.

Routine histological examination of strips of the abdominal skin and whole severed feet was performed on paraffin-embedded sections (6 μm), which were stained with haematoxylin and eosin, as well as with periodic acid Schiff, and Gomori methamine silver stain[2]. Near the ends of the toes, and in the crevices between the toes, the superficial epidermis contained rare fungal structures consistent with the zoosporangia of *B. dendrobatidis* (Fig. 1). These were often empty spherical structures (up to 8 μm in diameter) whose walls stained strongly with silver stains, but weakly with periodic acid Schiff stain. A few contained 4–6 basophilic zoospores, but discharge papillae were rarely seen. Associated changes in the epidermis included moderate diffuse epidermal hyperplasia and oedema. Sloughed empty zoosporangia and necrotic epithelial cells lay on the surface of the skin. Scattered degenerative

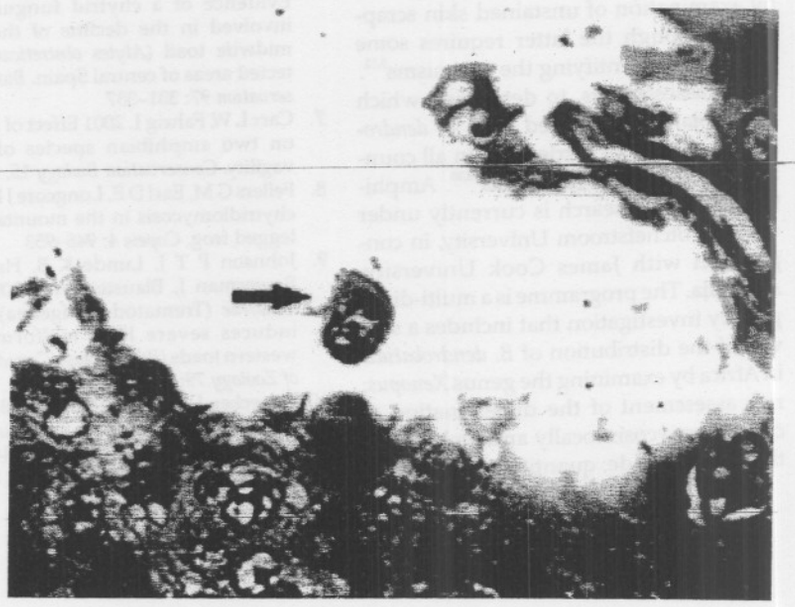


Fig. 1: **A zoosporangium, containing 4 zoospores** (arrow), **in a sloughed epithelial cell** (H&E, ×100).

[a]Department of Pathology, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa. E-mail: emily.lane@hixnet.co.za

[b]School of Environmental Sciences and Development, Potchefstroom University, Private Bag X6001, Potchefstroom, 2520 South Africa.

[c]CSIRO – Australian Animal Health Laboratory, Private Bag 24, Geelong, Victoria 3216, Australia.

*Author for correspondence.

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and necrotic epithelial cells and clefts filled with granular amphophilic cellular debris in the superficial epidermal layers were present. The epidermis and superficial dermis contained small numbers of heterophils. In addition, multifocal rare foci of acute mild necrotising purulent dermatitis were present, not usually in proximity to zoosporangia. These were characterised by large numbers of heterophils in the dermis admixed with small numbers of large pigment-laden cells and fibrin, and moderate heterophilic dermal leucostasis. No infectious agents were seen in or adjacent to these lesions. Remaining tissues showed no other lesions. A mild infestation of intestinal helminths was noted, which was not associated with enteritis.

The detection of cutaneous chytridiomycosis in *A. fuscigula* adds yet another amphibian species to the extensive list of free-ranging and captive hosts. It is not clear whether or not the chytrid infection caused the death of this frog, since the separate necrotising dermatitis may also have played a role. No other infectious agents or life-threatening lesions were noted in the frog. No infectious agent, including *B. dendrobatidis*, was seen in association with the necrotising dermatitis. *B. dendrobatidis* is often associated with other pathogens and has been considered the primary pathogen in a number of epizootics of amphibians[3,12,16].

This report highlights the importance of the value of *post-mortem* samples in the study of the epidemiology and distribution of chytridiomycosis in the subcontinent and the rest of Africa. Chytridiomycosis can be diagnosed by routine histology of skin specimens preserved in 10 % neutral buffered formalin, as well as by examination of unstained skin scrapings, although the latter requires some expertise in identifying the organisms[5,12].

Risk assessments, to determine which amphibians are infected with *B. dendrobatidis*, should be carried out in all countries, including South Africa[19,20]. Amphibian disease research is currently under way at Potchefstroom University, in conjunction with James Cook University, Australia. The programme is a multi-disciplinary investigation that includes a survey of the distribution of *B. dendrobatidis* in Africa by examining the genus *Xenopus*; risk assessment of the dissemination of chytridiomycosis locally and globally via the *X. laevis* trade; quantifying the factors that modify the interaction between *X. laevis* and *B. dendrobatidis*; and determining the level of genetic variation in *B. dendrobatidis* from Africa. Information on the epidemiology, pathogenesis and phylogeny of chytridiomycosis will help to elucidate its role as a pathogen of African amphibians.

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Annexure B

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Origin of the Amphibian Chytrid Fungus

Ché Weldon,* Louis H. du Preez,* Alex D. Hyatt,† Reinhold Muller,‡ and Rick Speare‡

The sudden appearance of chytridiomycosis, the cause of amphibian deaths and population declines in several continents, suggests that its etiologic agent, the amphibian chytrid *Batrachochytrium dendrobatidis*, was introduced into the affected regions. However, the origin of this virulent pathogen is unknown. A survey was conducted of 697 archived specimens of 3 species of *Xenopus* collected from 1879 to 1999 in southern Africa in which the histologic features of the interdigital webbing were analyzed. The earliest case of chytridiomycosis found was in a *Xenopus laevis* frog in 1938, and overall prevalence was 2.7%. The prevalence showed no significant differences between species, regions, season, or time period. Chytridiomycosis was a stable endemic infection in southern Africa for 23 years before any positive specimen was found outside Africa. We propose that Africa is the origin of the amphibian chytrid and that the international trade in *X. laevis* that began in the mid-1930s was the means of dissemination.

One of the biggest threats facing amphibian species and population survival worldwide is the disease chytridiomycosis, caused by the chytrid fungus, *Batrachochytrium dendrobatidis* (1,2). Chytridiomycosis was proposed as the cause of death in frog populations in the rain forests of Australia and Panama and was associated with the decline of frog populations in Ecuador, Venezuela, New Zealand, and Spain (3–6). Evidence for a country-wide decline in frog populations in South Africa is lacking (7), and local declines of several species have been ascribed to two main threats, habitat destruction and pollution (8). Chytridiomycosis is known in South Africa from infections in *Xenopus laevis, Afrana fuscigula*, and *Strongylopus grayii* (9–11). Through surveys of extant and archived specimens, *Batrachochytrium* has been found in every continent that has amphibians, except Asia (6,9,12,13). Since *B. dendrobatidis* has been recognized as an emerging pathogen, whose spread is facilitated by the international and intranational movement of amphibians (1), identifying its origin will be useful.

Some emerging infectious diseases arise when pathogens that have been localized to a single host or small geographic region go beyond previous boundaries (14). If *B. dendrobatidis* emerged in this fashion, we hypothesize that the source would meet the following criteria: 1) the hosts would show minimal or no apparent clinical effects, 2) the site would be the place of the earliest known global occurrence, 3) the date of this occurrence would precede any amphibian declines in pristine areas (i.e., late 1970s), 4) the prevalence in the source host or hosts would be stable over time, 5) no geographic spreading pattern would be observed over time in the region, 6) a feasible means of global dissemination of *Batrachochytrium* from the region of origin would be identified, and 7) *B. dendrobatidis* would show a greater genetic variation in the host region than in more recently invaded regions.

B. dendrobatidis is common in African frogs from Ghana, Kenya, South Africa, and Western Africa (12,15) and declines in frog populations are poorly documented in Africa (7,16). These factors, combined with the global trade in *X. laevis* and *X. tropicalis*, prompted us to investigate the likelihood that Africa was the origin of *Batrachochytrium* and that the trade in *Xenopus* spp. played a key role in its global dissemination. Within the *Xenopus* genus, *X. laevis* is distributed over the greatest area in sub-Saharan Africa. *X. laevis* occupies most bodies of water in savannah habitats from the Cape of Good Hope to Nigeria and Sudan (17,18).

We report the earliest case of the amphibian chytrid found in any amphibian and present epidemiologic evidence to support the hypothesis that *B. dendrobatidis* originated in Africa. In this article, chytridiomycosis refers to infection of amphibians by *B. dendrobatidis*.

Materials and Methods

A retrospective survey was conducted on archived specimens of the genus *Xenopus* housed in five southern Africa institutions, Bayworld (Port Elizabeth), Natal

*North-West University, Potchefstroom, South Africa; †CSIRO, Geelong, Australia; and ‡James Cook University, Townsville, Australia

Museum (Pietermaritzburg), National Museum (Bloemfontein), South African Museum (Cape Town), and Transvaal Museum (Pretoria). Specimens in these museums had been collected for archiving by a large number of persons for various purposes and had not been selected for a systematic survey of amphibian disease. Specimens were collected mainly from South Africa, Lesotho, and Swaziland. A piece (3 x 3 mm) of the interdigital webbing was removed from one hind foot of each specimen of *X. gilli*, *X. muelleri*, and *X. laevis*. Tissue was prepared for histologic examination with routine techniques (19). Sections were cut at 6 µm and stained with hematoxylin and eosin. Chytridiomycosis was diagnosed by using described criteria (20). Sections from the two specimens diagnosed as having chytridiomycosis with hematoxylin and eosin before 1971 (one collected in 1938, the other in 1943) were confirmed with the more specific immunoperoxidase test (21) to increase the confidence of the diagnosis. Measurements of sporangia were performed with a calibrated eyepiece and expressed as mean ± standard deviation (SD). Histologic slides were examined "blind," without reference to dates that the frogs were collected, to decrease any opportunity for bias in diagnosis.

Exact versions of chi-square tests were used to analyze bivariate associations between chytridiomycosis prevalence and host species, region in South Africa (southwestern, eastern, and central), and season. Bivariate time trends of prevalences were analyzed by exact chi-square tests for trend. Multivariate logistic regression models were applied to assess potential confounding effect of species, region, and season on the time trend of chytridiomycosis prevalence. Confidence intervals (CI) were calculated by using exact binomial probabilities. Longitudinal and latitudinal historical patterns of spread were analyzed with linear regression models.

Results

Zoosporangia with a diameter (mean ± SD) of 5.2 ± 0.72 µm (maximum 6 µm) were seen in the stratum corneum of the digital webbing of infected frogs (Figure 1). Most sporangia were empty spherical structures, but occasional sporangia were observed with developing stages, septa, or discharge papillae. The structures stained brown (indicating positivity) in the immunoperoxidase test with the specific anti-*Batrachochytrium* antibody (Figure 1). Lesions usually associated with chytridiomycosis, including hyperplasia of the epidermis and hyperkeratosis of the stratum corneum, were mild and localized to areas of infection.

Overall, chytridiomycosis prevalence from the survey was 2.7% (19 positives out of 697 specimens) and did not differ significantly across species (p = 0.7; Table 1). The earliest date for a chytridiomycosis-positive specimen was

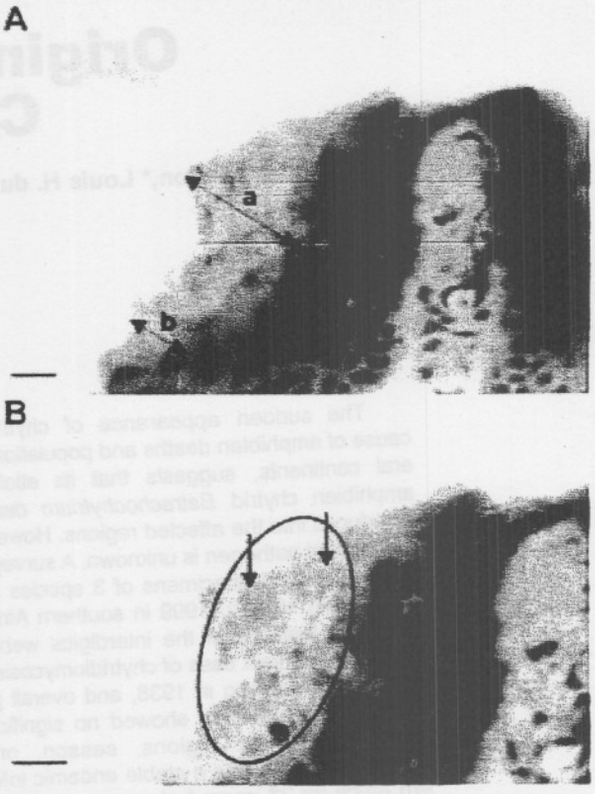


Figure 1. Micrographs of immunoperoxidase stained sections through the interdigital webbing of *Xenopus gilli*, showing the morphologic features and size of zoosporangia consistent with *Batrachochytrium dendrobatidis*. A) Arrow a indicates localized hyperplastic epidermal response; arrow b indicates an uninfected region of the epidermis. B) Arrows indicate two zoosporangia with internal septa. Circle indicates location of the infection in the stratum corneum. Bar, 10 µm.

1938 in an *X. laevis* collected from the Western Cape coastal lowland. This specimen is housed in the South African Museum, Cape Town (SAMZR 18927). The next earliest positive specimen detected was an *X. gilli* from 1943 (specimen number NMB 112, National Museum, Bloemfontein). The distribution of dates specimens were collected was greatly skewed to the latter half of the 20th century (Table 2). The breakdown for the time interval 1871–1940 is presented in order (decade, number of frogs infected/number of frogs examined) as follows: 1871–1880, 0/1; 1881–1890, 0/0; 1891–1900, 0/6; 1901–1910, 0/6; 1911–1920, 0/4; 1921–1930, 0/2; 1931–1940, 1/37. No statistically significant change of chytridiomycosis prevalence occurred over the decades since the 1940s (p = 0.36), or when the broader interval of pre-1971 is used as the baseline for the calculations (p = 0.22; Figure 2). No evidence for any trend in prevalence over time could be found using multivariate modeling where the odds ratios for the time intervals were adjusted

Table 1. Prevalence of chytridiomycosis in archived *Xenopus* spp. from southern Africa[a]

Species	No. examined	% positive (95% CI)	Earliest positive detected	Country
Xenopus laevis	583	2.6 (1.5–4.2)	1938	South Africa
X. meulleri	53	3.8 (0.5–13.0)	1991	Swaziland
X. gilli	61	3.3 (0.4–11.4)	1943	South Africa
Total	697	2.7		

[a]p = 0.7; CI, confidence interval.

for the potential confounders of species, season, and region. The multivariate odds ratios in these models were not significant and very similar to the bivariate findings, which indicate no confounding effects. The prevalence of chytridiomycosis in South Africa showed no significant change over time after 1940. No significant change of the geographic distribution of chytridiomycosis was detected after 1973. By 1973 the distribution of chytridiomycosis, as proved by positive specimens, covered already the area from 27° to 34° latitude and 18.25° to 32.5° longitude. This finding implies that positive specimens were detected from all regions of southern Africa by 1973. Infected frogs were found in 5 of the 9 provinces in South Africa, including the Western Cape (5 of 171), Northern Cape (2 of 22), Free State (6 of 141), Kwazulu-Natal (3 of 152), and Eastern Cape (1 of 137), as well as in Swaziland (2 of 42). Prevalence of *B. dendrobatidis* did not differ (p = 0.24) between the designated three broader regions with prevalences of 3.0% in the southwest, 3.8% in the central region, and 1.5% in the eastern region. Overall, the seasons (wet versus dry) when the specimens were collected were not significantly associated with prevalence (p = 0.22). Only in the eastern region, was a significantly higher prevalence found in the wet season than the dry season.

Discussion

Our study has extended the date for the earliest case of chytridiomycosis in wild amphibians by 23 years. The next earliest case outside South Africa was found in *Rana clamitans* from Saint-Pierre-de-Wakefield, Québec, Canada, in 1961 (22). After the case in Canada, the earliest cases from other countries follow sequentially over a period of 38 years from 1961 to 1999 (Figure 3).

X. laevis in the wild does not show clinical signs, nor has it experienced any sudden die-offs. Moreover, only subclinical chytrid infections have been observed among captive colonies of *X. laevis* (26,27). A frog of a related species, *X. tropicalis*, died in captivity from chytridiomycosis, it was suspected of having contracted the fungus from *X. laevis* (27). An ideal host for transmission of chytridiomycosis through international translocation would be a species of amphibian that does not become diseased or die from the infection; hence, *X. laevis* could take on the role of a natural carrier.

The sudden appearance of chytridiomycosis can best be explained by the hypothesis that *B. dendrobatidis* was recently introduced into new regions and subsequently infected novel host species (1). Dispersal of *B. dendrobatidis* between countries is most likely by the global transportation of amphibians (1,2,23,28,29). The World Organization for Animal Health has recently placed amphibian chytridiomycosis on the Wildlife Diseases List in recognition of this risk. If Africa is the source of *B. dendrobatidis*, a feasible route of dissemination by infected amphibians needs to be identified. Some members of the family *Pipidae* have been exported, in particular *Hymenochirus curtipes* and *X. laevis*, to North America and Europe (30).

In terms of a most likely candidate for spread from Africa, the number of frogs and geographic dissemination favor *X. laevis*. Soon after discovery of the pregnancy assay for humans in 1934 (30), enormous quantities of the species were caught in the wild in southern Africa and exported around the world. The pregnancy assay is based on the principle that ovulation in *X. laevis* is induced by injection with urine from pregnant women because of high levels of gonadotropic hormones in the urine (31,32). *X. laevis* was selected as the most suitable amphibian for investigating the mechanism of the mating reflex because of the relative ease with which the animal can be maintained in captivity (33). For 34 years, the trade in *X. laevis* in South Africa was controlled by the then Cape of Good

Table 2. Prevalence of chytridiomycosis in archived *Xenopus*, by time intervals[a]

Time interval	No. examined	No. positives	% positive (95% CI)
1871–1940	56	1	1.8 (0.0–9.6)
1941–1950	16	1	6.3 (0.2–30.2)
1951–1960	63	0	0.0 (0.0–5.7)
1961–1970	17	0	0.0 (0.0–19.5)
1971–1980	230	6	2.6 (1.0–5.6)
1981–1990	145	3	2.1 (0.4–5.9)
1991–2001	170	8	4.7 (2.0–9.0)
Total	697	19	2.7 (1.7–4.2)

[a]p = 0.36; CI, confidence interval.

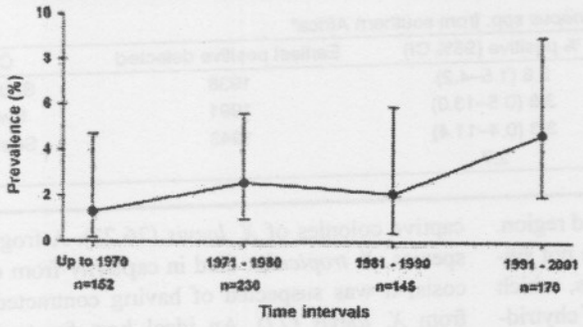


Figure 2. Historical time-trend of chytridiomycosis prevalence in southern Africa. No significant change was shown in the prevalence over time (p = 0.22, 95% confidence interval).

Hope Inland Fisheries Department (Western Cape Nature Conservation Board) at the Jonkershoek Fish Hatchery. As an indication of the numbers involved in this trade, 10,866 frogs were distributed in 1949, of which 3,803 (35%) were exported, and of the 20,942 frogs distributed in 1970, a total of 4,950 (24%) were shipped abroad (34,35). After the introduction of nonbiologic pregnancy tests, *X. laevis* became important as a model for the scientific study of immunity and later embryology and molecular biology. *X. laevis* could have carried the disease globally, particularly if the prevalence was similar to that seen in wild-caught *X. laevis* today. In the importing country, escaped frogs, the water they lived in (36), or both, could have come into contact with local amphibian species, and subsequent transmission of the disease could have followed. The establishment of feral populations of *X. laevis* in Ascension Island, the United Kingdom, the United States, and Chile in 1944, 1962, the 1960s, and 1985 (37), respectively, show that transmission could have become ongoing if these feral populations were infected.

Although we have demonstrated that *B. dendrobatidis* was in southern Africa since 1938, our studies provide no indication regarding whether this region was the original source within Africa. *B. dendrobatidis* has been found in wild frogs in Kenya and in frogs (*X. tropicalis* and *X. laevis*) wild-caught in Western Africa and detected after importation into the United States (12,26,27,38), which indicates that *B. dendrobatidis* is widely disseminated in Africa. *Xenopus* consists of 17 species that are found in sub-Saharan Africa, with a varying degree of sympatry between species (17). The overlap in the distribution and, in some cases, the sharing of habitats could facilitate transmission of *B. dendrobatidis* between these species. This finding would imply that chytridiomycosis could have originated elsewhere in Africa and spread within multiple host-region combinations. More detailed historical studies of archived African amphibians may indicate whether *B. dendrobatidis* was originally present in a small area of Africa from which it emerged to occupy large areas of the continent. Until the deficit in distribution data and comparative genetic studies is remedied, locating the source of the origin of *B. dendrobatidis* within Africa remains speculative. The relationship appears to have coevolved within an anuran host, and the opportunity to disseminate across the globe existed for *B. dendrobatidis* in southern Africa.

If *X. laevis* did carry *B. dendrobatidis* out of Africa as we propose, other amphibian species subsequently could have distributed it between and within countries. The American bullfrog, *Rana catesbeiana*, has been proposed as an important vector, mainly through international trade as a food item, but also within countries as populations established for the food trade escape and spread (29). The earliest current record for the occurrence of chytridiomycosis in *R. catesbeiana* is 1978 in South Carolina (38), 40 years after the first record in southern Africa, but details on the intensity of the search for chytridiomycosis in archived bullfrogs are not available. The transmission of chytridiomycosis globally may involve a series of key steps: 1) occurrence of *B. dendrobatidis* in an amphibian vector in southern Africa that is relatively resistant to disease (*X. laevis*), 2) sudden rise in 1935 of export trade in this vector because of technologic advances (*Xenopus* pregnancy test), 3) escape of the pathogen from the exported *Xenopus* to establish new foci in other countries (possibly expedited in some countries by establishment of feral populations of *X. laevis*), 4) transmission into other vector amphibians (food and pet trade), and 5) further transmission to other countries along different trade routes in key amphibian vectors that move in high numbers and become established in commercial populations and closely interact with wild

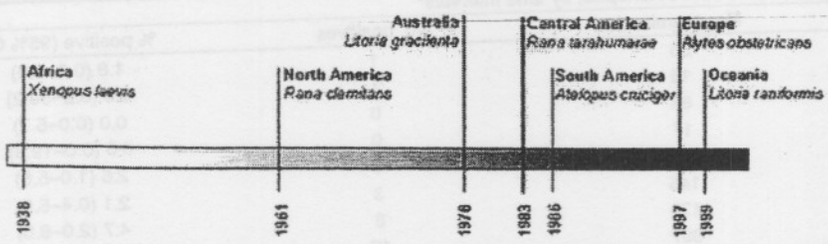


Figure 3. Time bar indicating when chytridiomycosis first appeared in the major centers of occurrence in relation to each other. Following a 23-year interruption in occurrences after the *Xenopus laevis* infection in 1938, records outside Africa appear with increasing frequency up until the present; North America (22), Australia (2,23), South America (5), Central America (24), Europe (6), Oceania (New Zealand) (25).

frogs, which likely leads to feral populations (food frogs *R. catesbeiana*). Spread through native amphibian populations with epidemic disease in some species could have occurred at any point after *B. dendrobatidis* entered a naïve native species.

We have provided epidemiologic evidence that Africa is the origin of the amphibian chytrid fungus. Support for six of the seven criteria proposed for the source of *B. dendrobatidis* has been demonstrated: 1) the major host (*X. laevis*) shows minimal or no apparent clinical effects, 2) site of the earliest global occurrence (1938), 3) this date precedes any amphibian declines in pristine areas, 4) the prevalence in the source host or hosts (*Xenopus* spp.) has been stable over time, 5) no geographic spreading pattern could be observed over time, and 6) a feasible means of global dissemination exists via the international trade in wild-caught *X. laevis*, which commenced in 1935 and continues today. Criterion 7, greater genetic diversity of *B. dendrobatidis* at the source, has not been investigated. A low level of genetic variation was shown for 35 strains of *B. dendrobatidis* and suggested that *B. dendrobatidis* was a recently emerged clone (39). The strains had been collected in North America, Australia, Panama, and Africa from wild and captive amphibians. Three strains isolated from captive *X. tropicalis* in United States had been imported from Ghana. Although these showed no significant differences from the U.S. strains (39), their assignment to Africa assumes no cross-infection had occurred within the importing facility. Future work on the genetic diversity of *B. dendrobatidis* in Africa compared with strains from regions outside Africa will add weight to the hypothesis if greater genetic diversity is found in African strains.

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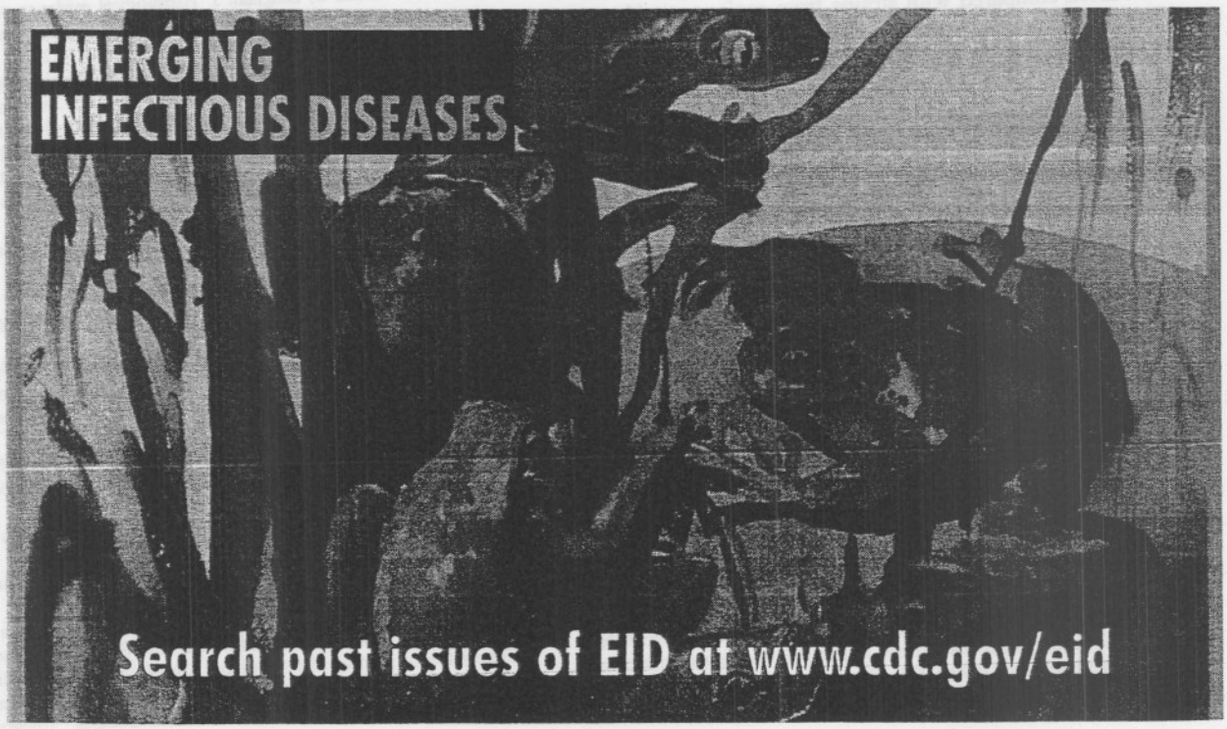
Mr. Weldon is a Ph.D. candidate and research assistant at the School of Environmental Sciences and Development, North-West University, South Africa. His research interests include the role of disease in amphibian declines, the effect of pesticides on amphibian biology, and the captive husbandry of *Xenopus*.

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Address for correspondence: Ché Weldon, School of Environmental Sciences and Development, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa; fax: 27-18-299–2503; email: drkcw@puk.ac.za



was isolated and cultivated from infected frogs and isolates were submitted for molecular analyses to help elucidate uncertainties concerning the origin of *B. dendrobatidis*. Additional objectives were to examine the macromolecular structures of *B. dendrobatidis* for the first time using confocal microscopy and to develop a technique that will enable investigators to quantify infection with chytridiomycosis in live amphibians. While these investigations were underway the Tanzanian endemic toad, *Nectophrynoides asperginis* suffered a major population crash and it was decided to investigate the possible involvement of chytridiomycosis in the decline event.

10.2 Origin and Dissemination of *B. dendrobatidis*

The screening of archived specimens allowed us to establish that *B. dendrobatidis* was not recently introduced into South Africa. The oldest record detected was 1938 in *X. laevis* from the Cape, thus making it the earliest case of *B. dendrobatidis* yet found anywhere in the world by 23 years. From the geographical distribution and prevalence data of *B. dendrobatidis* in three species of *Xenopus*, *X. laevis*, *X. meulleri* and *X. gilli* it was shown that chytridiomycosis was a stable infection since the 1930s and that the pathogen could not have been introduced and spread from a specific area. Of further significance was that the host, *X. laevis* does not become diseased or die from chytridiomycosis and could therefore take on the role of a natural carrier. These bits of epidemiological data lead to the formulation of the "out of Africa" hypothesis, namely that *B. dendrobatidis* originated in Southern Africa, co-evolved in one of several hosts, such as *X. laevis* and was disseminated to other parts of the world through the trade in *X. laevis* that commenced in the early 1930s (Weldon et al. 2004). The epidemiological evidence pointed to a southern African origin, but the involvement of the *X. laevis* trade in spreading the disease still lacked substantial evidence. In addition, a study of *B. dendrobatidis* from South Africa at the molecular level had not yet been conducted. The level of genetic variation in South African strains would have implications for an African origin because greater genetic variation would be expected from the source population.

With the cooperation of the Western Cape Nature Conservation Board (WCNCB) we were able to attain quantitative data on the export of *X. laevis*. Over the past seven years the trade consisted of approximately 10,000 frogs per annum that involved 124 import facilities from 31 countries. All the while, frogs were being collected and confined in ways that permitted an open spread of infectious diseases in the field and between sources and supply houses. Suppliers therefore had to be informed of those handling practices that promote the spread of amphibian diseases and of mitigation measures required to decrease the related risks. First the risks of *X. laevis* disseminating *B. dendrobatidis* had to be assessed before mitigation measures could be developed and to verify the role of *X. laevis* in the "out of Africa"

hypothesis. Chytridiomycosis screenings were carried out on *X. laevis* from wild populations utilised by suppliers, frogs obtained from supply houses and on feral populations. In doing so it was established that *B. dendrobatidis* was present in *X. laevis* intended for export and in feral frogs, thus demonstrating that *X. laevis* could spread chytridiomycosis through the scientific trade. The most effective way of intervening in the dissemination pathway is by containing the disease within the country through a management protocol that functions around controllable units based on the steps that structure the trade pathway. These control units include the handling of frogs in the field, husbandry practices at the supplier facilities and import/export regulations. The Veterinary Services Directorate of the Department of Agriculture could potentially play a much larger role in controlling the traffic of *B. dendrobatidis* in *X. laevis* because the responsibility for animal health certification resides within this directorate. Currently, animals intended for export are only quarantined and submitted to veterinary diagnostic testing if the import country requires it. Moreover, staff from Veterinary Services has not received formal training in the diagnosis of chytridiomycosis. Consequently infected animals are leaving the country unchecked.

Besides the unwanted export and consequent transmission of infectious disease, there are other conservation implications related to the *X. laevis* trade. Although *X. laevis* is very common in South Africa and not threatened by the numbers that are being removed from natural sources, irresponsible collection practices could easily lead to local extirpation of populations (Weldon 1999). Imported animals that escape from captivity have a good chance of establishing feral populations due to a high fecundity and the species' ability to tolerate a wide range of environmental conditions. Being generalist predators of the aquatic environment means *X. laevis* can compete with native amphibian and fish communities for prey, however, the nature of such inter-predator relationships remains inconclusive (Laferty and Page 1997, Measey 1998). The likelihood of chytridiomycosis transmitting to novel amphibian species is increased when *X. laevis* escape and establish feral populations. It is predicted that the risk of *X. laevis* spreading chytridiomycosis will gradually decrease when mitigation measures are put into practice and because the demand for the species has declined in recent years and continues on a downward trend. Transmission into other vector amphibians (food and pet trade) such as *Rana catesbeiana* (Mazzoni et al. 2003) could in turn spread the disease to other countries along different trade routes.

Despite the attempted isolation of *B. dendrobatidis* from a wide range of localities and frog species only five strains from two localities survived to the stock culture phase. These five strains were used in a population genetic study to establish whether the relationships and genetic variation of the known global *B. dendrobatidis* population is indicative of an African

origin. The analyses did not conclusively show that *B. dendrobatidis* originated in Africa. Genetic diversity in the South African strains was low, and genotypes differed little from those of putative Ghana isolates. However, it is too early to reject the "out of Africa" hypothesis because 1) strains from only two regions in South Africa were included in the test sample, 2) both these regions had distinct genotypes, 3) the data set showed a general bias towards North American strains and 4) the population structure was incomplete in terms of global representation. Regardless of the final outcome of analyses based on multiple strains from all known regions of occurrence, the important role of *X. laevis* as a carrier of the disease via the scientific and pet trade cannot be dismissed.

10.3 Occurrence of Chytridiomycosis in African Anura

We know from this survey and from previous work (Speare and Berger 2000, Reed et al. 2000, Morehouse et al. 2003) that *B. dendrobatidis* is widespread in Africa. Its known distribution includes southern, western and eastern Africa. The *Xenopus* survey did not detect chytridiomycosis north of Botswana despite the inclusion of 12 species and 19 countries in the survey. It was demonstrated that failure to detect infected individuals could be related to insufficient sample sizes. The known geographical distribution of chytridiomycosis in Africa is therefore likely to be an under-representation of the true distribution. A survey of archived specimens that focuses on a particular species or group of amphibians is most effective when the survey is guided by reports of population declines. Where no such reports are available, surveys would have to include large sample sizes from a number of localities within each region. The distribution of the search effort by country was reflected in the outcome of chytridiomycosis cases, as 80% of the cases were from South Africa from which the majority of specimens (archived and live) were examined. The distribution of chytridiomycosis in South Africa is not restricted to any specific region or species because new localities were found in every region that was surveyed and infected individuals were detected from all five families included in the survey. This resulted in South Africa being the only country on the continent for which detailed information on chytridiomycosis occurrence is available. The ability of *B. dendrobatidis* to infect any amphibian species is similar to what was known from infection patterns in other countries, but the omnipresence of chytridiomycosis in such a large area with little evidence of adverse host population effects is unusual.

Very little evidence was found to implicate *B. dendrobatidis* with host mortality. Only three cases of chytridiomycosis associated amphibian deaths were detected, all three in the Cape river frog, *Afrana fuscigula*. Even in this species, the incidences appear to be sporadic and associated with population recovery. There is more evidence that *A. fuscigula* is predominantly tolerant to chytridiomycosis and even able to clear the infection when maintained in captivity

than evidence for a highly susceptible species. Generally, it does not appear as if any species in South Africa is currently threatened by chytridiomycosis because none of our 25 threatened species were assigned their conservation status because of rapid population declines synonymous with the disease (see Harrison et al. 2001). The wide distribution of chytridiomycosis, low virulence to South African frogs and associated mild pathological reaction in the hosts fulfil the criteria for an endemic disease in support of the "out of Africa" hypothesis. In spite of the indications of an endemic disease, chytridiomycosis should still be regarded as a potential threat because the synergistic effect of infection with a range of co-factors has been known to be fatal to amphibians (Daszak et al. 1999, Bosch et al. 2001, Daszak et al. 2003, Burrowes et al. 2004). Those species that are characterised by a limited extent of occurrence or area of occupancy, such as the Cape platanna (*Xenopus gilli*) and the sympatric Micro frog (*Microbatrachella capensis*) are particularly threatened by becoming extirpated from certain areas in the event of an epidemic.

The decline of the Kihansi spray toad, *Nectophrynoides asperginis* was the first report of a rapid population decline of an amphibian species in Africa and the only to be associated with chytridiomycosis. Ample evidence was gathered to implicate *B. dendrobatidis* as the major cause of the decline following the introduction of the pathogen into Kihansi Gorge shortly before the population crash. However, it appears as if a cascade of events caused by the construction of the Lower Kihansi Hydropower Plant might have predisposed *N. asperginis* to a lethal *B. dendrobatidis* infection. There has been debate on whether the observed trend in the Kihansi spray toad population size was due to natural fluctuation or part of seasonal changes in abundance. Natural fluctuation is refuted because of the magnitude (>99% of individuals) and rapidity (3-4 weeks) of the decline and the lack of any subsequent recovery 22 months post-decline. The possibility of species extinction in the wild was proposed because no toads have been found in surveys following the decline. The possibility of remnant populations also was ruled out because of the strict niche specifications and extreme restricted range of the spray toads. A captive colony of *N. asperginis* in US zoos that was initiated in 2000 could be the final refuge of the species.

10.4 New Technology

In the past, various parameters, including degree of chytrid numbers, percentage of skin surface infected and layers of infected keratinaceous cells have been used to express the intensity of *B. dendrobatidis* infection during diagnostic examination of case reports or experimental transmission experiments (Nichols et al. 2001, Burrowes et al. 2004, Daszak et al. 2004, Hanselmann et al. 2004). A need therefore existed for a standardised technique by which *B. dendrobatidis* infection could be quantified. A simple technique was developed that

entails the counting of sporangia on sloughed skin that allows the expression of infection in terms of sporangium density. The quantification technique has additional applications in that it allows the selection of those parts of the amphibian foot that will provide the best chance of detecting infection through histology, and it allows the calculation of the probability of detecting infection through a given number of histological sections. The new quantification technique can make a positive contribution to comparative experimental and basic chytridiomycosis research in the fields of pathology, immunology and epidemiology. It was demonstrated that site preference on the foot is related to likelihood of transmission through contact in that those areas that are most in contact with the substrate or most likely to come in contact with other amphibians (e.g. during amplexus) have the highest sporangium density counts. The observation also was made that zoospores are not likely to infect hyperkeratotic skin, resulting in sporangium clusters that spread concentrically around an expanding core of empty, hyperkeratotic tissue. It was hypothesised that zoospores require skin tissue with normal anatomical arrangements to infect or that a local resistance factor to *B. dendrobatidis* may be involved.

10.5 Future Directions Regarding Chytridiomycosis in Africa

10.5.1 Legislation and Control

- Countries involved in the movement of amphibians should enforce control and restrictions over the trade with regards to chytridiomycosis. Member states of the OIE (World Animal Health Organization) have to comply with the requirements of this organization regarding quarantine restrictions and health certification of amphibians.
- Similarly, policy should be adopted to screen imported amphibians with specific diagnostic tests to determine the prevalence of *B. dendrobatidis.* Amphibians found sick or dead upon arrival should be submitted for examination of diseases.
- Disease prevention and management protocols that minimise the spread of diseases should be enforced on breeding facilities and supply houses of amphibians. Such protocols should include regular monitoring, treatment regimes and accreditation to facilities that comply with the standards set by the protocol.

10.5.2 Surveillance and Research

- All countries should conduct chytridiomycosis surveys so that the geographical distribution of the fungus on the continent can be mapped and to establish which areas within a country are chytrid-free and which are not.
- All countries should be regarded as infected and a rigorous pathogen prevention protocol should be adopted during field surveys.

- As far as possible the same sampling strategy (site definition, sampling techniques, sample size etc.) should be adopted for countrywide surveys to yield reliable and comparative results.
- Risk assessments should be carried out in those countries involved with the amphibian trade (e.g. *X. tropicalis* from Ghana, pet trade in Madagascar) to determine which species take on the role of carrier host and to devise mechanisms to curtail this risk.
- A centre for the study and coordination of amphibian diseases in Africa should be established. The centre should be responsible for innovative research, training of students and others, and should be involved with the development of disease management policy. The centre should not restrict its activities to chytridiomycosis, but expand its diagnostic capabilities to include necropsy under a dissecting microscope, virus cultures, aerobic bacterial cultures, parasite identification, and histological examinations. A database of case reports based on post-mortem examinations of specimens should be maintained.
- An emergency response mechanism should be put in place by the African amphibian disease centre that will allow rapid reaction to amphibian disease outbreaks. All movements of frogs should be restricted to and from a site of mass mortality at least until an investigation that includes a disease survey has been completed.
- In South Africa, threatened amphibian species should be regarded as high-risk species for acquiring chytridiomycosis and should receive high priority for chytridiomycosis screening.
- An amendment for the Red Data Book of South African frogs should be written that contains data on those already threatened species to which chytridiomycosis should be added as an additional threat.
- In the light of the importance of environmental factors to amphibian health, research concerning the causes of amphibian decline should follow a multidisciplinary approach, involving herpetologists, veterinarians, microbiologists, toxicologists etc.
- More isolates from more localities are needed to help elucidate the population structure of *B. dendrobatidis* through genetic analyses such as multi locus sequence typing.

10.5.3 Training and Education

- Collaboration and workshops should be arranged between countries with expertise in the diagnosis and management of chytridiomycosis and those lacking it. This will increase the number of able personnel in Africa that can participate in national surveys and allow countries to make use of their own expertise.
- Veterinarians and other pathologists responsible for animal health certification, such as the Veterinary Services of the Provincial Government of South Africa, should receive training in the diagnosis of chytridiomycosis.

- Individuals and organizations involved with the movement of amphibians within and between countries should be informed of the risks of disease dissemination and of the regional/national/international regulations governing these.
- The public should be informed of the role of diseases in amphibian declines and of the actions that promote the spread of disease through the use of television, radio, printed media, Internet, conventions etc.
- Groups that should receive special lectures regarding amphibian diseases because of their direct involvement with amphibians include staff from education departments, reptile and amphibian hobbyist clubs and angling clubs (in South Africa *X. laevis* is used as live bait).

10.6 Closing Statement

Collaboration with various international scientists has allowed the candidate to contribute to the wealth of new information and thinking with regards to amphibian diseases that has been accumulated since the discovery of *B. dendrobatidis*. There are numerous other chytridiomycosis research groups in the world already addressing important topics through various research disciplines that were not mentioned in the section on future research of this chapter. Through this international effort we come ever closer to understanding this eminent threat to global amphibian biodiversity.



Annexure A

Manuscript: Lane E.P., Weldon C. and Bingham J. 2003. Histological evidence of chytridiomycete fungal infection in a free-ranging amphibian, *Afrana fuscigula* (Anura: Ranidae), in South Africa. Journal of the South African Veterinary Association 74(1): 20-21.

Annexure C

Declaration form of landowner

YUNIBESITI YA BOKONE-BOPHIRIMA
NORTH WEST UNIVERSITY
NOORDWES UNIVERSITEIT

Private Bag X6001
Potchefstroom
2520
Tel (018) 299 2512
Fax (018) 299 2503

PERMISSION TO COLLECT FROGS

PROJECT TITLE (NORTH-WEST UNIVERSITY)

Chytridiomycosis, an emerging infectious disease of amphibians in South Africa

DECLARATION OF LAND OWNER

I ....., owner of private land ..... , hereby allow the permit holder, Mr. C. Weldon to collect the species of fauna stipulated by the permit No. ..... , issued by .....

Particulars of collection

Locality ..... Habitat: .....

Date of collection ..... No. of days collected .....

Day	Species	No. of males	No. of females
1			
2			
3			

Signature of land owner ..... Signature of permit holder .....

Thank you for your cooperation

Annexure D

Field specimen collection form

FIELD SPECIMEN COLLECTION FORM

Date ..... Time .....

Locality .....

GPS reading .....

Weather .....

No.	Species	SVL	Sex	Comments

Annexure E

Archived specimen collection form

ARCHIVED SPECIMEN COLLECTION FORM

Date .....

Source .....

No.	Catalog No.	Collection Date	Species	Locality	SVL	Sex	Collector

Annexure F

Questionnaire on the use of *Xenopus laevis* in the angling industry

QUESTIONNAIRE ON THE USE OF *Xenopus laevis* IN THE ANGLING INDUSTRY

A. Personal Details

Name

.....

Address

.....

..... Code .....

Tel ..... Fax .....

B. About *Xenopus*

1. What fish do you catch with *Xenopus*?

.....

2. How popular are *Xenopus* among anglers?

.....

3. Is there an angling season and does the demand for the frogs correlate with this season?

.....

4. How many frogs are sold during the peak months of the season?

.....

5. What size frog is ideal for angling?

.....

6. Do you provide frogs to the entire angling community of the region or do some anglers catch their own bait?

.....

7. Do you know of any one else who cells *Xenopus* commercially for angling?

.....

.....

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