# Aspects of amphibian chytrid infections in South Africa

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# Abstract

## Aspects of amphibian chytrid infections in South Africa

The waterborne pathogen *Batrachochytrium dendrobatidis* (Bd), amphibian chytrid, is implicated as being the causative agent for global amphibian declines. The fungus attacks the keratinized skin of adult and postmetamorphic animals and the keratinized mouthparts of tadpoles. Postmetamorphic animals seem to be more susceptible to Bd than tadpoles and adult frogs. Hypotheses exist that the origin of the fungus is in Africa. During the study different aspects of Bd infections in South African frogs were examined including the distribution of Bd, cultivation of Bd, preservation of cultures, the morphology of Bd as an infection as well as in culture and finally differences in host defense. Positive and negative localities for Bd were identified through surveys conducted in South Africa. These data will be contributed to the Bd Mapping Project and the African Bd Database in order to determine whether chytrid has any environmental preferences. Cultures obtained from the positive localities were maintained and cryopreserved for use in numerous experiments. In a future study, DNA extractions from the cultures will be analyzed using multilocus sequence typing in order to determine the sequence type of South African strains in comparison with global strains. This will provide important epidemiological information concerning the origin and control of Bd. The morphology of Bd was also examined using scanning electron microscopy and laser scanning confocal microscopy. Damage due to Bd infections was more severe on the larval mouthparts of *Amietia vertebralis* than that of *Hadromophryne natalensis.* The adverse effect of Bd is therefore not limited to postmetamorphic animals. Confocal microscopy uses fluorescent stains and lasers to examine specific structures within organisms. An especially effective stain used during confocal microscopy on Bd is Calcofluor White M2R. Due to its specificity this stain can be used as an effective screening tool for Bd in tissue. The role of antimicrobial skin peptides as a defense against Bd was also examined. *A. vertebralis* experiences die-offs due to chytrid, while *H. natalensis* does not experience the same effect in the presence of Bd. *H. natalensis* possess more antimicrobial skin peptides against Bd with a higher effectiveness than peptides extracted from *A. vertebralis.* This may explain the observed susceptibility of *A. vertebralis* to Bd. The relevance of this study is in order to identify areas in South Africa

in which the probability of finding Bd is high. This will help in the surveillance of Bd and in the identification of susceptible species to be monitored and protected against the fungus. The effect of Bd on frog species can also be determined by means of exposure experiment using cultures isolated during this study. Through the identification of peptides effective against Bd, predictions can be made with regard to the susceptibility of different frogs to Bd, improving our ability to protect the amphibian biodiversity in South Africa. With the use of confocal microscopy in the examination of Bd, we became the first group to use the method. By the identification of a stain with a high potential as a screening tool, we also contributed to the more efficient identification of Bd in tissue.

**Keywords:** *Batrachochytrium dendrobatidis,* Bd, amphibian chytrid, distribution, cultivation, antimicrobial skin peptides, laser scanning confocal microscopy, *Amietia vertebralis, Hadromophryne natalensis,* South Africa

# Opsomming

## Aspekte van amfibiër chytrid infeksies in Suid-Afrika

Die watergedraagde siekte, *Batrachochytrium dendrobatidis* (Bd), amfibiese chytrid, word verantwoordelik gehou vir 'n wêreldwye afname in amfibiërs. Die fungus val die gekeratiniseerde vel aan van diere wat pas deur metamorfose gegaan het sowel as volwasse amfibiërs, asook die monddele van paddavisse. Diere wat pas deur metamorfose gegaan het is meer vatbaar vir Bd as volwasse en larvale diere. Hipoteses bestaan dat die oorsprong van Bd uit Afrika is. Tydens hierdie studie is verskillende aspekte van Bd infeksies in Suid-Afrika bestudeer, insluitende die verspreiding van Bd, die kultivering daarvan, die preservering van kulture, die morfologie van Bd as 'n infeksie asook as kultuur en, uiteindelik, verskille in gasheerbeskerming teen Bd. Areas wat positief of negatief vir Bd is was geïdentifiseer tydens veldwerk in Suid-Afrika. Hierdie data sal tot die "Bd Mapping Project" en die "African Bd Database" gevoeg word om enige omgewingsvoorkeure vir Bd te identifiseer. Geïsoleerde kulture vanaf positiewe areas was onderhou en gekriopreserveer vir die gebruik in talle eksperimente. DNA ekstraksies vanaf die kulture sal geanaliseer word as 'n toekomstige studie met behulp van veelvoudige lokus volgordebepaling (MLST) om die volgordetipe vir Suid-Afrikaanse variëteite te bepaal asook te vergelyk met dié van internasionale variëteite. Inligting wat hieruit verkry word, sal belangrike epidemiologiese inligting verskaf oor die oorsprong en beheer van Bd. Die morfologie van Bd was ook bestudeer met skandeerelektronmikroskopie en laserskandeer konfokale mikroskopie. Die skade aangerig as gevolg van Bd infeksies was erger op die monddele van *Amietia vertebralis* paddavisse. Die effek van Bd is dus nie net beperk tot diere wat pas deur metamorfose gegaan het nie. Konfokale mikroskopie maak gebruik van fluoressente kleurstowwe en lasers om spesifieke strukture in organismes te bestudeer. Calcofluor White M2R is geïdentifiseer as 'n doeltreffende kleurstof in die diagnose van Bd op weefsel. Die rol van antimikrobiese velpeptiede as 'n beskerming teen Bd was ook bestudeer. *A. vertebralis* ondervind afnames as gevolg van Bd infeksies, terwyl *Hadromophryne natalensis* nie dieselfde patroon volg in die teenwoordigheid van Bd nie. *H. natalensis* beskik ook oor meer peptiede met 'n hoër effektiwiteit teen Bd as *A. vertebralis.* Dit mag die hoër vatbaarheid van *A. vertebralis* vir Bd verduidelik. Die doel van hierdie studie is

om areas in Suid-Afrika te identifiseer waar die kans om Bd te kry hoog is. Dit sal die monitering en identifikasie van vatbare spesies wat beskerm moet word teen Bd vergemaklik. Die effek van Bd op paddaspesies kan ook bepaal word tydens blootstellingseksperimente met kulture wat tydens hierdie studie geïsoleer is. Deur die identifisering van effektiewe peptiede teen Bd, kan waardevolle voorspellings oor die vatbaarheid van verskillende paddas vir Bd gemaak word. Sodoende kan die biodiversiteit van Suid-Afrikaanse amfibiërs behou word. Ons (AACRG) is ook die eerste groep wat gebruik maak van konfokale mikroskopie om Bd te bestudeer. Deur die identifisering van 'n spesifieke kleurstof as 'n potensiële diagnostiese hulpmiddel, dra ons ook by tot die korrekte identifikasie van Bd in weefsel.

**Sleutelwoorde:** *Batrachochytrium dendrobatidis,* Bd, amfibiese chytrid, verspreiding, kultivering, antimikrobiese velpeptiede, laserskandeer konfokale mikroskopie, *Amietia vertebralis, Hadromophryne natalensis,* Suid-Afrika

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# Chapter 1

# Introduction

Over the past five decades a global decline in the number of amphibian populations has been observed (Declining Amphibian Populations Task Force, 2004). Of the estimated 5 918 extant amphibian species about 130 have become extinct and another 1,896 species may be in imminent danger of extinction (IUCN, 2006). According to McCallum (2007), the current extinction rates are far higher than what is normally expected. This extinction rate for the next 50 years is 25 000-45 000 times that of the expected background extinction rate for amphibians, suggesting a global stressor(s) with possible human ties. Today amphibians are regarded as the most threatened vertebrate class.

Initial speculations attribute observed declines in amphibian species to adverse human influences including deforestation, wetlands degradation and draining, chemical pollution, acid precipitation, increased ultraviolet radiation, introduction of exotic species, harvesting by humans as well as natural population fluctuations (Blaustein *et al.*, 1994). In 1988, however, frog species living in or near pristine montane streams were experiencing declines without apparent evidence of the above mentioned causes (Berger *et al.*, 1998). Different hypotheses regarding the declines have been put forward, including that they were caused by a highly virulent waterborne pathogen occurring over a range of habitats but only became virulent to frogs in cool upland areas (Laurence *et al.*, 1996; Laurence *et al.*, 1997). Researchers speculated that the agent was a novel pathogen or an introduced exotic (Laurence *et al.*, 1997).

## 1.1 Discovery of *Batrachochytrium dendrobatidis*

Research on amphibian declines finally made a breakthrough when pathology revealed the presence of unknown round/oval fungal bodies with a distinct refractive wall in the skin of the dead animals, causing a disease named chytridiomycosis (Berger *et al.*, 1998). This unknown chytrid fungus infected the skin of the sharp-snouted day frog (*Taudactylus acutirostris*), waterfall frog (*Litoria nannotis*) and the common mist frog

(*Litoria rheocola*) which were dying in mass mortality events in tropical forests of Queensland and Panama during 1993. The presence of the fungus in itself raised a number of questions, namely whether the declines were caused by it, or by other environmental or anthropogenic causes. No correlations were found of the latter in the case of declines in high altitude species, since environmental degradation would affect the reproduction and nutritional status of frogs. That was not inconsistent with the fungal infections; therefore environmental degradation was ruled out as the cause of the mass mortalities, directing research to the unknown fungus (Berger *et al.*, 1999). Because of the aquatic nature of the fungus, it fitted perfectly into the hypothesis that the factor causing the declines in amphibian species was an infectious waterborne disease: declines were asynchronous, sudden, severe and spreading like a front. Adults and postmetamorphic animals of stream-dwellers with small clutch sizes and restricted geographic ranges were dying without any concomitant discernable environmental changes (Berger *et al.*, 1999).

Longcore *et al.* (1999) successfully isolated this fungus from the Blue poison dart frog (*Dendrobates auratus)*, describing the fungus as *Batrachochytrium dendrobatidis* (Bd), and belonging to the Chytridiomycota. The description was based on the presence of flagellar props, discoid cristae in the mitochondria and aggregated rather than dispersed ribosomes.

## 1.2 Bd as cause of declines

Infection prevalence and declines have been most severe in and around aquatic habitats, especially during the breeding season, which is consistent with a predominantly waterborne pathogen (Johnson, 2006). Associations with lotic streams seem to be a significant predictor of declining species. This indicated that the agent responsible for the declines was most probably waterborne and favoured flowing water (Kriger & Hero, 2007). Some of the more common pathogens that have been implicated in amphibian declines include *Saprolegnia,* a water mould causing amphibian egg mortalities (caused declines in *Bufo boreas* and *Rana cascadae*) (Daszak *et al.*, 2003); *Dermocystidium*-like mesomycetozoan fungi (Green *et al.*, 2002); Rana virus causing declines in the

salamander *Ambystoma tigrinum* as well as *Rana temporaria* (Daszak *et al.*, 2003); iridoviruses (Carey *et al.*, 1999); *Ribeiroia ondatrae* (Daszak *et al.*, 2003); *Basidiobolus ranarum*, a fungal pathogen implicated for declines of Wyoming toads (Rollins-Smith *et al.*, 2003); and secondary bacterial infections such as the opportunistic *Aeromonas hydrophila* (Green *et al.*, 2002). Only chytrid fungal epizootics are currently associated with population declines of multiple species (Green *et al.*, 2002; Skerratt *et al.*, 2007).

Fungal mass mortalities follow a certain pattern, namely: (1) a wide geographic area is affected; (2) mortalities of 50-100% can be found in populations; (3) declines are more pronounced at higher altitudes or cooler climates; (4) only some species decline; (5) metamorphic animals die; and (6) an infectious disease is the direct cause of the deaths (Carey *et al.*, 1999). It seems as if Bd infections follow this pattern: (1) Bd has been found in every continent occupied by amphibians, except Asia (McLeod *et al.*, 2008). (2) It has been predicted that within four to six months after Bd arrived at a site not previously occupied by the fungus, 50% of amphibian species and about 80% of individuals may disappear (Mendelson *et al.*, 2006). (3) Bd seems to grow better at lower temperatures. It can grow up to five months at 5°C or six months at 4°C, with an optimal range of 10-25°C (Piotrowski *et al.*, 2004). Infections occur in high altitude species (Daszak *et al.*, 1999, Berger *et al.*, 2004; Lips *et al.*, 2008) and infections increase during cooler months (Berger, 2004; Kriger & Hero, 2006a; Kriger & Hero, 2006b). (4) Certain species of frogs do not seem to die and are called reservoirs or carriers. These include *Rana catesbeiana* (Daszak *et al.*, 2004, Hanselmann *et al.*, 2004) and *Xenopus laevis* (Weldon *et al.*, 2004). (5) Only postmetamorphic animals die (Berger *et al.*, 1998; Parris & Cornelius, 2004) and (6) it is thought that Bd is the cause of the deaths. For these reasons Bd is considered a recently emergent infectious disease (EID) (Carey *et al.*, 2003). Tadpole deaths have also been observed (AACRG, unpublished results).

EIDs are diseases that have recently increased in incidence, impact or in geographic or host range, and that are caused by pathogens that have recently evolved and which have been newly discovered or are diseases that have recently changed their clinical presentation (Daszak & Cunningham, 2003). "Recently", in these terms, is considered to

constitute the past two to three decades (Daszak & Cunningham, 2003). In order to suggest that Bd is the cause of declines and die-offs, certain links must be established between the disease and the declines: (1) Koch's postulates must be fulfilled; (2) Bd must be identified as a causative pathogen during die-offs; (3) there must be pathological evidence that the disease caused the deaths; and (4) there must be clear evidence that the mortalities are the cause of the declines (Daszak *et al.*, 2003). Nichols *et al.* (2001) indicated that Bd fulfilled Koch's postulates after successfully isolating the fungus from frogs, culturing the fungus and reinfecting frogs, which caused chytridiomycosis. During multilocus sequence typing, it was shown that Bd has only five variable nucleotide positions among ten loci. Thus, there is a low level of genetic variation, consistent with the view that Bd is a recently emerged pathogen (Morehouse *et al.*, 2003).

## 1.3 Hypotheses on the origin of Bd

Bd was first recorded during retrospective histology on museum specimens in the United States of America in the 1960s, in Australia in the 1970s, in Central America and South America in the 1980s and in Europe in the 1990s (Drew *et al.*, 2006). The earliest case identified as Bd was in 1938 in *Xenopus laevis* collected from the Cape Flats from the South African Museum in Cape Town, South Africa (Weldon, 2005). It appears that Bd persisted as a stable endemic infection in southern African amphibians for at least 27 years before a positive specimen was found outside Africa. This evidence gave rise to the debate as to whether Bd is a novel or an endemic pathogen.

The Novel Pathogen Hypothesis (NPH) states that Bd has recently spread into new geographic areas and host species as a result of anthropogenically-mediated spread of Bd (Fisher & Garner, 2007). The pathogen then infected naïve host individuals that are highly susceptible to infection (Rachowicz *et al.*, 2005). The NPH suggests that the focus should be placed on the identification and control of agents spreading Bd (Rachowicz *et al.*, 2005). The rate of spread cannot be only due to the movement of frogs, but must be facilitated by birds, insects or human intervention in order to explain the observed rate of spread (McCallum, 2005). It has also been shown that there are different modes of dissemination for the spread of Bd, including its own motility

(flagella), rain, spreading by waterbirds, spreading by fish, soil movement, as well as contaminated feet or vehicles (Johnson & Speare, 2005). The mode most implicated for the emergence of Bd is anthropogenic introduction, also called pathogen pollution (Daszak & Cunningham, 2003; Morgan *et al.,* 2007). Various observations support this suggestion. One of the biggest culprits seems to be global trade which is spreading infected animals worldwide (Mazzoni *et al.,* 2003; Weldon *et al.,* 2004; Fisher & Garner, 2007). Bd has been identified in the pet trade, zoo animal translocation, the food trade, laboratory animal trade and released biocontrol animals (Daszak & Cunningham, 2003). DNA sequence phylogeny also strongly suggests that recent mixing (mediated by pathogen pollution) between populations has occurred (Daszak & Cunningham, 2003). The pattern of declines is also consistent with the introduction of virulent pathogens into a naïve population (Daszak & Cunningham, 2003). Epidemic fronts of introduction (Lips *et al.,* 2006), little genetic diversity found during multilocus sequence typing (Morehouse *et al.,* 2003) and infected amphibians in trade are factors that can be mentioned in support of the NPH. If Bd originated in Africa, however, the NPH does not quite explain the recent outbreaks of Bd associated with mortality in other African anurans sympatric with *Xenopus* (Hopkins & Channing, 2003; Weldon & Du Preez, 2004; Rachowicz *et al.,* 2005). Even though it may seem as if Bd is a new pathogen that is spread worldwide to new localities by means of carriers, this cannot be firmly concluded (Rachowicz *et al.,* 2005). A study by Morgan *et al.* (2007) in Sierra Nevada in California showed that although Bd was novel to some areas, it was showing signs of endemism due to the fact that no two sites shared the same genotype, and, furthermore, some sites contained several related genotypes with evidence of recombination. However, the most parsimonious explanation - with supporting evidence - for global declines in amphibians and the emergence of Bd, is the introduction and spread of Bd among naïve populations of frogs (Skerratt *et al.,* 2007).

Bd might have been endemic to the localities that were facing declines with some other factor being the ultimate causal agent of these declines. This factor, for example, may be an environmental stressor such as temperature change (McCallum, 2005). This gives rise to the Endemic Pathogen Hypothesis (EPH). According to this hypothesis, the

emergence of Bd is caused by amphibian hosts becoming more susceptible to pre-existing infections as a consequence of changes in the environment (Fisher & Garner, 2007). The endemic pathogen hypothesis investigates and manages cofactors, synergies and context dependence of factors (Rachowicz *et al.,* 2005). Environmental change could directly affect the ability of hosts to express behaviours or select microhabitats that normally reduce their susceptibility (Rachowicz *et al.,* 2005). According to Pounds *et al.* (2006) temperature changes are the main cause of declines among high altitude species since these species have fewer refuges to rid them of Bd. Some frogs can rid themselves of infection by basking in the sun, thereby causing a similar effect to induced fever in endotherms (Pounds *et al.,* 2006). However, due to global warming, temperatures are rising to the optimal temperature for the growth of Bd. The EPH suggests that Bd has been present in the environment but has entered new host species or has increased in pathogenicity because of environmental changes. These changes, in turn, caused a change in the immunological, ecological and/or behavioural parameters of the host or parasite, thus causing a shift from a benign association to a parasitic relationship (Rachowicz *et al.,* 2005). Support for the EPH is the presence of Bd in global amphibian populations for decades (Drew *et al.,* 2006; Weldon *et al.,* 2004; Rachowicz *et al.,* 2005) and the association between amphibian condition, global warming and the presence of Bd (Fisher & Garner, 2007). There are a large number of controversies about the EPH, also called the climate-linked epidemic hypothesis. The spread of Bd from its point of origin shares a pattern with many known emerging infectious diseases and is not climate-driven, even though climate has the potential to pose a threat to host-pathogen systems (Belden & Harris, 2007; Lips *et al.,* 2008). Multilocus sequence typing showed a low geographical structuring and host specificity of genotypes, and as such does not support the hypothesis that Bd has emerged from a pre-existing relationship between the fungus and amphibians through climatic change or other abiotic factors. If Bd was present in the environment and there were any pre-existing relationships, there would have been some geographical population structuring (Morehouse *et al.,* 2003; Morgan *et al.,* 2007). There is also no evidence of any environmental changes prior to mass mortalities in north-eastern Queensland, Puerto Rico, the central Colorado Rockies or Panama that would have

increased the pathogenicity of Bd (Carey *et al.*, 2003). There is thus a lack of evidence of the direct effect of climate change on declines.

## 1.4 Global distribution and extent of infection of frogs with Bd

Today, Bd affects about 200 species of frogs worldwide (Kriger & Hero, 2006b; Skerratt *et al.*, 2007). More than 40 amphibian families have been found to be infected with Bd (Fisher, pers. comm.) with no Apoda families yet found to be infected. The clearest, and most documented, evidence of disappearing species include *Atelopus* (Harlequin frog) and *Bufo periglenes* (Golden toad) (Lips *et al.*, 2005), *Rheobatrachus silus* (Gastric brooding frog) and *Taudactylus diurnus* (Southern day frog) in Australia (McDonald *et al.*, 2005) with local extinctions of *Nyctimystes dayi, Litoria nannotis* and *Litoria rheocola* (McDonald *et al.*, 2005). *Taudactylus acutirostris* (the sharp-snouted day frogs) was the first documented case of extinction due to Bd (Schoegel *et al.*, 2006).

Bd is present in global habitats in the following areas:

- Oceania – Australia (Berger *et al.*, 1998), New Zealand (Waldman & Van De Wolfshaar, 2001), Tazmania (Pauza & Driessen, 2008)
- Europe – Britain (Cunningham *et al.*, 2005; Garner *et al.*, 2005), Spain (Bosch *et al.*, 2001), Italy (Mutschmann *et al.*, 2000 ; Stagni *et al.*, 2002)
- North America – Canada and the USA (Muths *et al.*, 2003), including Hawaii (Beard & O'Neill, 2005)
- Central America – Mexico (Lips *et al.*, 2004; Hale *et al.*, 2005), Costa Rica, Panama, Puerto Rico (Lips *et al.*, 2005; Puschendorf *et al.*, 2006a; Puschendorf *et al.*, 2006b)
- South America – Equador, Venezuela, Uruguay (Garner *et al.*, 2006), Brazil (Toledo *et al.*, 2006)
- Africa – Swaziland, Lesotho, Kenya, Tanzania, Democratic Republic of the Congo, South Africa (Hopkins & Channing, 2003; Weldon *et al.*, 2004; Channing *et al.*, 2006; Smith *et al.*, 2007; Greenbaum *et al.*, 2008)

The only continent with amphibians where Bd has not been found, is Asia (McLeod *et al.*, 2008), although more exhaustive searches are needed to be absolutely sure.

In South Africa Bd has been reported mostly in the Northern, Eastern and Western Cape Provinces (Hopkins & Channing, 2003; Lane *et al.*, 2003), with detection of a more limited nature in the Free State, Lesotho, Limpopo Province and Kwazulu-Natal (Weldon, 2005). During this study one of the objectives was to identify other areas with Bd infection in South Africa.

These and other areas with Bd were incorporated into a new initiative started in 2007 called the "Bd Mapping Project". The Bd Mapping Project is a dedicated system aimed at collecting, mapping and modelling the prevalence of infection in order to aid in the control of Bd. A webpage was set up to include global surveillance data of Bd incorporated into interactive web-based Google maps, which can be viewed by different audiences - including scientists, policy makers and the public. Information highlighted include the Bd isolate position, epidemiological data, the amphibian species it was isolated from, as well as the Bd genotype, if available (http://www.spatialepidemiology.net/bd/). Several issues are addressed within the site, for instance the spread, epidemiology and evolution of Bd. In investigating the evolutionary history of Bd, the importance of determining the genotype of Bd through multilocus sequence typing has been demonstrated by Morehouse *et al.* (2003). By comparing the genetic differentiation of strains from North America and the rest of the world, it was found that North American strains may form a distinct gene pool. Bd may also have spread from this area. Strains from Panama and Australia have the same multilocus sequence typing, meaning that the Bd from these areas has either dispersed recently or was introduced into these areas (Morehouse *et al.*, 2003). But due to the small differences between the genotype of the strains from different areas of the world and those of North America, the origin of Bd cannot be proven without a doubt (Morgan *et al.*, 2007).

Information gathered through the Bd Mapping Project might shed some light on the preferences of Bd, for instance in terms of altitude, temperature and rainfall, as well as environmental correlations between Bd and declines. The origin of this pathogenic fungus can also be determined, leading to better understanding of the fungus and higher conservation probabilities in the future.

## 1.5 Morphology and life-cycle of Bd

*Batrachochytrium dendrobatidis* forms part of the phylum Chytridiomycota, class Chytridiomycetes and the order Chytridiales, and is nestled within the "Chytridium clade" according to the phylogenetic tree (James *et al.,* 2000). All chytrids have a zoospore with a single, posteriorly directed flagellum (James *et al.,* 2000). Chytrids are microscopic heterotrophic fungi, occurring in soil and water, degraders and saprobes of substrates like chitin, plant detritus and keratin. Because of the unwalled flagellated zoospores, chytrids require water for dispersal and are considered aquatic (Berger *et al.,* 1998). Members of Chytridiomycota are known to infect algae, fungi, vascular plants, rotifers, nematodes and insects (Sparrow, 1960; Karling, 1977; Longcore *et al.,* 1999), but Bd, however, is the first member to infect a vertebrate (Berger *et al.,* 1998). Bd is known to infect amphibians, especially Anura (frogs, toads) and Caudata (salamanders) (Davidson *et al.,* 2003), but is yet to be found in Apoda (caecilians).

Bd is keratinophilic, meaning it attacks keratin. Therefore it does not occur in areas not containing keratin, such as the conjunctiva, nasal cavities, mouth, tongue and intestines of adult frogs (Berger *et al.,* 1998; Marantelli *et al.,* 2004). Most commonly, the fungus occurs in the epidermis of the digits and ventral surface of adult frogs, with the pelvic patch ("drinking patch") and innermost digit the best places to detect Bd (Puschendorf & Bolanos, 2006). Weldon and Du Preez (2006) found that the tubercles of digits and toe-tips are most heavily infected in *Amietia fuscigula.* In tadpoles only the keratin-rich mouthparts are infected. The developing feet of tadpoles only showed a light infection at Gosner stage 42 as the tail is resorbed. At stage 45 sporangia becomes established all over the body (Lamirande & Nichols, 2002; Speare, 2006) as the epidermis becomes more keratinised. Even though this is the case, it is uncertain whether Bd really degrades

keratin. Bd produces non-specific proteases, able to degrade milk, gelatine and snakeskin. This may help the fungus to survive saprobically on proteins in the environment (Piotrowski *et al.*, 2004). The reason why keratinised cells are infected may be because these cells are dead and therefore easy to invade (Piotrowski *et al.*, 2004).

During the life-cycle of Bd the following events occur: mature zoosporangia release zoospores through their discharge papillae which are dispersed by water to a susceptible host (Berger, 2001) (Fig. 1.1). The majority of zoospores swim less than 2m using their flagella and then encyst (Piotrowski *et al.*, 2004). The flagellum is resorbed and a thickened wall forms. A young sporangium or germling then forms with fine, branched rhizoids that can parasitise on adjacent sporangia. With infections in the skin, one cannot see the rhizoids. This is because Bd occurs in epidermal cells and attachment is therefore not necessary (Berger, 2001). The contents of the maturing zoosporangia will then become more complex and multinucleated through mitotic divisions, finally forming mature rounded, flagellated zoospores after cleaving. Bd is inoperculate (James *et al.*, 2000), forming discharge papillae. Thin septae divide the thalli into compartments, each with zoospores ready to be released (Berger, 2001).

The immature stages of Bd occur in the deeper viable cells of the host (Berger, 2001). Chytrid is clearly evolved to live in the dynamic tissue of the epidermis. As the cell matures, the sporangia develop at such a rate that the mature sporangia's discharge papillae usually open onto the distal surface of the *stratum corneum* (Berger, 2001; Berger *et al.*, 2005a). This also means that the old, empty stages are shed with the *stratum corneum*. The mechanism of how the zoospores reach the deeper cells is still unknown. It is thought that the zoospore encysts on the surface of the epidermis and then injects the nucleus and contents through a germ tube (Longcore *et al.*, 1999; Berger *et al.*, 2005a). According to James *et al.* (2000) Bd has endogenous development, meaning the zoospore cyst enlarges to form a reproductive structure (zoosporangium) and the nucleus remains within the zoospore cyst during development, therefore giving no evidence of a germ tube forming.

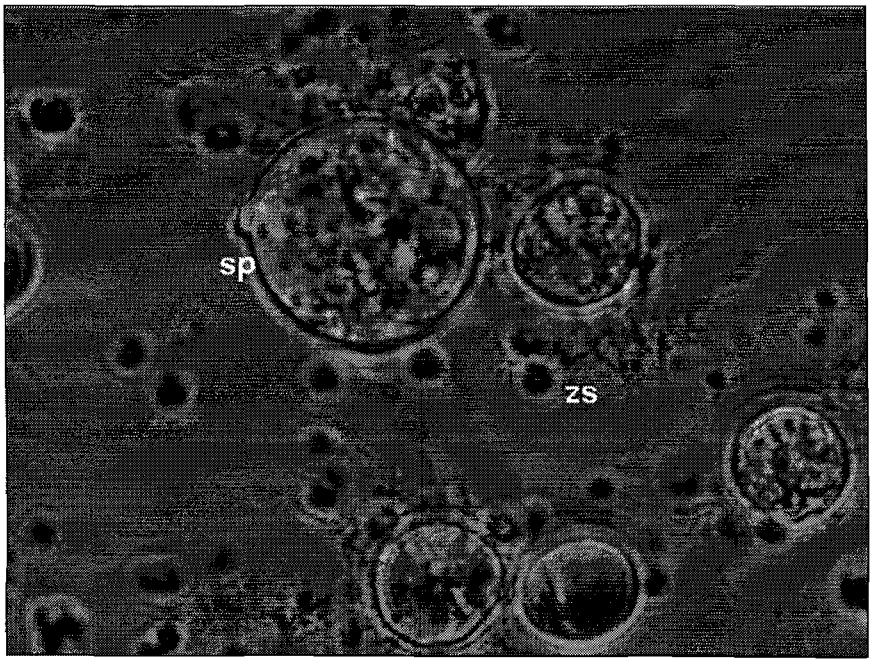


**Figure 1.1 The morphology of *Batrachochytrium dendrobatidis* in broth culture. Abbreviations: sp, zoosporangia; zs, zoospore**

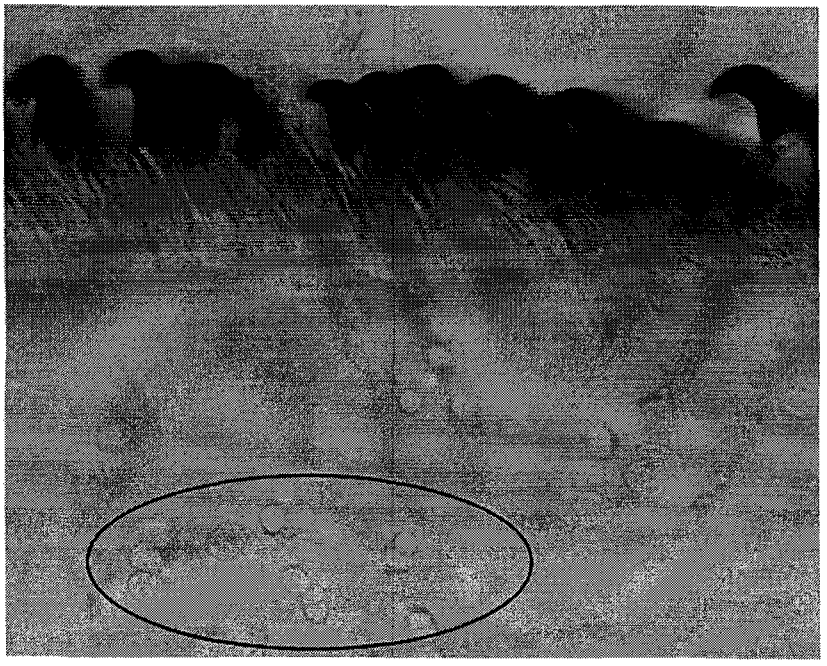

**Figure 1.2 *Batrachochytrium dendrobatidis* infection of the keratinised mouthparts of an *Amietia angolensis* tadpole. The clusters of sporangia are indicated.**

Observations with light microscopy reveal that Bd sporangia seem to "cluster" together *in vivo* to form semi-circular clusters (Weldon & Du Preez, 2006) (Fig. 1.2). It has been suggested that the reasons for this phenomenon are that zoospores are attracted to foci of infection or zoospores are released and infect adjacent cells immediately (Berger *et al.*, 2005a; Weldon & Du Preez, 2006). The grouping of sporangia is also seen in cultures – individual zoospores/zoosporangia die, but survive in colonies. The group effect of growing thalli is uncommon in fungi (Berger, 2001).

## 1.6 Pathology and clinical symptoms of Bd

### 1.6.1 Pathology of chytridiomycosis

Bd infects the skin, specifically the *stratum corneum* and *stratum granulosum* (Longcore *et al.*, 1999; Pessier *et al.*, 1999; Daszak *et al.*, 1999; Oullet *et al.*, 2004; Garner *et al.*, 2005; Puschendorf & Bolanos, 2006). Histological changes visible under the microscope include parakeratotic hyperkeratosis, irregular hyperplasia, minimal spongiosis, acanthosis, disordered epidermal cell layers, skin erosion and occasional ulcerations of the skin (Berger *et al.*, 1998; Berger *et al.*, 1999; Carey *et al.*, 2003; Berger *et al.*, 2004; Oullet *et al.*, 2004; Berger *et al.*, 2005a; Berger *et al.*, 2005b; Carey *et al.*, 2006). Hyperkeratosis may appear because of an increased turnover of cells and premature keratinization and death of infected cells (Berger *et al.*, 2005a). Epidermal cell layers become disordered with epidermal thickness changing by either diffusing in areas or thickening to about 10 cell layers in others (Longcore *et al.*, 1999; Berger *et al.*, 2005a). There is minimal inflammatory response visible (Oullet *et al.*, 2004). Individual epidermal cell pyknosis and vacuolation may occur in the *stratum basale* or more superficial layers. Occasionally, these vacuolated cells appear to form vesicles, lifting the epidermis and causing erosion, causing an inflammatory response (Berger, 2001). Because of the consistent skin lesions associated with Bd infection, Oullet *et al.* (2004) suggest that Bd is a parasite, rather than a saprobe. Secondary skin infections may occur due to environmental pathogens trapped in the excess skin (Pessier, 2002).

### 1.6.2 Clinical signs of Bd

Clinical signs of Bd are not a clear indication of infection since they only manifest during the final stages of infection. Not only this, but not all dying animals exhibit the signs (Kriger *et al.,* 2007). The clinical signs of Bd are non-specific and are manifested as behavioural changes, neurological signs and skin lesions (Speare, 2006). The Cape river frog (*Amietia fuscigula*) infected with Bd has been observed to display unusual neurological behaviour by climbing into shrubs in broad daylight and without signs of fear (Du Preez, unpublished data). Behavioural changes occurred about two to three days before death in *Mixophyes fasciolatus* (Berger *et al.,* 2004) and include lethargy, inappetence, sitting unprotected during the day with hind legs abducted (Berger *et al.,* 1999; Speare 2006), sitting out of water during experiments and decreased rates of respiration in *Bufo boreas* froglets (Carey *et al.,* 2006). The decrease in respiration may reflect a gradual inhibition of metabolism by pathological changes in the skin caused by Bd (Carey *et al.,* 2006). Frogs in early stages of becoming symptomatic display some escape activity and can initially right themselves after turning them over (Berger *et al.,* 1999; Berger *et al.,* 2005b; Speare 2006). Lesions include skin discoloration like the reddening (hyperaemia) of the ventral skin, excessive sloughing, erosion and ulceration. Increased shedding may be a host mechanism to reduce infection loads or a way in which Bd manipulates the host to increase keratinised substrate (Woodhams *et al.,* 2007b). Erythema of ventral skin and congestion of internal organs has also been noticed in *Litoria caerulea* (White's tree frog) during terminal chytridiomycosis (Berger *et al.,* 2005b), but is rarely seen. Sometimes neurological signs such as slow responses to tactile stimuli occur (Berger *et al.,* 1999; Blaustein *et al.,* 2005). Dying frogs become rigid and tremble with extension of hind limbs and flexion of forelimbs (Berger *et al.,* 1999; Nichols *et al.,* 2001). Weight-loss and deterioration in the health of frogs have been identified as sub-lethal effects (Retallick & Miera, 2007).

### 1.6.3 Stage-specific nature of Bd infections

The pathology and clinical signs of Bd seems to be stage-specific (Briggs *et al.,* 2005). Because of their low susceptibility to chytridiomycosis, tadpoles are considered to be reservoirs (Daszak *et al.,* 1999; Briggs *et al.,* 2005). Deaths in tadpoles are rarely seen

but differ between species (Blaustein *et al.*, 2005), with clinical signs only developing when the end of metamorphosis is approached (Lamirande & Nichols, 2002). These signs include distended bodies, skin ulcerations, internal bleeding and various developmental abnormalities (Waldman & Van De Wolfshaar, 2001). One outstanding feature of Bd infections in pre-hindlimb tadpoles is the presence of oral deformities (Burger *et al.*, 2000). These deformities, in the form of depigmentation of upper jaw sheaths (Rachowicz., 2002; Knapp & Morgan, 2006) and the lack of labial tooth rows (Burger & Snodgrass, 2000), can be used as an indication of Bd infections (Fellers *et al.*, 2001; Rachowicz *et al.*, 2006; Knapp & Morgan, 2006), but there are some disputes about the relevance of oral depigmentation due to Bd since it can also be caused by other factors such as low temperature and contaminants as well (Altig, 2007; Burger & Snodgrass, 2000; Rachowicz, 2002; Padgett-Flohr & Goble, 2007). Tadpoles at Gosner stage 35 have higher infections (Smith *et al.*, 2007) and those with longer larval stages will be more susceptible to depigmentation associated with Bd infections (Knapp & Morgan, 2006). This may mean the following: (1) infections with Bd may be dependent on a certain level of larval development; (2) becoming clinically infected is time-dependent and is also dependent on the amount of exposure to Bd before reaching a critical magnitude; or (3) infected tadpoles have reduced foraging efficiency due to oral deformities, leading in turn to a reduction in growth which accelerated larval development (Smith *et al.*, 2007). The only hypothesis which is supported by experiments is the time-dependence of Bd infections. It can take as long as two weeks for adult frogs to become infected (Nichols *et al.*, 2001), but up to seven weeks to infect tadpoles (Rachowicz & Vredenburg 2004). Furthermore, no loss in foraging efficiency was detected in the current study (Smith *et al.*, 2007).

Bd infections increase in subadults, killing two to three week-old postmetamorphic *Taudactylus acutirostris*, *Litoria nannotis* and *Litoria rheocola* (Berger *et al.*, 1998; Beard & O'Neill, 2005). These froglets exhibit skin lesions over their feet, hind legs and ventrum, with smaller lesions on the head and dorsum. Lesions consist of foci of acanthosis and hyperkeratosis with the presence of Bd thalli in the keratinised layers of the skin (Lamirande & Nichols, 2002). Carey *et al.* (2006) demonstrated that a threshold

number of thalli is necessary to cause death. This threshold number is directly related to the surface area of the animal, meaning that it will be reached quicker and with fewer thalli needed in a small animal than for a larger animal. Therefore, not only the size of froglets, but also the possibility that the epidermis of smaller frogs may be penetrated easier than those of larger frogs, may cause the early demise of froglets. Peptide defences may also be less defined in froglets (Carey *et al.*, 2006).

### 1.6.4 Killing mechanism of Bd

The mechanism whereby Bd is able to kill frogs is unknown, but different hypotheses exist. It may be that Bd releases proteolytic enzymes or other active compounds that are absorbed through the permeable skin of frogs. Another hypothesis is also that the damage to the skin can cause disruptions in the oxygen, water or electrolyte balance of the frog (Berger *et al.*, 1999; Berger *et al.*, 2005a; Carey *et al.*, 2006).

i. Voyles *et al.*, (2007) found that *Litoria caerulea* had reduced plasma osmolality, sodium, potassium, magnesium, chloride concentrations and blood pH when they are infected with Bd. According to this hypothesis, Bd kills amphibians by disrupting normal epidermal functioning, leading to osmotic imbalance through the loss of electrolytes. Electrolyte reductions may explain the neurological signs such as muscle tetany in the terminal stages of infection (Voyles *et al.*, 2007).

ii. Epidermal hyperplasia may also impair cutaneous respiration and osmoregulation (Berger *et al.*, 1998). Carey *et al.* (2006) noted a decrease in respiration in infected frogs. This may reflect a gradual inhibition of metabolism by pathological changes in the skin caused by Bd. It is not believed that Bd kills amphibians by blocking oxygen uptake through the skin, because that would lead to an increase in lung ventilation to compensate for the disruption in respiration.

iii. Due to the dissolution of the cytoplasm during infection, toxicity is also suggested (Berger *et al.*, 1998; Parris & Cornelius, 2004; Berger *et al.*, 2005a). Deaths of tadpoles due to Bd have also been attributed to a possible toxin released during infections (Blaustein *et al.*, 2005).

iv. Frogs from the same species, held under the same conditions, exhibited different lethal and sub-lethal effects during exposure experiments. This can be ascribed to Bd strain differences (Retallick & Miera, 2007).

## 1.7 Factors influencing natural infections of Bd

The persistence and virulence of Bd can be regulated by a number of processes and factors. Only a few key factors will be discussed in this section:

### 1.7.1 Climatic factors

Terrestrial processes include, among other things, the growth of Bd in the soil (the persistence of the fungus in the environment), as well as the reproduction of post-metamorphic frogs on land and the seasonal variation in the immune responses in post-metamorphic frogs. Aquatic processes would include the growth on Bd on tadpoles or stream substrates as well as the waterborne zoospore's dispersal ability. Both these processes are in some way regulated by climate (Kriger & Hero, 2006b).

The factors most typically associated with the outbreak of Bd are high altitude, low temperature and wet climates (Woodhams & Alford, 2005; Drew *et al.*, 2006). Low temperatures can affect the adaptive immunity by delaying graft rejection responses, while also decreasing or increasing antimicrobial peptide production in frogs (Carey *et al.*, 1999; Rollins-Smith, 2001), whereas environmental temperatures over 30°C seem to exclude Bd infections (Briggs *et al.*, 2005; Drew *et al.*, 2006). The majority of upland habitats infected with Bd seem to fall between 20-25°C (Ron, 2005). At lower altitudes the increase in temperature seems to decrease the growth of Bd (Kriger & Hero, 2006b). It has also been shown that increased temperatures can be used to rid frogs of Bd in the laboratory (Woodhams *et al.*, 2003; Berger *et al.*, 2004). Even though air temperature has a higher prediction value for diseases (Kriger & Hero, 2006b), water temperature will also affect the pathogenicity of Bd. Changes in regional or local climate may directly or indirectly alter pathogen development and survival rates, disease transmission and host susceptibility (Harvell *et al.*, 2002; Lips *et al.*, 2008). Bd responds to decreasing temperatures with life-history trade-offs (Woodhams *et al.*, 2008). At lower temperatures the fecundity of the zoosporangia increases because the maturation rate slows and infectivity increases through the production of more zoospores. Zoospores will settle and develop faster at 17-25°C, while at 7-10°C a greater number of zoospores per zoosporangium forms. These zoospores remain infectious for longer. A sudden drop in temperature in Bd cultures also induced the release of zoospores. This may be in order to

compensate for the lower growth rate at lower temperatures. Life-history trade-offs will therefore allow Bd to maintain its fitness across a broad range of temperatures (Woodhams *et al.*, 2008).

Studies have shown that the declines in amphibian populations are linked to temperature and moisture variations caused by climate change due to the El Nino phenomenon (Carey *et al.*, 2003). Synergistic effects of changes in humidity and temperature can influence the life history of Bd and the amphibian host, as well as the response of the host to the pathogen. For example, environmental changes may cause some enzootics to expand their distribution rates (Lips *et al.*, 2008). Disease prevalence peaked in early spring and dropped in late summer and early autumn (Oullet *et al.*, 2004; Kriger & Hero, 2006b). The odds of observing an infected frog were 4.3 times greater in the cool, dry season than in the warm, wet season (Woodhams & Alford, 2005). Infection prevalence was higher in tadpoles than in adults during the dry season but was higher in adults than in tadpoles during the wet season (Woodhams & Alford, 2005). This variation in season can affect Bd because of its thermal requirements, changes in host immunity, and interactions with species and life-history (Woodhams & Alford, 2005; Kriger & Hero, 2006b). Climate can also indirectly affect the host through habitat and breeding alteration, environmental contamination, promotion of infectious disease and other challenges (Carey *et al.*, 2003; Lips *et al.*, 2008). Studies showed that increased temperatures experienced in various parts of the world caused earlier breeding in some frog species (Carey *et al.*, 2003). This may pose the advantage of increased time to grow and store energy before hibernation, but the disadvantage of this phenomenon is death due to low temperatures associated with early breeding.

Evidence of the direct effect of climate change on Bd infections is still lacking. The closest to a correlation was made by Pounds *et al.* (1999) who showed that increased mist coverage due to climate change caused declines in amphibians in the Costa Rican highland forests (Collins & Storfer, 2003; Carey *et al.*, 2003). The chytrid-thermal-optimum hypothesis states that increased cloud cover at higher altitudes due to warming will increase the minimum temperature and decrease the maximum, thereby altering the

daytime radiant heating of microenvironments. This causes an environment preferred by Bd, which potentially increases its pathogenicity (Pounds *et al.*, 2006; Bosch *et al.*, 2007).

In order to determine with a measure of certainty whether climate change has an effect on amphibians, more information is needed about whether amphibians are changing their distributional patterns in response to defined climate change and how climate change affects the reproductive success and incidence of infectious disease (Carey *et al.*, 2003).

### 1.7.2 Persistence and reservoirs

The persistence of a disease will greatly be influenced by the difference in the susceptibility of the tadpole stage relative to the post-metamorphic stage (Briggs *et al.*, 2005). If the susceptibilities of the two stages are similar, the disease will be more persistent, but with the low levels of susceptibility of tadpoles, Bd cannot persist. The success of Bd therefore lies in the fact that the fungus is a saprobe, able to survive in the environment without a host, and having carriers/reservoirs.

Infectious diseases that are frequency-dependent utilise reservoir hosts or can survive in the abiotic environment, are most likely to cause extinctions (De Castro & Bolker, 2005; Smith *et al.*, 2006). Reservoir-hosts will be less susceptible to Bd under the same conditions - under which other species have declined - and should still exist where endangered species have either declined or disappeared (McCallum, 2005). It has been said that tadpoles are reservoirs due to their low susceptibility to Bd (Daszak *et al.*, 1999). Because tadpoles can act as reservoirs they might cause periodic outbreaks of Bd in host populations (McCallum, 2005). The fact that they do not lose their infections during metamorphosis also increases the persistence of Bd in aquatic habitats (Briggs *et al.*, 2005). Avirulent infections in carrier species such as *Xenopus laevis* and *Rana catesbeiana* also increase the persistence of Bd in habitats with declines (Woodhams & Alford, 2005). Because Bd is a saprobe it can grow in an aquatic habitat with or without a host (Briggs *et al*, 2005). In these conditions, Bd can remain infectious for between three to six weeks in a sterile aquatic environment (Johnson & Speare, 2005) and for longer than seven weeks in lake water (Johnson & Speare, 2003). Walker *et al.* (2007)

found that Bd can be detected in the natural environment outside its amphibian host at densities of up to 262 zoospore equivalents per litre. The turbidity of the water also had a positive correlation with the concentration of Bd, suggesting that more complex water matrices may favour the survival of the fungus (Walker *et al.*, 2007).

Another factor increasing the persistence of Bd in nature is the question of whether or not Bd has a resting spore stage to survive adverse conditions. Multilocus sequence typing studies indicated that Bd reproduces clonally, supporting the lack of a sexually produced resting stage (Morehouse *et al.*, 2003; Berger *et al.*, 2005a). James *et al.* (2000) however suggest differently, asserting that during endogenous development the enlarged zoospore cyst can form a reproductive structure, usually a zoosporangium, but that it might be a resting spore. Morgan *et al.* (2007) also argue that the genetic variance of Bd cannot be explained by clonal reproduction alone, but must be because of sexual recombination. Sexual reproduction in other chytrids typically results in thick-walled, resistant sporangia (Morgan *et al.*, 2007). No such structure has been observed before in *B. dendrobatidis*, until Di Rosa *et al.* (2007) found an apparently new form, possibly a resting zoospore. Whether this is true or the form is merely a saprobic form is still to be determined (Mitchell *et al.*, 2008). The implication of a resting spore stage will be that Bd can persist longer in the environment, even in adverse conditions, because of host independence (Mitchell *et al.*, 2008).

### 1.7.3 Immunity, peptides and skin bacteria

The primary reasons for patterns of infection prevalence at the landscape level can be attributed to variations in environmental conditions, but the immune function of frogs also influences host-pathogen dynamics (Woodhams & Alford, 2005).

Frogs have two sets of immune systems – one used during the tadpole stage and another one that functions after metamorphosis (Rollins-Smith, 1998). As a fully aquatic animal, tadpoles need an immune system competent against pathogens in the water, but during metamorphosis they acquire new molecules that are specific to adults. These include adult haemoglobin, urea cycle enzymes, adult-type keratin and vitellogenin. If the immune system remained the same during the entire life-cycle of the frog (from tadpole

to adult frog), the tadpole, undergoing its metamorphosis, might see these new molecules as antigens and generate a destructive response against them. Therefore, during metamorphosis, a new immune system is incorporated into the tadpole along with all these new antigens in order to recognise and tolerate them (Rollins-Smith, 1998). This might explain the differences in the susceptibility of tadpoles and post-metamorphic frogs to Bd: newly metamorphosed frogs may become susceptible to Bd because they express keratin in the skin where Bd will attack and their immune defence mechanism may not have recovered from the reorganisation of metamorphosis (Rollins-Smith, 1998).

Protection of the post-metamorphic skin is primarily achieved through innate defences consisting of epithelial barriers, phagocytic cells, natural killer cells and antimicrobial peptides (Rollins-Smith, 2001). The innate immunity is rapid and non-specific, protecting the frog until the adaptive immunity sets in (Carey *et al.,* 1999; Rollins-Smith, 2001). The phagocytic cells and natural killer cells are cytotoxic to pathogens. Alternative pathways and membrane-attack complexes may also form during the protection of the innate immunity (Carey *et al.,* 1999; Rollins-Smith, 2001). The adaptive immune system is highly specific, forming memory cells to antigens (Carey *et al.,* 1999). However, no research has been conducted on the adaptive immunity against fungal infections.

The first defence against pathogens are antimicrobial peptides (Carey *et al.,* 1999). Mucous cells secrete mucopolysaccharides that keep the skin moist while granular glands secrete bioactive peptides (Rollins-Smith, 2001). With the help of norepinephrine and other methods to induce peptide release, researchers were able to study the properties of these peptides and their role in the immunity of the frog (Rollins-Smith, 2001; Rollins-Smith *et al.,* 2005).

Peptides form part of the defence system and the regulation of dermal physiological action (Apponyi *et al.,* 2004) and consist of 10 to 46 amino acids with linear amphipathic helical peptides (Rollins-Smith, 2001; Rollins-Smith *et al.,* 2002a). They are active against gram positive and negative bacteria, fungi, protozoa and viruses (Rollins-Smith,

2001). The mechanism by means of which peptides are able to kill pathogens is still unclear, but it is thought that one or more of the following mechanisms are used: (1) fatal membrane depolarisation, (2) membrane pore formation leading to loss of intracellular contents, (3) induction of hydrolases, (4) disturbance of membrane function and (5) specific damage to critical intracellular targets (Chinchar *et al.*, 2004).

The role of peptides in the protection of frogs against Bd has been studied thoroughly. In 2002, Rollins-Smith *et al.* (2002a) gave the first direct evidence that antimicrobial peptides in the skin can operate as a first line of defence against Bd. Several peptides have been identified with an antimicrobial action against Bd (Table 1).

**Table 1: Some antimicrobial peptides known to have defensive properties against the amphibian chytrid, *Batrachochytrium dendrobatidis*.**

| **Peptide** | **Minimal inhibitory concentration (MIC)** | **Reference** |
|---|---|---|
| Brevinin-1TRa | 12.5 μM | Rollins-Smith *et al.*, 2002c |
| Brevinin-2Ob | 6.25 μM | Rollins-Smith *et al.*, 2002b |
| Caerulein precursor fragment (CPF) | 12.5 μM | Rollins-Smith *et al.*, 2002a |
| Cecropin A-temporin A hybride peptide (CATA) | 47 μm | Wade *et al.*, 2001 |
| Dermaseptin-L1 | 23 μM | Rollins-Smith *et al.*, 2002a; Conlon *et al.*, 2007 |
| Esculentin-1A | 12.5 μM | Rollins-Smith *et al.*, 2002b |
| Esculentin-2L | 12.5 μM | Rollins-Smith *et al.*, 2002b |
| Esculentin-2P | 25 μM | Rollins-Smith *et al.*, 2002b |
| Magainins | 50 - 100 μM | Rollins-Smith *et al.*, 2002a |
| Palustrin-3A | 6.25 μM | Rollins-Smith *et al.*, 2002b |

| | | |
|---|---|---|
| Peptide with amino terminal glycine and carboxyl terminal leucinamide (PGLa) | 100 μM | Rollins-Smith *et al.*, 2002a |
| Phylloseptein-L1 | 25 μM | Conlon *et al.*, 2007 |
| Ranalexin | 9 - 12.5 μM | Rollins-Smith *et al.*, 2002a; Rollins-Smith *et al.*, 2002b |
| Ranatuerin-1 | 50 μM | Rollins-Smith *et al.*, 2002b |
| Ranatuerin-2P | 100 μM | Rollins-Smith *et al.*, 2002b |
| Ranatuerin-2TRa | 50 μM | Rollins-Smith *et al.*, 2002b |
| Ranatuerin-6 | › 100 μM | Rollins-Smith *et al.*, 2003 |
| Temporin-1Ob | 25 μM | Rollins-Smith *et al.*, 2002b |
| Temporin-1P | 50 μM | Rollins-Smith *et al.*, 2003 |
| Temporin-A | 23 - 66 μM | Rollins-Smith *et al.*, 2003; Wade *et al.*, 2001 |

Peptides seem to be more effective against the zoospores of Bd than the zoosporangia (Rollins-Smith, 2001; Rollins-Smith *et al.*, 2002c; Rollins-Smith *et al.*, 2005) and are also stronger when working in combination, synergistically against Bd (Rollins-Smith *et al.*, 2002a).

The phenomenon that frogs still die because of Bd even though they possess antimicrobial peptides remains a mystery. One reason why peptides may not be effective against Bd is because the zoospores mainly attach to the ventral surfaces of frogs and these areas may not be effectively reached by the skin secretions (Apponyi *et al.*, 2004). Another possibility is the variation among species in the distribution of granular glands (Woodhams *et al.*, 2007b). Some species have granular glands evenly distributed on all skin surfaces while others may have areas of concentrated skin glands (Apponyi *et al.*, 2004). Some areas of frog skin do not have a mucous layer within which the peptides can spread. The ventral surfaces of the toes of *Litoria caerulea* lack granular glands (Berger *et al.*, 2005b), and therefore the toes and inguinal region of frogs are targets for Bd due to

the lack of peptides (Woodhams *et al.*, 2007b). If Bd infections of the skin do affect skin peptide defences, it is not depleting the skin peptide stores (Woodhams *et al.*, 2007c). However, infection may disrupt skin structure and block granular gland discharge or regulate the synthesis of individual peptides. The regulation of certain peptides correlates with the infection intensity (Woodhams *et al.*, 2007c), proving that the regulation of peptides is not only influenced by genetic or environmental factors, but also by infection status and history. During stress, corticosteroid is produced. During metamorphosis and breeding, this substance can reach such high levels that it can suppress the immunity of the sub adult frog (Rollins-Smith, 2001). Host resistance is modulated by certain factors (Carey *et al.*, 1999). Natural modulators such as developmental and temperature changes can act synergistically to increase the susceptibility of animals to pathogens. As mentioned previously, the immune system changes during metamorphosis, causing a developmental change that influences immunity. The conditions under which tadpoles metamorphose can affect the immunocompetence of the frog after metamorphosis. Smaller tadpoles are usually immunocompromised (Rollins-Smith, 1998; Carey *et al.*, 1999). Frog immune systems are also more effective at higher temperatures (Cooper *et al.*, 1992; Maniero & Carey, 1997; Carey *et al.*, 1999; Oullet *et al.*, 2004). It has been shown that peptides inhibit Bd at temperatures of 10 - 22°C (Berger *et al.*, 2004). Low temperatures may benefit Bd by decreasing the immunity of frogs and decreasing the amount of peptides and bacteria found on the skin (Berger *et al.*, 2004; Woodhams *et al.*, 2008). Frogs' immunity is the lowest during winter when their white blood cell counts also decrease (Berger *et al.*, 2004). Low temperatures also lower epidermal cell turnover and lower the ability of the frogs to metabolize toxins (Berger *et al.*, 2004). Other factors playing a role are man-made modulators, psychosocial stressors such as crowding, UV light, xenobiotics (Carey *et al.*, 1999) and conditions of hydration (Rollins-Smith *et al.*, 2005). The exact role of antimicrobial peptides is also still debated. The process of obtaining peptides through mild electrical stimulation and norepinephrine injections may also suppress innate and adaptive immunity, making the relative contribution of peptides hard to determine (Chinchar *et al.*, 2004).

However, species with peptides with low levels of activity against Bd are not declining. This may be due to variations in the virulence of Bd, the effectiveness of the peptides and population-level variations in skin shedding rate, life history and behaviour (Harris *et al.*, 2006). Peptides are therefore not the only reason for the immunity of these frogs against Bd. Certain bacteria found on the skin of frogs also help to inhibit Bd by helping to defend them against Bd (Woodhams *et al.*, 2007c). Bacteria that have been isolated and that have inhibitory functions against Bd include: *Arthrobacter spp, Bacillus spp, Kitasatospora spp, Peanibacillus spp, Pedobacter spp, Pseudomonas spp, Streptomyces spp* and *Lysobacter spp.* Most of these bacteria were isolated from salamanders (Harris *et al.*, 2006). These microbes can also prevent the colonisation of pathogens and keep their numbers low due to competition (Belden & Harris, 2007). Probiotics can be a cure or prevention in the fight against Bd (Belden & Harris, 2007).

### 1.7.4 Habitat and behaviour

The worldwide impact of Bd is determined by the interactions between Bd, the environment and the amphibian host; the variations in location, host community, the types of Bd present (virulence) and time (Retallick & Miera, 2007). Another reason why some species decline while others do not may be due to interspecific differences in behaviour (Rowley & Alford, 2007). Certain behavioural differences will increase the frequency with which frogs come in contact with Bd in infected water, environmental substrates and through social behaviour (Woodhams *et al.*, 2008). Social species of frogs, such as *Litoria nannotis*, are more in contact with water and experience more declines than *L. genimaculata* or *L. lesueri.* Adult female frogs are also less likely to become infected with Bd because they spend less time in the water than mating males and only enter a breeding site for one night of the year. They also tend to hibernate alone (Carey *et al.*, 2006). Species associated with water are also more vulnerable to Bd infections than terrestrial species (Williams & Hero, 1998; McDonald & Alford, 1999; Hero *et al.*, 2005; Lips, 2006; Kriger & Hero, 2007). During a study focusing on the prevalence of Bd in five different guilds of breeders, Bd was not detected on any frog inhabiting ephemeral waterbodies and only detected on one terrestrial-breeding frog (Kriger & Hero, 2007). Frogs in permanent waterbodies were more likely to be infected with Bd than frogs in ephemeral or terrestrial habitats; frogs in streams were also most

likely to become infected with Bd within the guild of frogs in permanent waterbodies (Kriger & Hero, 2007).

## 1.8 Are all frogs going to croak?

The devastation Bd wreaks in amphibians can be explained by a population pyramid model (Figure 1.3) (Daszak *et al.*, 1999). According to the population pyramid model host ecological traits and biological traits of Bd combine to cause the declines experienced worldwide. The host ecological traits are, as discussed, naïve populations of frogs in high altitude streams with low fecundity (small clutch sizes) (Lips *et al.*, 2005). The susceptibility of these frogs makes them especially vulnerable to the biological traits of Bd. Increased virulence and persistence due to carriers and reservoirs, together with the fact that Bd can survive outside a host, make their cool habitat a suitable environment in which to grow and infect a variety of hosts. All these traits give rise to local declines (Daszak *et al.*, 1999; Daszak *et al.*, 2003).

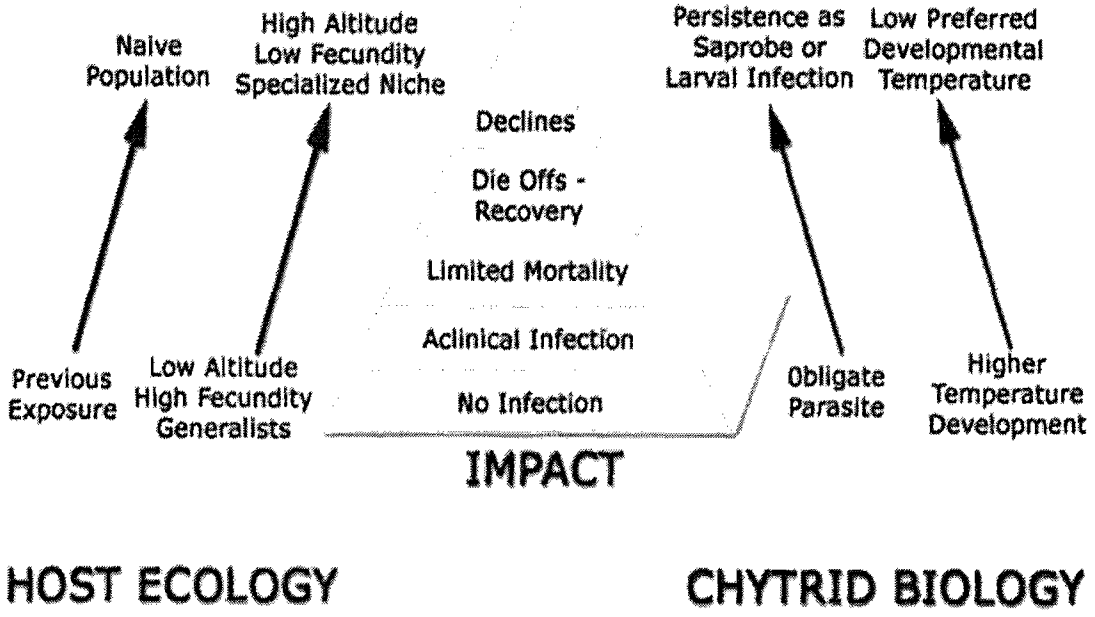


**Figure 1.3 Population pyramid model showing the different outcomes for populations infected with a fungal disease like *Batrachochytrium dendrobatidis* (Daszak *et al.*, 1999)**

However, all is not lost. It has been shown that frogs can become resistant even if Bd is still present, causing populations to recover from declines (Berger *et al.,* 1999). Bd can persist in an endemic state in healthy frogs once an epidemic wave has passed through an amphibian population (Retallick *et al.,* 2004; Briggs *et al.,* 2005; McDonald *et al.,* 2005; Carey *et al.,* 2006). *Taudactylus eungellensis* declined and almost disappeared during the mid-1980s, but now persists with stable Bd infections. This could be either because Bd was not responsible for the declines or the frogs have evolved a resistance to Bd, or Bd has become less virulent (Retallick *et al.,* 2004). Some researchers are still uncertain as to whether Bd is the cause of global amphibian declines (Burgin *et al.,* 2005; McCallum, 2005), but considering all the evidence, Bd seems to be the only explanation for global declines and the disappearance of frog populations and species (Skerratt *et al.,* 2007).

**Objective 1: Identification of areas where Bd is present in South Africa.**

1.a) Contribute to the information on the distribution of Bd in South Africa.

**Objective 2: The establishment and maintenance of *Batrachochytrium dendrobatidis* (Bd) cultures in the laboratory.**

2.a) To provide DNA standards to the National Zoological Gardens molecular laboratory.

2.b) Make cultures available for future multilocus sequence typing analysis.

2.c) To successfully preserve isolated cultures using cryopreservation.

**Objective 3: Determining the role of skin peptides in the immune defense of certain frog species to Bd.**

3.a) Determine whether there are any differences in the antimicrobial peptide makeup of *Amietia vertebralis*[1] and *Hadromophryne natalensis*[2] (Fig. 1.4)

3.b) Determine whether the differences (if any) may explain the phenomenon that *A. vertebralis* dies but not *H. natalensis.*

**Objective 4: Examination of the morphology and development of *Batrachochytrium dendrobatidis.***

Study aspects of the morphology of Bd infections of tadpoles and in culture by means of laser scanning confocal microscopy (LSCM) as well as scanning electron microscopy (SEM).

**Objective 5: Providing a superficial evaluation of the viability of using F10 SC as a treatment for *Batrachochytrium dendrobatidis.***

Study the morphological effects of F10SC veterinary disinfectant on frogs and as a treatment against Bd.

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[1] *Amietia vertebralis* – formerly known as *Strongylopus hymenopus*

[2] *Hadromophryne natalensis* – formerly known as *Heleophryne natalensis*

**Figure 1.4** ***Amietia vertebralis*** **(left) and** ***Hadromophryne natalensis*** **(Photos: Leon Meyer)**

## Chapter outlay

Chapter 1 contains an introduction and brief overview of *Batrachochytrium dendrobatidis* (Bd), as well as objectives to be achieved during this study.

In Chapter 2 localities sampled during this study are listed. Material and methods used for various aspects are also described, ranging from the isolation, cultivation and preservation of Bd in the laboratory, a protocol to extract antimicrobial skin peptides from frogs, various microscopy methods used to examine Bd, including laser scanning confocal microscopy and scanning electron microscopy, and finally a treatment regime for chytridiomycosis using F10SC.

Results obtained from the above-mentioned aspects are shown in Chapter 3. New data pertaining to the distribution of Bd in South Africa, as well as the extraction of DNA from cultures, will contribute to the "Out of Africa" Hypothesis as well as to two epidemiological databases. Results from the peptide study may explain the differences in susceptibility between two South African frog species. Images resulting from the novel method of using confocal microscopy to study Bd are compared between different probes and probe combinations. The adverse reaction of frogs to F10SC is described.

In Chapter 4 the following are discussed: the importance of adding new Bd-positive localities and isolating and preserving cultures from them; the role of antimicrobial peptide studies in the identification and control of susceptible frog species; the efficiency of the different fluorescent probes, problems associated with the process, as well as the implication of the results on Bd research. Advice into the alterations of the F10SC protocol or the use of alternative treatments is provided in this chapter.

Concluding remarks from the previously mentioned chapters are shortly documented in Chapter 5.

# Chapter 2

# Material and methods

## 2.1 Collection of animals

Tadpoles and adults of available frog species were collected from suitable habitats with the use of dip nets and catching by hand (Fig. 2.1). If adults were not available, tadpoles and sub-adults were caught. The study was not dependent on the age of the animals or the species collected, but rather their distribution. Animals were collected from areas that were lacking data on the distribution of *Batrachochytrium dendrobatidis* (Bd).

**Figure 2.1 Field sampling: Frogs were collected by hand and tadpoles by using a dip net.**

Areas sampled in order to collect specimens include:

- Mpumalanga Province – Mac-Mac Falls, Sabie, Rainbow Falls, Hazyview, Nelspruit, Middelburg
- Free State Province – Memel, Heilbron, Bethlehem
- KwaZulu-Natal – Royal Natal National Park, Boston, Winterton, Vernon Crooks (Scottburgh), Newcastle
- Limpopo Province – Pietersburg, Magoebaskloof, Tzaneen, Waterberg and along the Luvuvhu River
- North-West Province – Koppies, Swartruggens
- Western Cape Province - Cape Peninsula (Silvermine)

- Northern Cape Province – Jan Kempdorp

The animals were transported to the North-West University where postmetamorphic animals were housed in plastic containers with water and damp gravel; and tadpoles in aerated aquarium tanks until they were screened for Bd or used in experiments. Frogs were fed every second day on lab-raised crickets.

## 2.2 Application of isolated Bd

### 2.2.1 Culturing of Bd

Information on the date, species and locality of the host were obtained and the Bd status of the frogs was noted in a spreadsheet. The isolation process consisted of a series of steps as shown in Figure 2.2. Tissue samples were removed from the webbing of frogs or the mouthparts of tadpoles and screened for Bd with a dissecting microscope at 20X or 40 X. Bd can be recognised as round walled bodies (10-20 μm) inside superficial epidermal cells (Fig. 2.2.c). The Bd from infected skin was then transferred to a petri dish containing mTGh-agar and antibiotics (8 g tryptone, 2 g gelatin hydrolysate, 10 g agar, 1 L distilled water) for cultivation (Berger *et al.,* 2001). The skin was cleaned by drawing and pushing it through nutrient agar to remove bacteria, yeast and fungal spores. The cleaned skin was placed on a fresh plate of mTGh-agar using sterile techniques that minimise contamination with airborne fungal spores. The agar plates were then sealed with Parafilm and labelled to indicate the allocated number of the isolate as well as the date. The plates were then incubated at 22°C and the development checked by inverting the culture plate on the stage of a compound microscope with the low power objective.

The plates were checked daily for contamination during the first few weeks of incubation. Fungal and bacterial contaminants were removed with a flame-sterilised scalpel, taking care to remove all fungal hyphae and bacteria. After increased zoospore activity around the colonies was observed, the colonies were transferred to tryptone agar. Aseptic methods were used to transfer chytrid colonies in groups to a plate of tryptone agar (16 g tryptone, 10 g agar and 1 liter distilled water) after which they were incubated for one to two weeks. If the colony grew without any contaminants, it was transferred to nutrient broth in a screw-capped 250 ml culture flask containing tryptone broth (10 g tryptone and 1 liter distilled water). It was found that it is unnecessary to include the tryptone agar

step in the cultivation process. During the later isolations, the colonies were directly transferred from mTGh-agar to tryptone broth. After about four days the broth cultures were transferred to a 5 °C fridge.

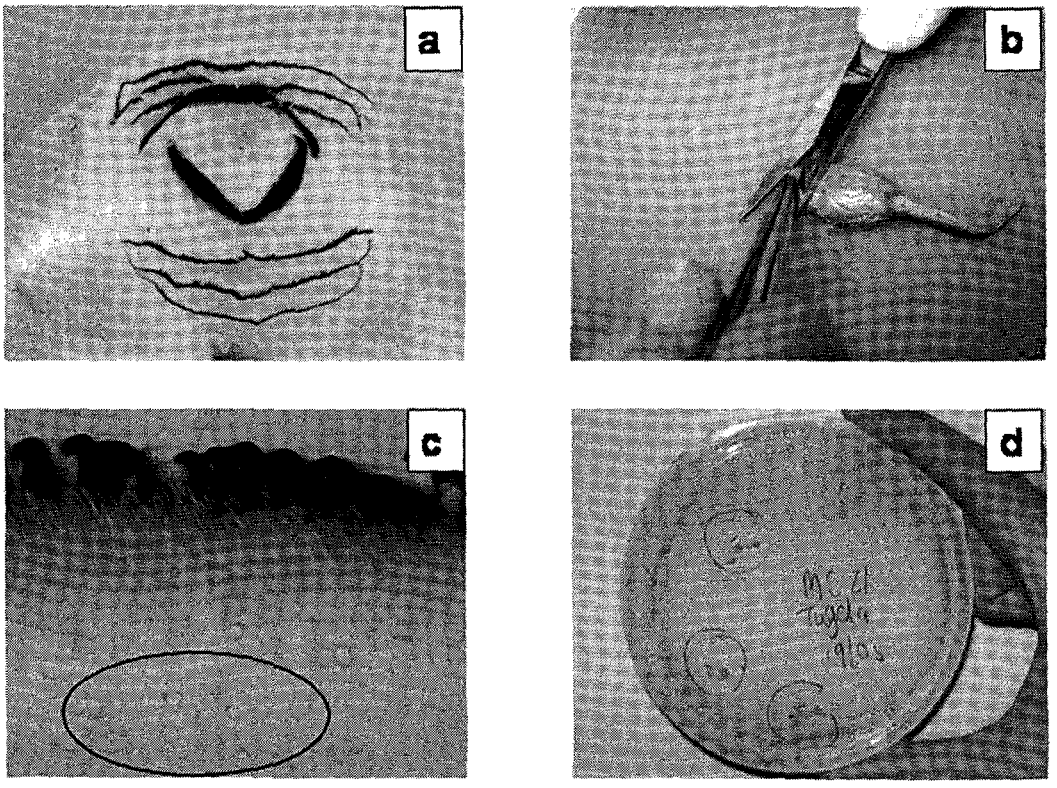


**Figure 2.2 The isolation process consists of surgically isolating the keratinised mouthparts (a and b) from the tadpole and inspecting it for the spherical sporangia of *Batrachochytrium dendrobatidis*, as indicated in (c). The infected areas were isolated, cleaned and then transferred to mTGh agar (d).**

## 2.2.2 DNA extraction from cultures

Cultures suitable for DNA extraction were selected on the criteria of numerous active zoospores, the locality from which the culture was obtained, as well as the species from which they were isolated. The best isolates from the same locality but from different species were used. Otherwise, one culture from the same species and the same locality was used in the DNA extraction. The procedure for DNA extraction was conducted at the NRF-funded laboratory situated at the National Zoological Gardens.

In order to extract the DNA, a DNeasy Blood and Tissue Kit (Qiagen) was used. Before the complete procedure, 96 – 100 % ethanol was added to AW1 and AW2 in order to prepare the working solutions of the buffers according to the protocol. The water bath was also heated to 56°C beforehand.

a) Cultures showing good growth (broth had a whitish colour) were used during the experiment. Each culture was done in duplicate.
b) An amount of 1.5 mL culture was placed in a 1.5 mL Eppendorf tube and centrifuged for five minutes at 5000 rcf to ensure pellet formation. The supernatant was then removed, sterilised and discarded.
c) Pellet was resuspended in 200 µL PBS (50 mM potassium phosphate, 150 mM NaCl) buffer.
d) 20 µL Proteinase K was then added to the tube. Proteinase K prevents enzymes from destroying the DNA during extraction.
e) 200 µL Buffer AL was then added. Buffer AL is a lysing buffer. The solution was then mixed and vortexed for 15 seconds.
f) Samples were then incubated at 56°C for 10 minutes.
g) After the incubation, 200 µL of -20°C 96 - 100% ethanol was added to the samples and mixed using a vortex.
h) Tubes were then placed in the freezer for five minutes to allow the DNA to precipitate.
i) Samples were transferred using a pipette to labelled DNeasy spin columns which were centrifuged at 6000 rcf for 1 minute.
j) The upper filter part of the spin column was transferred to a new collection tube.
k) 500 µL of a wash buffer, Buffer AW1, was added to the column and centifuged at 6000 rcf for one minute.
l) The filtered column was transferred to a new collection tube and washed again using 500 µL Buffer AW2 and centrifuged at 16 000 rcf for three minutes.
m) The filtered column was then transferred for a last time to a labelled 1.5 mL Eppendorf, after which 200 µL Buffer AE was added to the column in order to elude the solution.

n) The samples were then incubated for five minutes at room temperature and centrifuged at 6000 rcf for one minute. The filtered column could then be discarded, leaving the extracted DNA in the Eppendorf.

o) Extracted DNA was then analysed using a NanoDrop in order to test the amount of DNA in the samples.

p) Extracted DNA was kept at -80°C.

### 2.2.3 Cryopreservation of cultures

In order to ensure the continued survival of cultures, it is necessary to passage cultures every fourth day to a week when kept in an incubator or every two to three months when the cultures are held at 5°C. This method may prove to be too time and reagent-consuming and therefore another method, called cryopreservation, can be implemented. This long-term storage technique helps to ensure the genetic integrity of the isolates and allows for the recovery of cultures from storage at -80°C and in liquid nitrogen over an extended period (Boyle *et al.*, 2003). The protocol used to cryopreserve our cultures is based on the paper by Boyle *et al.* (2003) and modified by Megan Johnson (2003).

a) Broth cultures were transferred to new broth and incubated for three to four days in order for an active growth phase to develop. The active growth phase can be described as zoospore activity and an increase in mature, zoospore releasing zoosporangia.

b) A cell scraper was used to loosen cells from the walls of the flask and the culture was then transfered to a centrifuge tube. It was then centrifuged at 2 500 rpm for 10 minutes after which the supernatant was discarded.

c) The pellet was resuspended in 1 mL cryo-media consisting of 10% DMSO (dimethyl sulphoxide) and 10% foetal calf serum in tryptone broth.

d) Media containing the culture was aliquoted into a labelled cryo tube and placed in a cryocontainer for a minimum of four hours.

e) The cryotube was then placed into liquid nitrogen. It can survive for up to nine months in this state (Boyle *et al.*, 2003).

## 2.3 Microscopy examination of Bd

The ultrastructure of Bd in culture and tissue was described using both Scanning Electron Microscopy FEI Quanta 200 ESEM at 10 kV and Confocal Laser Scanning Microscope (LCSM) Nikon D-eclipse C1 *si* equipped with a violet diode laser of 400-450 nm, a He/Ne laser of 543 nm and an argon ion laser with a wavelength of 457-514 nm, as well as a Confocal Laser Scanning Microscope (CLSM) Nikon PCM2000 only equipped with a He/Ne laser with an excitation wavelength of 543 nm and an argon ion laser of 457-514 nm.

Standard procedure was used as protocol for SEM examination. Fluorescines were used to stain the fungus for the confocal procedure. The different developmental stages were subsequently examined.

### 2.3.1 Scanning electron microscopy

In order to describe the infection of Bd on the mouthparts of tadpoles, the mouthparts of *Amietia vertebralis* (Phophung river frog) and *Hadromophryne natalensis* (Natal Cascade frog) tadpoles were surgically removed and fixed in Todd's solution for more than 24 hours. The mouth was fixed in a position that kept the labial toothrows intact with the mouth gaping. Tissues were further fixed in 2 % osmium tetraoxide solution and an acetone series. Hereafter, tissues were critical-point-dried and sputter-coated with 20 nm layer of gold/palladium (20/80).

### 2.3.2 Laser scanning confocal microscopy

Cultures grown by means of the above-mentioned culturing process (2.1.2) were used for this technique. 1 ml of culture was placed in an Eppindorph tube and 100 µL distilled water was added. The solution was then placed in a centrifuge for seven minutes at 5000 rpm. This caused the sporangia to form a pellet at the bottom of the tube and the zoospores to be suspended in the supernatant. The supernatant was then removed and placed in a separate vial. The pellet was resuspended in distilled water and the stain was added. Some stains needed an incubation period before the preparation could be viewed. A drop of this solution was placed on a microscope slide and examined at 60 X

magnification. Images were saved as ids-files (image cytometric data files) and saved as either tiff- or bmp-files on EZ2000 for Confocal Microscope PCM2000 software.

The following stains were used:

- **LysoTracker Green**

1 µL LysoTracker was added to the specimen and incubated for 15 minutes in the dark. LysoTracker is a fluorescine that specifically stains hydrogen ions (highlights differences in pH). It can be used to stain areas green were acids accumulate, for instance lysosomes.

- **MitoTracker Red**

1 µL MitoTracker was added to the specimen and incubated for 15 minutes in the dark. MitoTracker is a fluorescine that stains areas with a membrane potential, such as that of mitochondria, red.

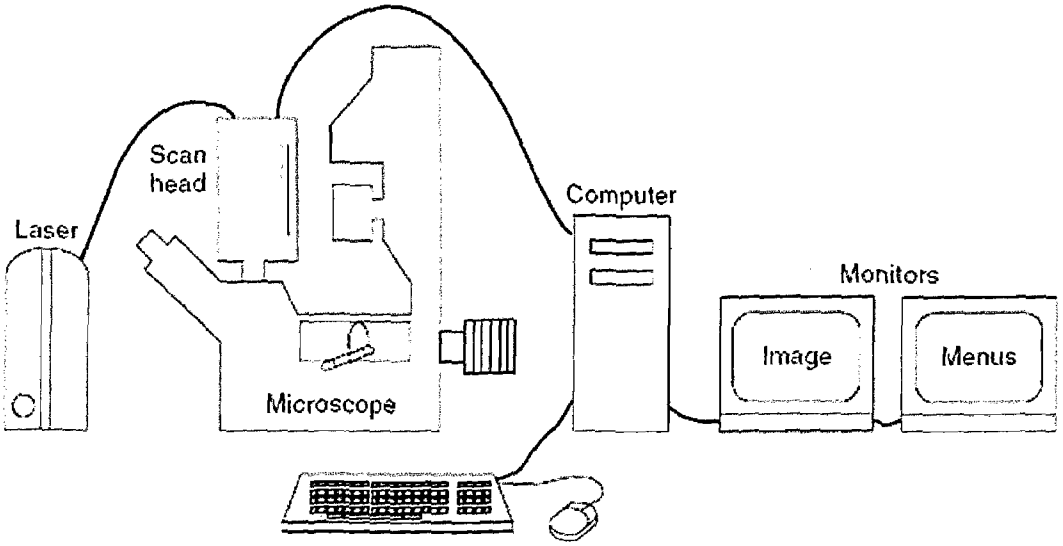


**Figure 2.3 The basic components of a confocal laser scanning microscope. A laser is scanned across the specimen by the scan head. The scan head directs the fluorescent signals to the pinhole and the photomultiplier tube (PMT). The image is then built up electronically on the monitor from signals received by the PMT. The second monitor displays software menus for image acquisition and processing. (Image from Murphy, 2001)**

- **Nile red**

5 µL Nile Red was added to the culture. No incubation period was needed. This stain is specific for phospholipids and stains red.

- **Acridine orange**

5 µL Acridine Orange was used to stain the culture and incubated for 15 minutes. It stains ssRNA red and dsDNA green. Acridine orange can also be used to stain areas with a low pH such as lysosomes (Canonico & Bird, 1969), staining it orange.

- **Calcofluor White M2R**

5µL Calcofluor White M2R stain was added to the culture along with 50 µL distilled water, 50 µL 10 mM HEPES and 50 µL 2% D-(+)-glucose. The mixture was then incubated for 30 minutes in the dark. Calcofluor is a stain specifically for yeast and fungi, staining the chitin in the cell walls blue.

- **Alexa Fluor 488 Phalloidin**

1 µL Alexa Fluor 488 Phalloidin stain was used on culture for an incubation period of 15 minutes. Phalloidon is a fluorescine commonly used to stain actin filaments green, but can also be used to stain other specimens.

- **FUN1**

5µL FUN1 was added to the culture along with 50 µL distilled water, 50 µL 10 mM HEPES and 50 µL 2% D-(+)-glucose. The mixture was then incubated for 30 minutes in the dark. FUN1 is a stain used to identify living and dead cells in yeast and fungal cultures. Dead cells have a diffused yellow colour while living ones are bright yellow containing red intravacuolar structures.

## 2.4 Skin peptide collection and analysis

Skin peptides of two species of frogs *A. vertebralis* and *H. natalensis* were extracted for comparison of their anti-fungal properties. Frogs were collected in April 2008 at Royal Natal National Park. Skin secretions (containing the antimicrobial peptides) were collected from each frog species for the analysis of the peptides found in each frog species. The protocol used for the collection of the skin peptides was received from

Louise Rollins-Smith[3] and is a modification of the Rollins-Smith *et al.* (2002b) protocol. Among the analyses done by Rollins-Smith were the relative peptide concentrations eluted from the Sep-Pak cartridges which were analysed, as well as Bd anti-growth inhibition assays for both the peptides from *H. natalensis* and *A. vertebralis.* Mass spectrometry (MALDI-TOF) analysis was also conducted in order to analyse the profile from each sample.

### 2.4.1 Peptide collection

The following reagents were prepared beforehand:

- **Amphibian Phosphate-Buffered Saline (APBS)**

6.6g NaCl

1.15g $Na_2HPO_4$ (anhydrous) or 2.17g $Na_2HPO_4.7H_2O$

0.2g $KH_2PO_4$

One litre of distilled water was added to the salts and the pH set to 7.4.

- **Norepinephrine-bititrate salt** (Sigma N-5785) for 10 nmole/gram body weight subcutaneous injection

a) 13.5 mg norepinephrine-bitrate salt was weighed in a 50 ml tube using a sensitive analytical balance.

b) 40 ml APBS was added to the norepinephrine powder, and filter-sterilised (0.22micron syringe filter) before being injected into frogs

c) The frogs were then injected with 0.01 ml per gram frog.

The solution can be stored frozen (-20 °C), wrapped in foil to limit light exposure.

- **Collecting Buffer**

2.92g NaCl

2.05g Na Acetate

Fill to 1L HPLC-grade water (Fisher W5-4, 4L)

The solution can be stored on the shelf.

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[3] Louise Rollins-Smith: Departments of Microbiology and Immunology & Pediatrics, Vanderbilt University, Medical Center, Nashville, TN 37232, USA

- **HCl**

  a) Dilute the HCl to 50% to prevent breathing toxic fumes. Combine 20 ml HCl with 20 ml HPLC water in a 50 ml centrifuge tube.
  The HCl can be stored on the shelf.

- **Buffer A**

  1 ml HCl

  1 L HPLC water

  This was used for preparing Sep-Pak filters, keeping filters moist while stored in tubes.

  The solution can be stored on the shelf.

In order to collect the peptides, the following steps were performed:

a) Ten *A. vertebralis* and seven *H. natalensis* were weighed in a plastic bag using a pezola. The injectable amount of norepinephrine was calculated from the frog's weight (0.01 ml / g).

b) The norepinephrine was injected into the dorsal lymph sac of the frogs using a 1 ml syringe with 27.5 G needle. A microsyringe was used for the smaller *A. vertebralis* individuals.

c) After injection, the frogs were kept in the collecting buffer for 15 minutes.

d) During this time, Sep Pak Plus C-18 cartridges were activated by flushing two connected cartridges with 10 ml methanol, followed by 10 ml Buffer A. The cartridges were kept moist and stored in approximately 2 ml Buffer A during the procedure.

e) Frogs were removed from the collecting buffer, dried and weighed for a second time. 1 ml of the HCl solution was added to the collecting buffer immediately after removal of the frogs.

f) Next, the frogs were swabbed for the detection of *B. dendrobatidis*.

g) The collecting buffer with the released peptides and HCl were then pushed through the two connected cartridges using a 50 ml syringe. A new syringe was used for each frog's peptides.

h) Sep Pack cartridges, along with the swabs, were then sent to Louise Rollins-Smith's laboratory at Vanderbilt University in the United States of America.

### 2.4.2 MALDI-TOF MS analysis of the skin peptide mixture

The naturally occurring skin peptides within the Sep-Pak cartridges were analysed using MALDI-TOF (matrix-assisted laser desorption ionisation time-of-flight mass spectrometry). The different peptides in the peptide mixture were then shown as a MALDI-TOF mass spectrum.

### 2.4.3 Bd anti-growth inhibition assays

The inhibition of Bd by the peptides was done by exposing zoospores in broth to HPLC-grade water with or without diluted peptides (Conlon *et al.*, 2007). A positive control (maximal growth) of broth cultures without any added peptides, and a negative control (maximal growth inhibition) in which the cultures were treated by temperature shock (60°C for 10 minutes) to kill cells, were used to determine the minimal inhibitory concentration at which no growth was detectable.

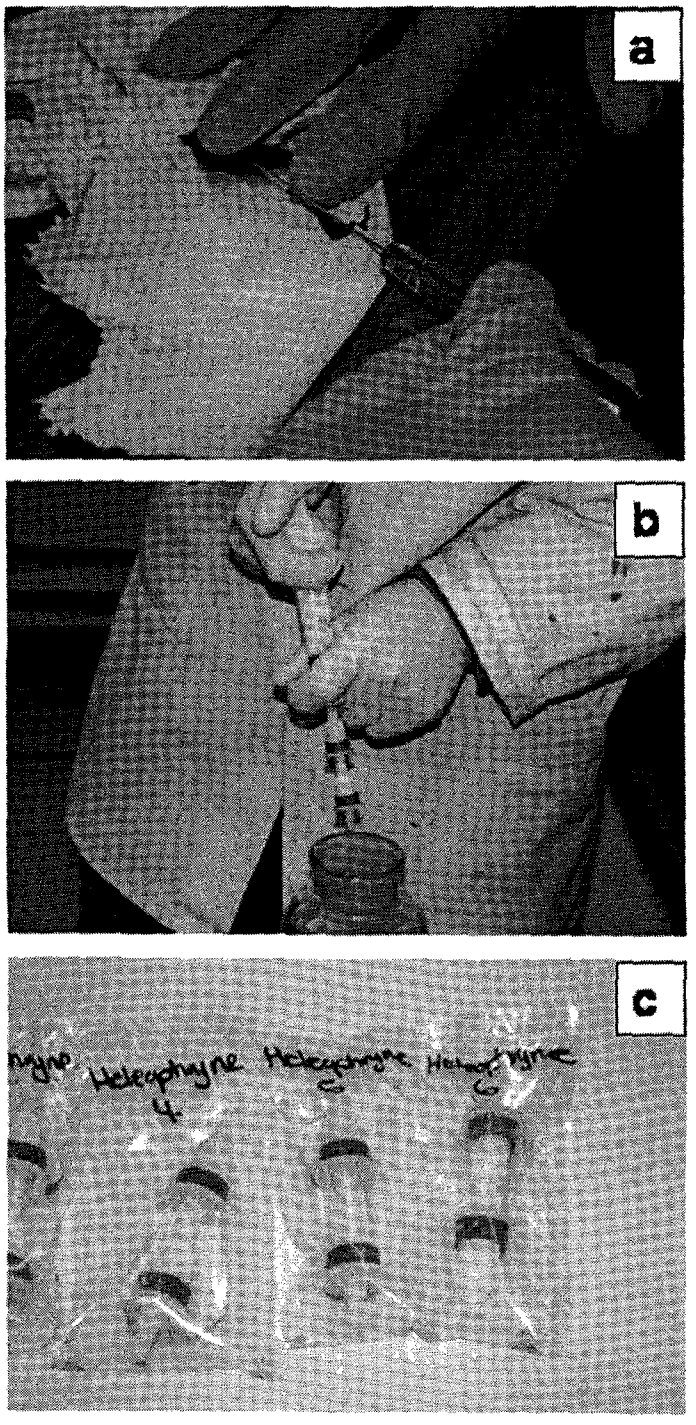


**Figure 2.4 (a) Norepinephrine is injected in the vicinity of the dorsal lymph sack in order to stimulate peptide excretion. In (b) the extracted peptides, along with buffers, are pushed through the Sep Pak cartridges, which are then packaged and ready to be sent to the lab (c).**

## 2.5 Treatment of B. dendrobatidis with F10 veterinary disinfectant

Numerous disinfectants have been identified to cure frogs of Bd. Some of these disinfectants have only been tested on *in vitro* cultures of Bd. One of these products is F10 Super Concentrate. The product is described as biodegradable, non-toxic and non-irritating. In Webb *et al.* (2007) F10SC, a veterinary disinfectant containing quaternary ammonia and biguanidine compounds, was used to kill Bd culture *in vitro*. The compound was still effective against Bd at concentrations of 1:3 500 for an exposure time of ten minutes. In the article, the effectiveness of F10SC against Bd *in vivo* was contemplated, leading to this experiment.

**Figure 2.5 Frogs were housed in containers containing both water and gravel during the experiment (seen at back). Exposures to F10 for 30 minutes were conducted in smaller containers as seen at the front of the picture.**

20 *Amietia vertebralis* and six *Hadromophryne natalensis* were exposed to either a 1:3 000 or 1:3 500 F10SC dilutions for 30 minutes for four consecutive days. This exposure schedule was decided upon since Bd seems to have a life-cycle spanning four to five days (Berger *et al.*, 2005a). Exposures were done by placing the animals in plastic containers with a volume of 50 mL F10SC. The dilution volume was sufficient to partially submerge the frogs.

After 30 minutes, the frogs were removed, rinsed with clean water and placed inside a plastic container containing 200 mL aged water as well as gravel, forming a terrarium. The frogs were held in a room with the temperature regulated at 20°C.

# Chapter 3

# Results

## 3.1 Localities and species sampled for Bd

Currently the Bd Mapping Project for South Africa has a total number of 39 samples, consisting of 27 sites and 14 species from which only five samples have been genotyped. Localities that overlap between the known areas already in the Project and those sampled during this study comprise the following:

- Free State, Harrismith
- Some areas in Limpopo Province (exact localities not indicated on the web site)

The following new localities and Bd infected species can now be added:

- Kwazulu-Natal, Vemvaan Upper – *Amietia vertebralis*, Hadromophryne natalensis**
- Kwazulu-Natal, Ribbon Falls Lower – *Amietia vertebralis*, Amietia angolensis*
- Kwazulu-Natal, Winterton – *Kassina senegalensis**
- Kwazulu-Natal, Scottburgh – *Hyperolius pusillus*, Hyperolius marmoratus*, Ptychadena oxyrhynchus**
- Mpumalanga, Sabie – *Amietia angolensis,*
- Mpumalanga, MacMac Falls – *Amietia angolensis*
- Mpumalanga, Tugela – *Amietia vertebralis**
- Mpumalanga, Hazyview – *Amietia angolensis, Phrynobatrachus natalensis**
- Mpumalanga, Nelspruit – *Phrynobatrachus natalensis*, Hyperolius marmoratus**
- Mpumalanga, Middelburg – *Amietia fusgicula, Xenopus laevis*
- Free State, Memel – *Amietia angolensis, Amietia fuscigula, Xenopus laevis, Cacosternum sp.*
- Western Cape, Silver Mine – *Amietia fuscigula*
- Limpopo Province, Magoebaskloof – *Hadromophryne natalensis*, Amietia angolensis*
- Limpopo Province, Waterberge – *Amietia angolensis*
- Limpopo Province, Luvuvhu river (Louis Trichardt) – *Kassina senegalensis*, Hyperolius marmoratus/semidiscus*, Cacosternum boettgeri, Amietia angolensis, Xenopus laevis*
- Northern Cape, Jan Kempdorp – *Xenopus laevis, Amietia angolensis, Amietia fuscigula*

*New species added to South Africa's account in the Bd Mapping Project

Frogs were collected predominantly in the northern areas of South Africa, including the Limpopo Province, Mpumalanga Province, Kwazulu-Natal, and the Free State Province and minimally in the Northern and Western Cape Provinces. The species sampled from these localities are listed in Table 3.1, and consist of the following families:

- Arthroleptidae (*Leptopelis natalensis*)
- Bufonidae (*Amietiophrynus gutturalis*)
- Heleophrynidae (*Hadromophryne natalensis*)
- Hyperoliidae (*Hyperolius marmoratus, Kassina senegalensis, Hyperolius pusillus, Hyperolius tuberilinguis, Afrixalus fornasinii*)
- Pipidae (*Xenopus laevis*)
- Phrynobatrachidae (*Phrynobatrachus natalensis*)
- Ptychadenidae (*Ptychadena oxyrhynchus*)
- Pyxicephalidae (*Amietia angolensis, Amietia fuscigula, Cacosternum sp., Amietia vertebralis*)

The majority of the material collected consists of tadpoles as they are easier to catch and the effect on amphibian populations is less destructive. An exception was *Xenopus laevis*, from which only sub-adults and adult frogs were collected as the tadpoles have no keratinised mouthparts and thus no Bd. On tadpoles Bd is only associated with keratinised mouthparts.

Various habitats of frogs were sampled, including streams, rivers and riverbanks, ephemeral pools, vleis, ponds and dams. Figure 3.1 clearly shows that Bd seems to be situated mainly in streams or flowing water. The data may be interpreted in this manner since most of the animals were caught from streams or rivers, although the fraction of infected frogs in relation to the total number of infected animals was the highest in stream-breeding frogs. Of the 201 animals caught in this habitat, 103 were found to be infected with Bd using microscopic screening of isolated mouthparts or webbing, while only 11 of the 137 animals caught in dams were infected using the same technique.

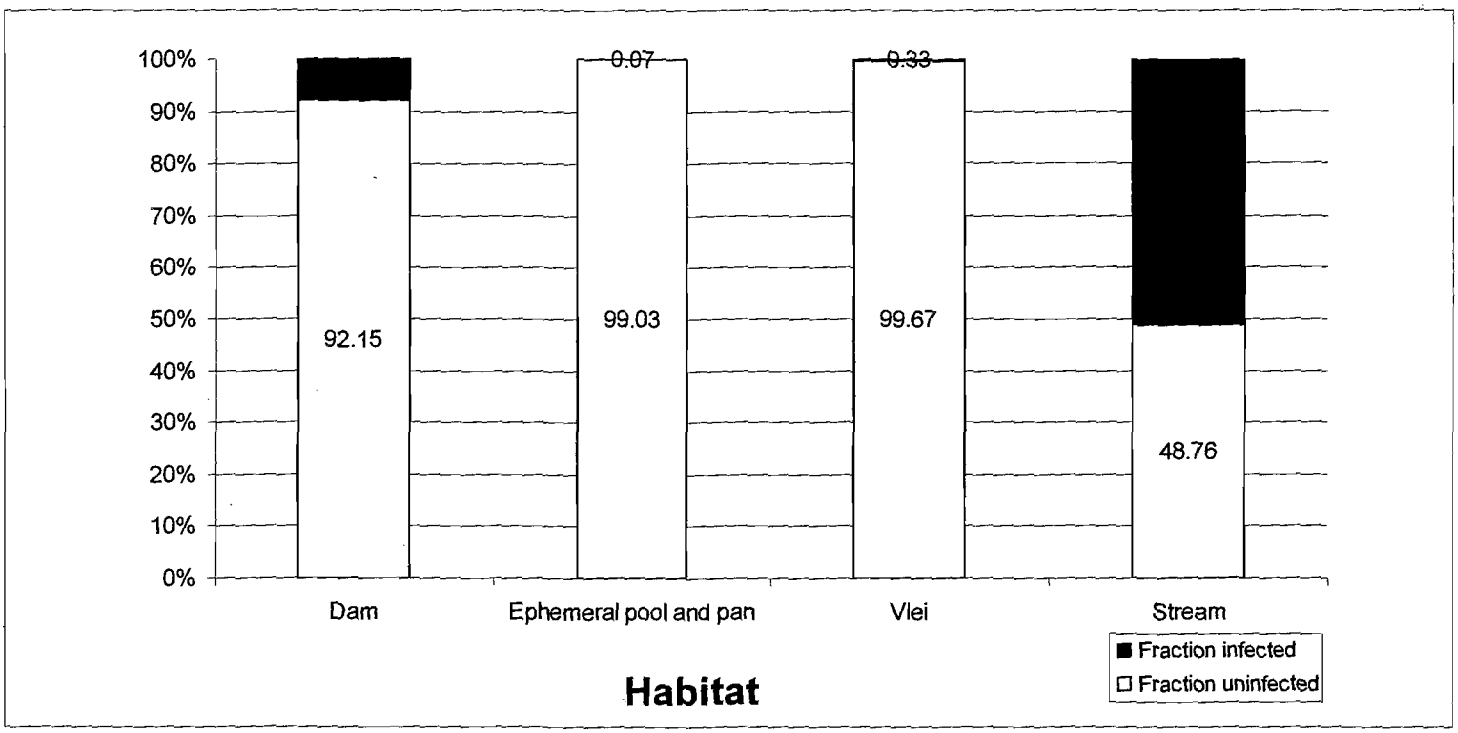


**Figure 3.1 The fraction of *Batrachochytrium dendrobatidis* infected frogs collected from different habitats.**

As can be seen from Table 3.1, the only family of frogs collected during the study with a 100% infection percentage was Heleophrynidae. *Hadromophryne natalensis* tadpoles were collected during April 2007 at Vemvaan in the Royal Natal National Park and transported alive to the North-West University.

**Table 3.1 Summary of the species sampled during the study, as well as the infection fraction and percentage of frogs infected with *Batrachochytrium dendrobatidis* per family.**

| Family | Species | Infection fraction | Prevalence per family as percentage |
|---|---|---|---|
| Bufonidae | *Amietiophrynus gutturalis* | 0/1 | 0 |
| Heleophrynidae | *Hadromophryne natalensis* | 15/15 | 100 |
| Hyperoliidae | *Hyperolius marmoratus* | 7/33 | 16 |
| | *Kassina senegalensis* | 4/33 | |
| | *Hyperolius pusillus* | 3/15 | |
| | *Hyperolius tuberilinguis* | 0/4 | |
| | *Afrixalus fornasinii* | 0/2 | |
| Pipidae | *Xenopus laevis* | 20/112 | 18 |
| Pyxicephalidae | *Amietia angolensis* | 40/107 | 39 |
| | *Amietia fuscigula* | 8/31 | |
| | *Cacosternum* | 2/21 | |
| | *Amietia vertebralis* | 25/31 | |
| Arthroleptidae | *Leptopelis natalensis* | 0/4 | 0 |
| Phrynobatrachidae | *Phrynobatrachus natalensis* | 3/6 | 50 |
| Ptychadenidae | *Ptychadena oxyrhynchus* | 2/2 | 100 |

In Figure 3.2 the localities sampled from the provinces are indicated with different coloured stars. The dark blue and yellow stars indicate localities sampled for Bd during this study. The yellow stars indicate localities from which successful cultures were isolated. It is clear that a large part of South Africa has not been sampled yet. In Table 3.2 all positive sites for Bd are indicated, whether or not any cultures were successfully isolated.

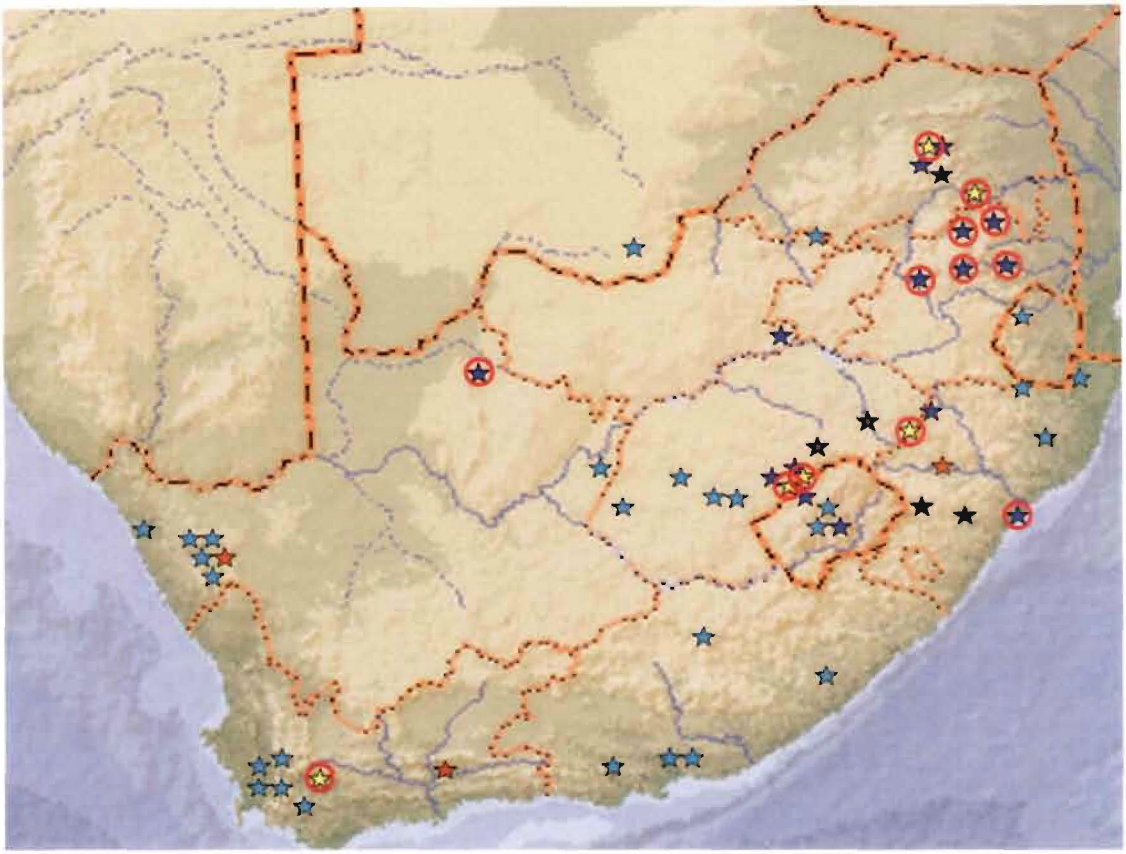

**Figure 3.2 Field sampling for Bd in South Africa. The light blue stars (★) indicate localities sampled in previous studies, while the dark blue stars (★) represent sampling done in this study. The orange stars (★) are cultures isolated during previous studies, with the yellow stars (★) indicating the localities from which cultures were isolated during this study. Stars encircled with red are positive localities identified during this study.**

**Table 3.2 Collection data for *Batrachochytrium dendrobatidis* sampling. The last column shows from which species cultures were successfully isolated.**

| Species | Locality | Habitat | Fraction infected | GPS reading | Altitude (m) | Culture |
|---|---|---|---|---|---|---|
| *Amietia vertebralis* | Vemvaan Upper | stream | 3/3 | S28.74913° E028.87186° | 3031 | X |
| *Amietia angolensis* | MacMac | stream | 3/3 | S25.01393° E030.83127° | 1286 | |
| *Amietia angolensis* | Rainbow Falls | stream | 1/1 | S28.76317° E028.91848° | 1250 | |
| *Amietia angolensis* | Sabie | stream | 1/2 | S25°05'08" E30°46'42" | | |
| *Amietia vertebralis* | Ribbon Falls Lower | stream | 12/15 | S28.76268° E028.91794° | 2963 | X |
| *Hadromophryne natalensis* | Vemvaan | stream | 3/3 | S28.72230° E028.91598° | 2321 | |
| *Amietia vertebralis* | Tugela | stream | 10/13 | S28.74185° E028.91460° | 1809 | |
| *Amietia angolensis* | Memel | stream | 10/24 | S2772390° E029.52393° | 1807 | |
| *Amietia fuscigula* | Memel | stream | 2/5 | S2772390° E029.52393° | 1807 | X |
| *Xenopus laevis* | Memel | stream | 2/2 | S2772390° E029.52393° | 1807 | |
| *Cacosternum* | Memel | ephemeral pool | 1/20 | S27.72094° E029.52163° | 1787 | |
| *Amietia sp* | Bethlehem | dam | 0/6 | S28.29671° E028.30458° | 1664 | |
| *Amietia angolensis* | Boston Upper dam | dam | 0/1 | S29.70132° E030.01444° | 1333 | |
| *Hyperolius marmoratus* | Boston Upper dam | dam | 0/5 | S29.70132° E030.01444° | 1333 | |
| *Xenopus laevis* | Boston Upper dam | dam | 0/5 | S29.70132° E030.01444° | 1333 | |
| *Phrynobatrachus natalensis* | Winterton Alpine View | dam | 0/1 | S28.752194° E029.619611° | | |
| *Kassina senegalensis* | Winterton Alpine View | dam | 0/5 | S28.752194° | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | E029.619611° | | |
| *Kassina senegalensis* | Vernon Crooks Loop Road | dam | 0/19 | S28.75071° E028.86821° | 425 | |
| *Hyperolius pusillus* | Vernon Crooks Loop Road | dam | 3/15 | S28.75071° E028.86821° | 425 | |
| *Ptychadena oxyrhynchus* | Vlei at Vernon Crooks | vlei | 2/2 | S30.26072° E030.61093° | 425 | |
| *Leptopelis natalensis* | Vlei at Vernon Crooks | vlei | 0/4 | S30.26072° E030.61093° | 425 | |
| *Hyperolius tuberilinguis* | Vernon Crooks Loop Road | dam | 0/4 | S28.75071° E028.86821° | 425 | |
| *Afrixalus fornasinii* | Vernon Crooks Loop Road | dam | 0/1 | S28.75071° E028.86821° | 425 | |
| *Phrynobatrachus natalensis* | Vernon Crooks Loop Road | dam | 0/1 | S28.75071° E028.86821° | 425 | |
| *Hyperolius marmoratus* | Vernon Crooks Loop Road | dam | 1/1 | S28.75071° E028.86821° | 425 | |
| *Kassina senegalensis* | Rondepan (Polokwane) | dam | 0/14 | S23.77177° E029.39526° | 1238 | |
| *Amietia fuscigula* | Cape - Silver mine | dam | 3/19 | S34.09091° E018.42114° | 274 | X |
| *Amietia angolensis* | Picasso (Haenertsburg) | stream | 0/10 | S23.93994° E029.94115° | 1371 | |
| *Xenopus laevis* | Tom Naude (Polokwane) | dam | 0/1 | S23.88600° E029.45783° | 1251 | |
| *Amietia angolensis* | Magoebaskloof | stream | 12/18 | S23.81647° E030.02381° | 1102 | X |
| *Xenopus laevis* | Polokwnae 1 | dam | 0/13 | S23.93063° E029.53474° | 1274 | |
| *Xenopus laevis* | Bypass S (Polokwane) | dam | 0/15 | S23.94190° E029.41277° | 1264 | |
| *Hadromophryne natalensis* | Magoebaskloof | stream | 12/12 | S23.81647° E030.02381° | 1102 | X |
| *Hyperolius marmoratus* | Tom Naude (Polokwane) | dam | 0/4 | S23.88600° E029.45783° | 1251 | |
| *Hyperolius marmoratus* | Prison Dam (Tzaneen) | dam | 0/1 | S23.83330° | 788 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | E030.13711° | | |
| *Amietia angolensis* | Newcastle | stream | 0/14 | S27.69016° E029.86317° | 1293 | |
| *Xenopus laevis* | Swartruggens | dam | 0/2 | S25°39'08" E26°31'37" | | |
| *Xenopus laevis* | Koppies | dam | 0/1 | S27.20085 E027.51591 | | |
| *Phrynobatrachus natalensis* | Hazyview2 | dam | 2/3 | S25.10707° E031.07805° | 757 | |
| *Hyperolius marmoratus* | Hazyview2 | dam | 0/6 | S25.10707° E031.07805° | 757 | |
| *Amietia* | Hazyview1 | stream | 1/1 | S25.02076° E031.01168° | | |
| *Hyperolius marmoratus* | Nelspruit | dam | 2/6 | S25.42699° E030.90656° | 696 | |
| *Phrynobatrachus natalensis* | Nelspruit 1 | stream | 1/1 | S25.44614° E030.88477° | 614 | |
| *Amietia fuscigula* | CR Swart (Middelburg) | stream | 0/1 | S25.78141° E029.46848° | 1433 | |
| *Amietia fuscigula* | Martiens (Middelburg) | stream | 1/2 | S25.60857° E029.71210° | 1678 | |
| *Amietia angolensis* | Glass (Sabie) | stream | 2/5 | S25.08943° E030.77788° | 949 | |
| *Amietia angolensis* | Waterberge Site 1 | stream | 2/3 | S24°18' E28°33' | | |
| *Amietia angolensis* | Waterberge Site 2 | stream | 1/11 | S24°18' E28°33' | | |
| *Xenopus laevis* | Martiens (Middelburg) | pan | 2/2 | S25.60857° E029.71210° | 1678 | |
| *Kassina senegalensis* | S22°E30° (Luvuvhu) | stream | 4/5 | S22°E30° | | |
| *Hyperolius marmoratus/semidiscus* | S22°E30° (Luvuvhu) | stream | 3/7 | S22°E30° | | |
| *Cacosternum boettgeri* | S22°E30° (Luvuvhu) | stream | 1/1 | S22°E30° | | |
| *Amietia angolensis* | S22°E30° (Luvuvhu) | stream | 5/6 | S22°E30° | | |
| *Amietia angolensis* | Jan Kemp 1 | dam | 2/3 | S27.97318° E024.82823° | 1140 | |
| *Amietiophrynus gutturalis* | Ganspan Jan Kemp | dam | 0/1 | S27.93676° | 1039 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | E024.76174° | | |
| *Amieta fuscigula* | Jan Kemp 1 | dam | 1/3 | S27.97318° E024.82823° | 1140 | |
| *Xenopus laevis* | S22°E30° (Luvuvhu) | stream | 7/7 | S22°E30° | | X |
| *Afrixalus fornasini/Hyperolius* | S22°E30° (Luvuvhu) | stream | 0/1 | S22°E30° | | |
| *Hyperolius* | S23°E30° (Luvuvhu) | stream | 1/3 | S23°E30° | | |
| *Xenopus laevis* | S23°E30° (Luvuvhu) | stream | 0/3 | S23°E30° | | |
| *Xenopus laevis* | Heilbron | dam | 0/10 | S27.23.967° E027.46133° | | |
| *Xenopus laevis* | Jan Kemp 1 | dam | 7/19 | S27.97318° E024.82823° | 1140 | |
| *Xenopus laevis* | Jan Kemp 2 | dam | 2/12 | S27.96510° E024.82896° | 1140 | |
| *Xenopus laevis* | Drakensberge (Pressure Pool) | ephemeral pool | 0/20 | S28.68393° E028.92298° | 1801 | |

**Table 3.3 Sites positive for the presence of *Batrachochytrium dendrobatidis*.**

| Locality | Species | Habitat | Fraction infected | GPS reading |
|---|---|---|---|---|
| Vemvaan Upper | *Amietia vertebralis* | stream | 3/3 | S28.74913° E028.87186° |
| | *Hadromophryne natalensis* | stream | 3/3 | S28.74913° E028.87186° |
| Ribbon Falls Lower | *Amietia vertebralis* | stream | 12/15 | S28.76268° E028.91794° |
| | *Amietia angolensis* | stream | 1/1 | S28.76268° E028.91794° |
| Sabie | *Amietia angolensis* | stream | 1/2 | S25°05'08" E30°46'42" |
| MacMac | *Amietia angolensis* | stream | 3/3 | S25.01393° E030.83127° |
| Tugela | *Amietia vertebralis* | stream | 10/13 | S28.74185° E028.91460° |
| Memel | *Amietia angolensis* | stream | 10/24 | S2772390° E029.52393° |
| | *Amietia fuscigula* | stream | 2/5 | S2772390° E029.52393° |
| | *Xenopus laevis* | stream | 2/2 | S2772390° E029.52393° |
| | *Cacosternum* | ephemeral pool | 1/20 | S27.72094° E029.52163° |
| Vernon Crooks Loop road | *Hyperolius pusillus* | dam | 3/15 | S28.75071° E028.86821° |
| | *Hyperolius marmoratus* | dam | 1/1 | S28.75071° E028.86821° |
| Vlei by Vernon Crooks | *Ptychadena oxyrhynchus* | vlei | 2/2 | S30.26072° E030.61093° |
| Kaap Silver mine | *Amietia fuscigula* | dam | 3/19 | S34.09091° E018.42114° |
| Magoebaskloof | *Amietia angolensis* | stream | 12/18 | S23.81647° E030.02381° |
| | *Hadromophryne natalensis* | stream | 12/12 | S23.81647° |

| | | | | |
|---|---|---|---|---|
| | | | | E030.02381° |
| Hazyview 1 | *Amietia* | stream | 1/1 | S25.02076° E031.01168° |
| Hazyview 2 | *Phrynobatrachus natalensis* | dam | 2/3 | S25.10707° E031.07805° |
| Nelspruit (Jakkalsnels) | *Hyperolius marmoratus* | dam | 2/6 | S25.42699° E030.90656° |
| Middelburg (Martiens) | *Amietia fuscigula* | stream | 1/2 | S25.60857° E029.71210° |
| | *Xenopus laevis* | pan | 2/2 | S25.60857° E029.71210° |
| Sabie | *Amietia angolensis* | stream | 2/5 | S25.08943° E030.77788° |
| Nelspruit 1 | *Phrynobatrachus natalensis* | stream | 1/1 | S25.44614° E030.88477° |
| Waterberge Site 1 | *Amietia angolensis* | stream | 2/3 | |
| Waterberge Site 2 | *Amietia angolensis* | stream | 1/11 | |
| S22°E30° (Luvuvhu) | *Kassina senegalensis* | stream | 4/5 | S22°E30° |
| | *Hyperolius marmoratus/semidiscus* | stream | 3/7 | S22°E30° |
| | *Cacosternum boettgeri* | stream | 1/1 | S22°E30° |
| | *Amietia angolensis* | stream | 5/6 | S22°E30° |
| | *Xenopus laevis* | stream | 7/10 | S22°E30° |
| | *Hyperolius* | stream | 1/3 | S23°E30° |
| Jan Kemp 1 | *Amietia angolensis* | dam | 2/3 | S27.97318° E024.82823° |
| | *Xenopus laevis* | dam | 7/9 | S27.97318° E024.82823° |
| | *Amietia fuscigula* | dam | 1/3 | S27.97318° E024.82823° |
| Jan Kemp 2 | *Xenopus laevis* | dam | 2/12 | S27.96510° E024.82896° |

**Table 3.4 Summary of the cultures isolated during the study.**

| Locality | Species | GPS reading | Culture code |
|---|---|---|---|
| Vemvaan Upper | *Amietia vertebralis* | S28.74913° E028.87186° | VemvaanU/Shymenopus/07/MCT2/P15 |
| | *Amietia vertebralis* | S28.76268° E028.91794° | RFL/Shymeopuns/07/MCT4/P12 |
| Memel | *Amietia angolensis* | S2772390° E029.52393° | Memel2/Aangolensis/08/MCT6/P8 |
| Silver Mine Kaap | *Amietia fuscigula* | S34.09091° E018.42114° | Silvermine/Afuscigula/08/MCT8/P9 |
| | *Amietia fuscigula* | S34.09091° E018.42114° | Silvermine/Afuscigula/08/MCT10/P9 |
| Magoebaskloof | *Amietia angolensis* | S23.81647° E030.02381° | Magoebaskloof/Aangolensis/08/MCT11/P9 |
| | *Amietia angolensis* | S23.81647° E030.02381° | Magoebaskloof/Aangolensis/08/MCT12/P9 |
| | *Hadromophryne natalensis* | S23.81647° E030.02381° | Magoebaskloof/Hnatalensis/08/MCT13/P7 |
| | *Hadromophryne natalensis* | S23.81647° E030.02381° | Magoebaskloof/Hnatalensis/08/MCT14/P7 |
| | *Hadromophryne natalensis* | S23.81647° E030.02381° | Magoebaskloof/Hnatalensis/08/MCT15/P7 |
| | *Hadromophryne natalensis* | S23.81647° E030.02381° | Magoebaskloof/Hnatalensis/08/MCT16/P7 |
| Luvuvhu | *Xenopus laevis* | S22° E30° | Luvuvhu2230/Xlaevis/08/MCT17/P8 |
| | *Xenopus laevis* | S22° E30° | Luvuvhu2230/Xlaevis/08/MCT18/P8 |

## 3.2 Application of isolated Bd

### 3.2.1 Cultivation of Bd

Only living animals were used in the screening process and later for the cultivation process. This is due to the relatively sensitive nature of Bd cultivation. Bd is killed by alcohol, which eliminates preserved animals and tadpoles from the isolation process, since living Bd is necessary for starting a culture. Dead animals also posed a problem since bacteria and other pathogens increase on the carcass when death occurs, and contaminate - and therefore complicate - the cultivation process. Animals were euthanased by MS222 (tricaine methane sulfonate), which has been proven not to kill or interfere with Bd in any way (Webb *et al.*, 2005), before the isolation of Bd proceeded.

When the infected tissue was isolated during the isolation process, the isolate on the mTGh-agar was giving a shortened number. The isolates were incubated at 22°C, the optimum temperature for Bd growth (Piotrowski *et al.*, 2004). The product of the

isolation process is growing Bd colonies around the isolated mouthparts on the mTGh-agar.

As soon as the growth around and on the tissue was visible to the eye in the form of observable sporangia and high zoospore activity (Fig. 3.3.a), the isolate was transferred either to tryptone agar or tryptone broth (Fig. 3.3.c). The isolate was only transferred to tryptone broth once it was certain that there were no contaminants present in and around the isolate. This was checked with the use of a compound microscope. The periphery of the isolate was inspected for any foreign growths, such as those of filamentous fungi or spherical and bacilli bacteria.

As soon as the isolate was transferred to tryptone agar or broth, and grew successfully (Fig. 3.3.d), it was allocated a new shortened code, beginning with MCT. The numbering system suggested by Berger *et al.* (2005c) was used. With this, cultures were numbered as follows:

**Locality isolated from / Species / Year / Shortened code**

For example, Bd isolated from the common river frog (*Amietia angolensis*) from Heilbron during 2008 was given the shortened code of MCT44. The numbering of this culture will therefore be:

**Heilbron / Aangolensis / 08 / MCT44**

The total number of times the culture has been passaged to new nutrient media was also indicated and placed last. For example, if the above-mentioned culture has been transferred ten times, it would be indicated as follows:

**Heilbron / Aangolensis / 08 / MCT44 / 10**

It is important to transfer the culture to new broth every few months or days, depending on the manner in which it was stored. Cultures incubated at 22°C must be transferred as soon as growth seems to be too high. According to Speare *et al.* (2001), this transferal must take place every four to five days. During this study, transfers varied between five days and two weeks, depending on the rate of growth. Transfers must be done every three months if the cultures were stored at 5°C (Fig. 3.3.e). After three months, no or very little movement was seen in cultures, but they did grow when transferred to a new broth. I would recommend that cultures are transferred every nine to ten weeks if kept at 5°C to ensure growth. Large amounts of broth and culture can be stored in Schott bottles. The ideal ratio between broth and culture (broth: culture) used during this study was 4:1 or 1:10, depending on how fast one wants to transfer. The higher the amounts of broth in relation to culture, the slower the nutrients were depleted. The culture was then transferred after longer periods. In small culture flasks (50 $cm^3$) 10 ml broth and 1 ml culture or the isolated tissue was added. In the larger culture flasks (250 $cm^3$) 40 ml broth and 10 ml culture were added.

From time to time broth cultures became contaminated. During these times, tryptone agar containing antibiotics (Penicillin G and Streptomycin sulfate) were used to rid the cultures of the contamination. 1 ml of the contaminated culture was spread on the tryptone agar plate and left open to dry in a laminar flow cabinet. After a few days, pure Bd colonies were isolated from the plate and transferred to new tryptone broth, forming a new pure culture.

It must be added that successful culture cannot be assured, regardless of the initial infection intensity. As seen in Table 3.2, many seemingly positive specimens yielded no cultures.

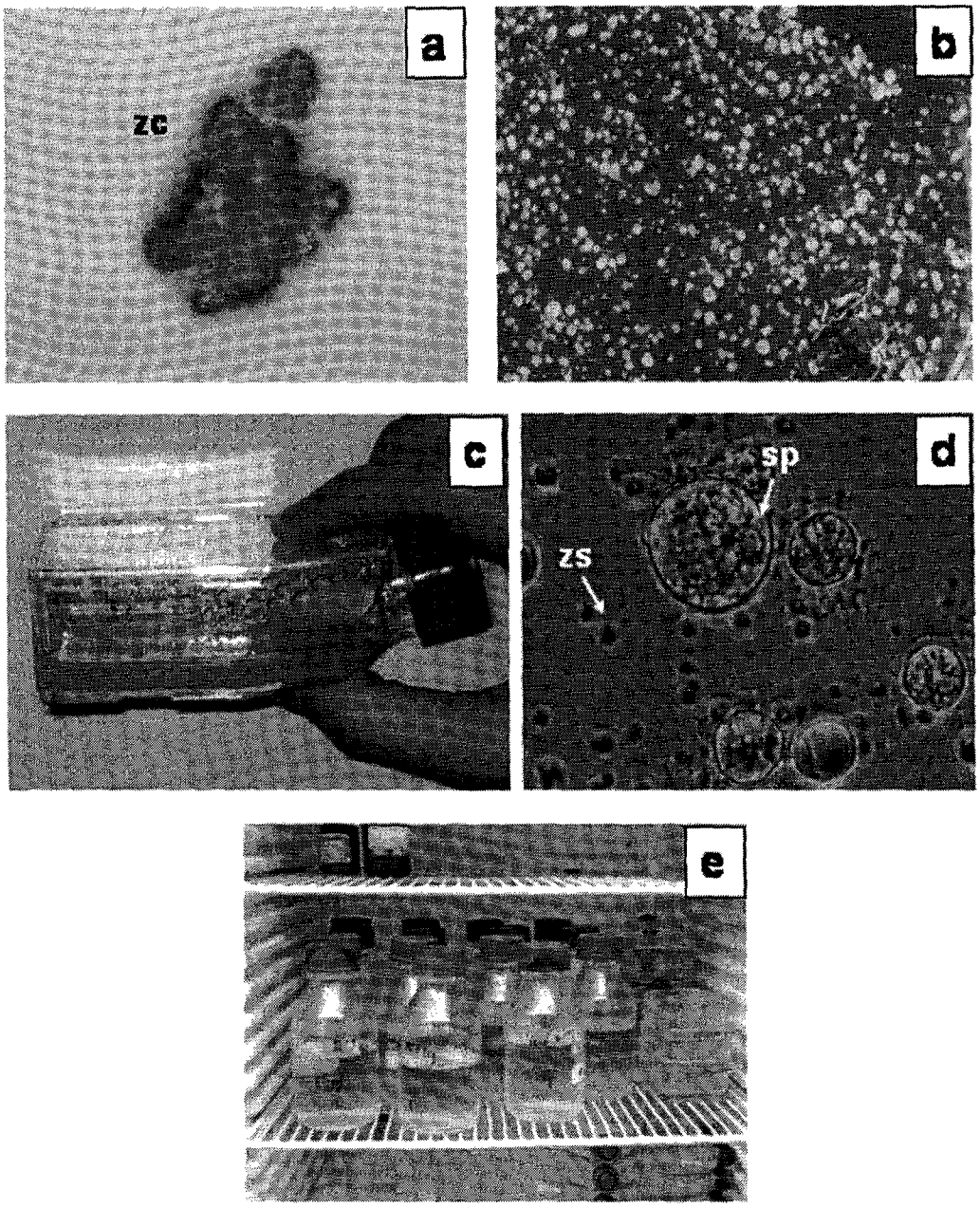


**Figure 3.3 As soon as a cloud of zoospores were seen around the isolated tissue (a) and the culture on mTGh-agar had a whitish colour (b), it was transferred to tryptone broth (c) in a culture flask. Good growth within the broth consisted of numerous released, motile zoospores as well as mature, zoospore-filled sporangia (d). Broth cultures were passaged to larger containers, such as Schott bottles, and kept at 5°C long term (e). Larger red-capped flask can also be seen in (e). Abbreviations: zc, zoospore cloud; zs, zoospores; sp, sporangia**

### 3.2.2 Extraction of DNA from Bd cultures

DNA was extracted from the cultures at the NRF molecular laboratory situated at the National Zoological Gardens. This DNA is currently being used as standards for the detection and quantification of Bd through real-time PCR during procedures done at the laboratory at National Zoological Gardens. The DNA is also available for future use to determine its relatedness to other global strains through multilocus sequence typing. This may shed light on the origin and diversification of Bd.

The purity and the lack of protein contamination of a culture are indicated by the A260/280 value obtained by means of a NanoDrop. Values between 1.7 and 2.0 indicate optimal purity (Table 3.5). Of the 26 cultures from which DNA were extracted, 14 falls within this range and can therefore be used in future experiments with a high degree of confidence.

Currently, DNA extracted from MCT15 (isolated from a *Hadromophryne natalensis* tadpole from Magoebaskloof) is used as a positive standard during real-time PCR at National Zoological Gardens by Desire Dalton[4].

### 3.2.3 Cryopreservation of cultures

The following cultures were cryopreserved using a 10% foetal calf serum and 10% dimethyl sulfoxide mixture in tryptone broth:

MCT2, MCT4, MCT6, MCT8, MCT9, MCT10, MCT11, MCT12, MCT13, MCT14, MCT15 and MCT17

These are the same cultures we used in the DNA extraction process mentioned under 3.2.2. The selection of cultures to be cryopreserved depended on which cultures were used in the DNA extraction and consisted of cultures from different species and from different localities. It was considered important to store the cultures from which we had gathered the most information.

[4] Dr Desire Dalton: Laboratory technician at the NRF funded laboratory situated at National Zoological Gardens

**Table 3.5 The results of the A260/A80 values, as well as the concentrations of the different cultures from which DNA was extracted.**

| DNA number | A260 | A280 | A260 / A280 | Conc. (ng.µL) |
|---|---|---|---|---|
| MCT 2 a | 0.665 | 0.134 | 1.96 | 6.7 |
| MCT 2 b | 0.138 | 0.089 | 1.55 | 6.9 |
| MCT 2 c | 0.268 | 0.134 | 2.00 | 13.4 |
| MCT 2 d | 0.365 | 0.202 | 1.80 | 18.3 |
| MCT 4 a | 0.246 | 0.117 | 2.10 | 12.3 |
| MCT 4 b | 0.157 | 0.082 | 1.90 | 7.8 |
| MCT 6 a | 0.119 | 0.066 | 1.82 | 6.0 |
| MCT 6 b | 0.096 | 0.049 | 1.98 | 4.8 |
| MCT 8 a | 0.137 | 0.085 | 1.62 | 6.9 |
| MCT 8 b | 0.088 | 0.042 | 2.08 | 4.4 |
| MCT 9 a | 0.220 | 0.092 | 2.40 | 11.0 |
| MCT 9 b | 0.165 | 0.062 | 2.68 | 8.3 |
| MCT 10 a | 0.225 | 0.112 | 2.01 | 11.3 |
| MCT 10 b | 0.383 | 0.202 | 1.89 | 19.1 |
| MCT 11 a | 0.068 | 0.022 | 3.06 | 3.4 |
| MCT 11 b | 0.188 | 0.101 | 1.87 | 9.4 |
| MCT 12 a | 0.160 | 0.072 | 2.23 | 8.0 |
| MCT 12 b | 0.225 | 0.089 | 2.53 | 11.3 |
| MCT 13 a | 0.142 | 0.074 | 1.93 | 7.1 |
| MCT 13 b | 0.148 | 0.078 | 1.91 | 7.4 |
| MCT 14 a | 0.112 | 0.058 | 1.94 | 5.6 |
| MCT 14 b | 0.193 | 0.091 | 2.12 | 9.7 |
| MCT 15 a | 0.089 | 0.049 | 1.83 | 4.4 |
| MCT 15 b | 0.183 | 0.085 | 2.15 | 9.2 |
| MCT 17 a | 0.114 | 0.061 | 1.86 | 5.7 |
| MCT 17 b | 0.145 | 0.093 | 1.56 | 7.2 |

## 3.3 Microscopical examination of Bd

### 3.3.1. Scanning electron microscopy

Infection of Bd on the mouthparts of tadpoles of *Amietia vertebralis* and *Hadromophryne natalensis* was inspected with the use of scanning electron microscopy. Infected areas can be identified under the light microscope as pigmented darker areas between the labial tooth rows, making identification of infected *H. natalensis* tadpoles easy in the field (Smith *et al.*, 2007). However, not all infections can be seen as pigmented areas - as *Rana muscosa* Bd infected tadpoles tend to lose pigmentation due to the infection (Vredenburg & Summers, 2001), as do *A. vertebralis.* The study of the infection between the species was conducted using SEM, to observe whether differences also existed in the appearance of infection under high magnification.

Infections of the mouthparts of *H. natalensis* tadpoles are predominantly situated in the area between toothrows, as indicated by the bracket in Fig. 3.4 (a). Infections were predominantly associated with the first three rows (keratodonts) of the lower oral disc but only the first two keratodonts are visible in (a). As soon as the magnification increased (b-c), the damage caused by Bd infections becomes evident. Infected areas had a corroded appearance with skin sloughing from the skin surface (b). Bulging infected cells are seen in (c) and are mature zoosporangia underneath the skin. The depressions (c) indicate positions where mature sporangia were shed with superficial skin layers. The depressions can be seen as imprints of the shed sporangia on the skin surface. These depressions may also be formed due to collapsing empty sporangia within the epithelial cell. When the jaw sheath (rostrodont) was examined, peeling from the structure was noticed (d). Whether the peeling resulted due to normal processes or because of Bd infections, is uncertain. Bd is known to infect keratin-rich areas such as the jaw sheath.

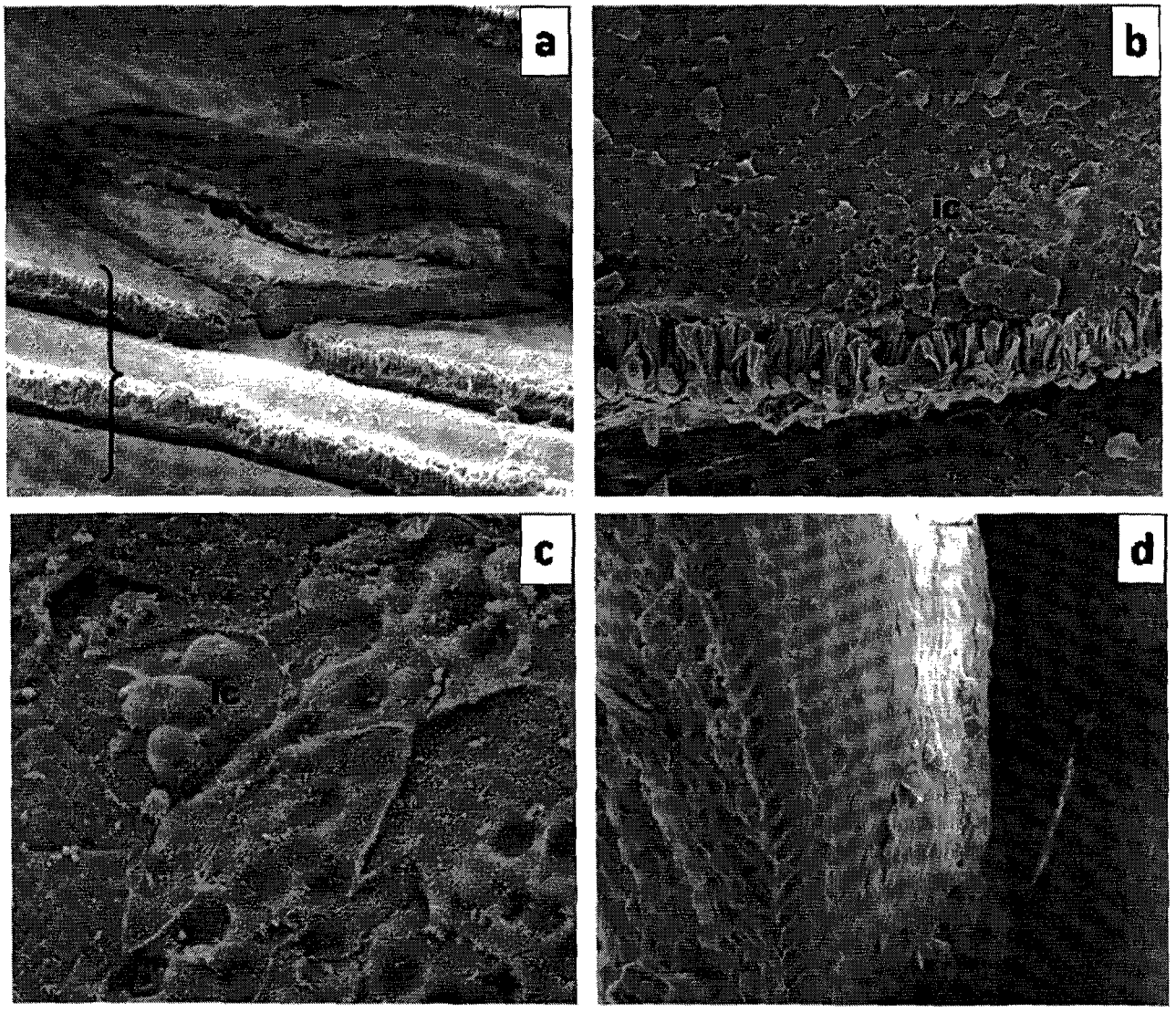


**Figure 3.4 Bd infections on the mouthparts of *Hadromophryne natalensis*. Abbreviation: ic – infected cells**

Bulging epithelial cells as seen in Figure 3.4 (c) contain mature sporangia which develop one or more discharge papilla from which zoospores are expelled. In Figure 3.5 such a structure can be seen protruding from the host skin.

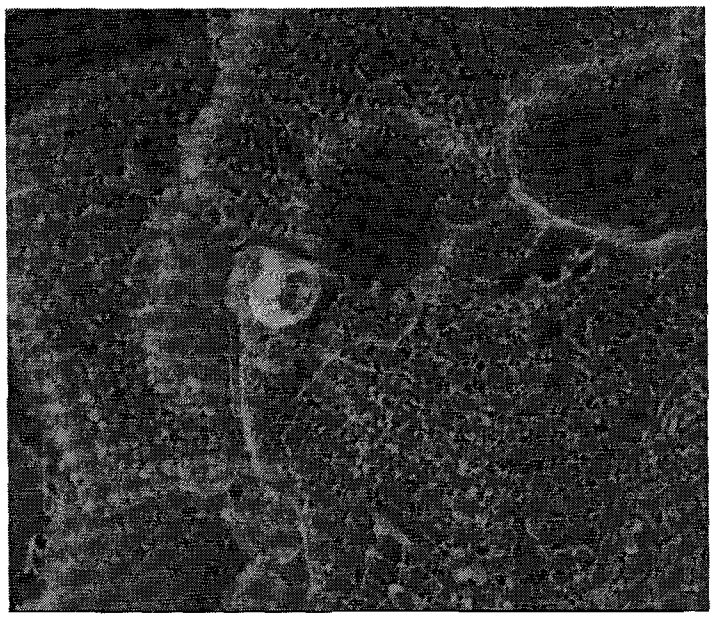

**Figure 3.5 A discharge papillae, through which *Batrachochytrium dendrobatidis* sporangia will discharge mature zoospores, is clearly seen in this image.**

Bd infections in *A. vertebralis* appeared more severe than those in *H. natalensis*. In Figure 3.6(a) the epithelial cells from which the sporangia were shed are clearly visible as an eroded area between the toothrows. Debris, in the form of mucus and food, lodged between the labial toothrows made the clear examination of the infection more difficult. The debris is not due to infection. Nonetheless, from the image it can be seen that the infections are situated between teethrows, similar to that of *H. natalensis*. Due to the fact that *A. vertebralis* only has four rows of teeth on the lower oral disc, the entire mouth seemed to be infected with Bd. The bulging cells in the epithelium, as seen in (b), are mature sporangia within the epithelial cells and have a similar appearance to Bd infections in *H. natalensis*. In (c) the loss of keratodonts due to severe Bd infections is clearly visible and is indicated by the asterisk. The coarse appearance of the surrounding tissue around the gap in the labial tooth rows is most likely due to Bd infection. The Bd infection on the jaw sheath of the tadpole, in (d), also shows the same bulging cells as cell situated between labial toothrows and are due to the presence of mature sporangia within the epithelial cells. The presence of infected areas was more severe at the corners of the jaw sheath (d). Note the peeling of the jaw sheath where infected epithelial cells are dislodging.

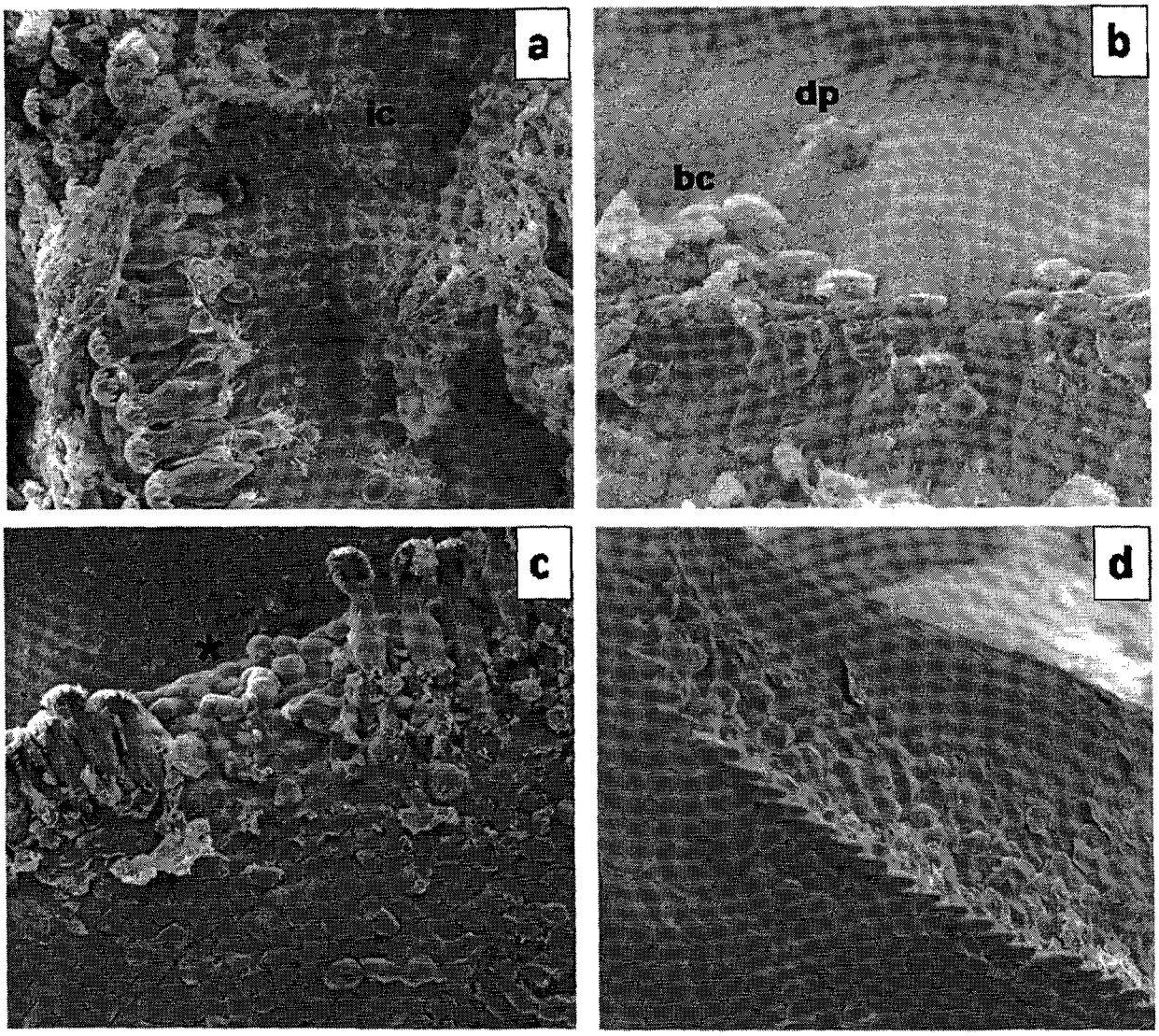


**Figure 3.6 *Batrachochytrium dendrobatidis* infection on the mouthparts of *Amietia vertebralis*. Abbreviations: ic, infected cell; bc, bulging cells; dp, discharge papilla**

### 3.3.2 Laser scanning confocal microscopy

Bd possesses several morphological forms during its life-cycle. These include the motile zoospore, encysted zoospores that form germlings, developing sporangia and mature sporangia with zoospores. Zoospores are released through the discharge papillae of the mature sporangium, thereby completing the life-cycle. Within tissue, the entire suite of life-cycle stages is not always clearly discernable. Only discharge papillae on the surface of the skin and sometimes zoospores are visible, depending on the specificity of stains

used. During this study different fluorescent stains were used to examine Bd with a laser scanning confocal microscope as described in the Material and Methods chapter.

**Bd in culture**

- **The zoospore**

When the zoospore was stained with both LysoTracker Green and MitoTracker Red distinct lysosomes could be discerned (Fig. 3.7). LysoTracker Green stains lysosomes and other acidic structures bright green while MitoTracker Red stains mitochondria bright red. In Figure 3.7, three images of the same zoospore show the presence of two clear lysosomes associated with mitochondria. Both the green and red filters are used in (a) and shows the presence of two distinct yellow bodies. In (b) only the green filter is used and shows the clear presence of lysosomes. These lysosomes might be used during the infection process when the zoospore penetrates the host tissue. In (c) only the red filter is activated and shows two to three bright red mitochondria, clearly visible in the front part of the zoospore. The resulting yellow colour, where the mitochondria and lysosomes overlap, as seen in (a), is due to co-localisation.

During the examination of a zoospore stained with Nile Red the same results were obtained as those by Berger *et al.* (2005a). According to Longcore *et al.* (1999) a key feature of Bd is the presence of numerous lipid droplets within the zoospores. Nile Red has the ability to stain phospholipids, and therefore lipid droplets, a bright red. More lipids present within the structure correspondingly resulted in a brighter red obtained. In Figure 3.8 the bright red lipid droplet present in the zoospore is clearly visible. The less defined plasma membrane around the zoospore is also visible.

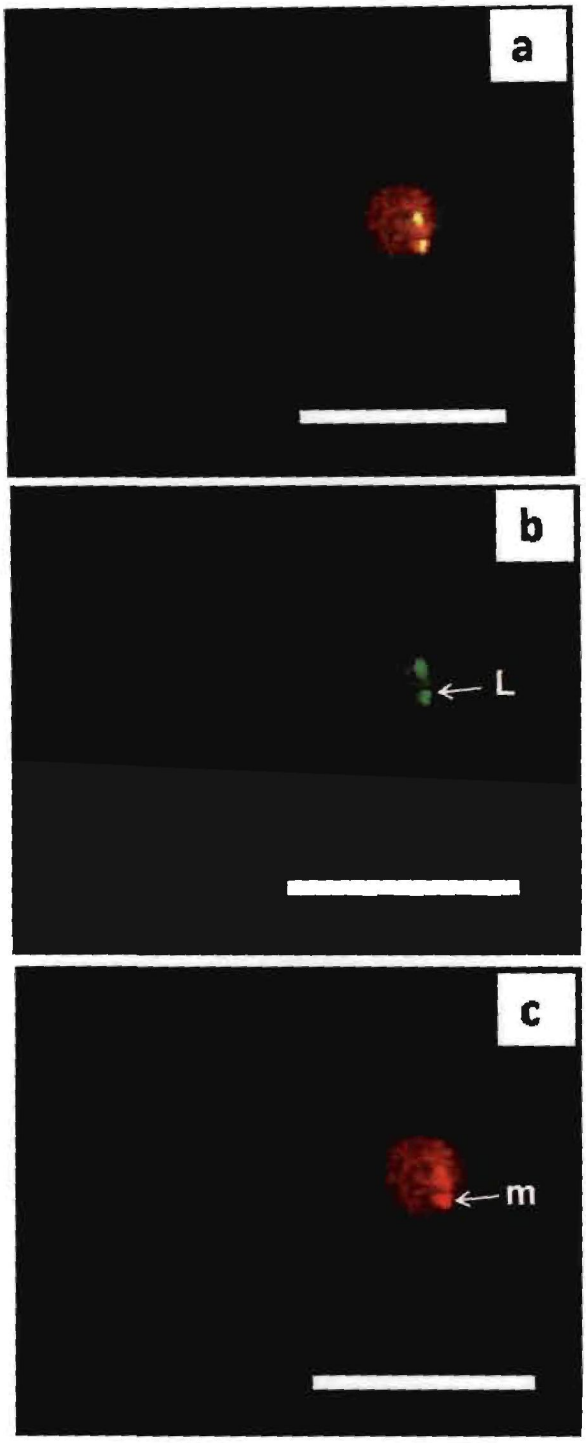


**Figure 3.7 Zoospore series stained with LysoTracker Green and MitoTracker Red with the alternating activation of the green and red filters. In (a) both the red and green filters are activated. In (b) the green filter is activated and in (c) the red filter. Abbreviations: m, mitochondrion; L, lysosomes (Scale bar: 5 μm)**

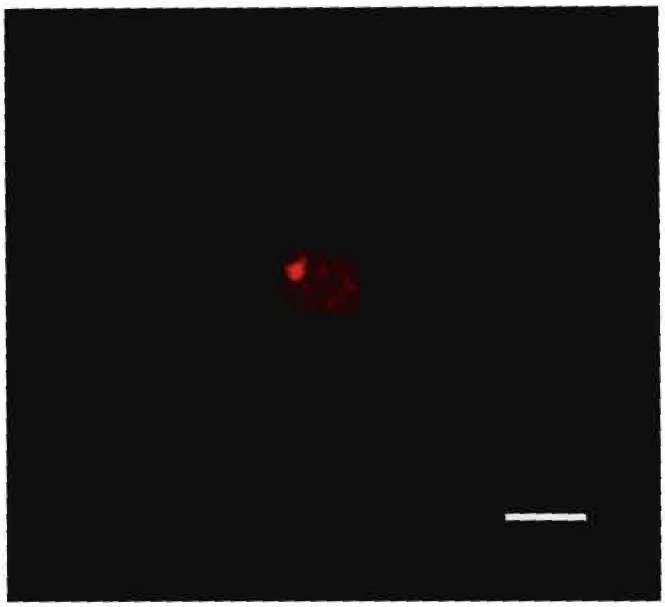

**Figure 3.8 A zoospore stained with Nile Red showed a clear lipid globule indicated by a bright red dot. The plasma membrane is also visible as a diffused red halo around the periphery of the zoospore (Scale bar: 3 μm)**

- **Sporangia**

In this section the results achieved with both developing and mature sporangia will be shown.

Calcofluor White M2R (Calcofluor) is part of a Yeast Viability Kit, but can be used to specifically stain the chitin in the cell wall of fungi. In Figure 3.9 the cell wall is stained blue. In Figure 3.9 (a), the image was focused on a higher plane, giving a three dimensional quality to the sporangia. In (b) the focus is deeper, slicing through the sporangia. Some sporangia develop septa during development. The compartments formed by the septa can be seen as separate sporangia. This type of development is termed colonial growth and when sporangia do not form any divisions, it is termed monocentric growth (Longcore *et al.*, 1999; Berger *et al.*, 2005a). In (a), the definite septa can be seen from above. In (b) an open discharge papilla is clearly visible.

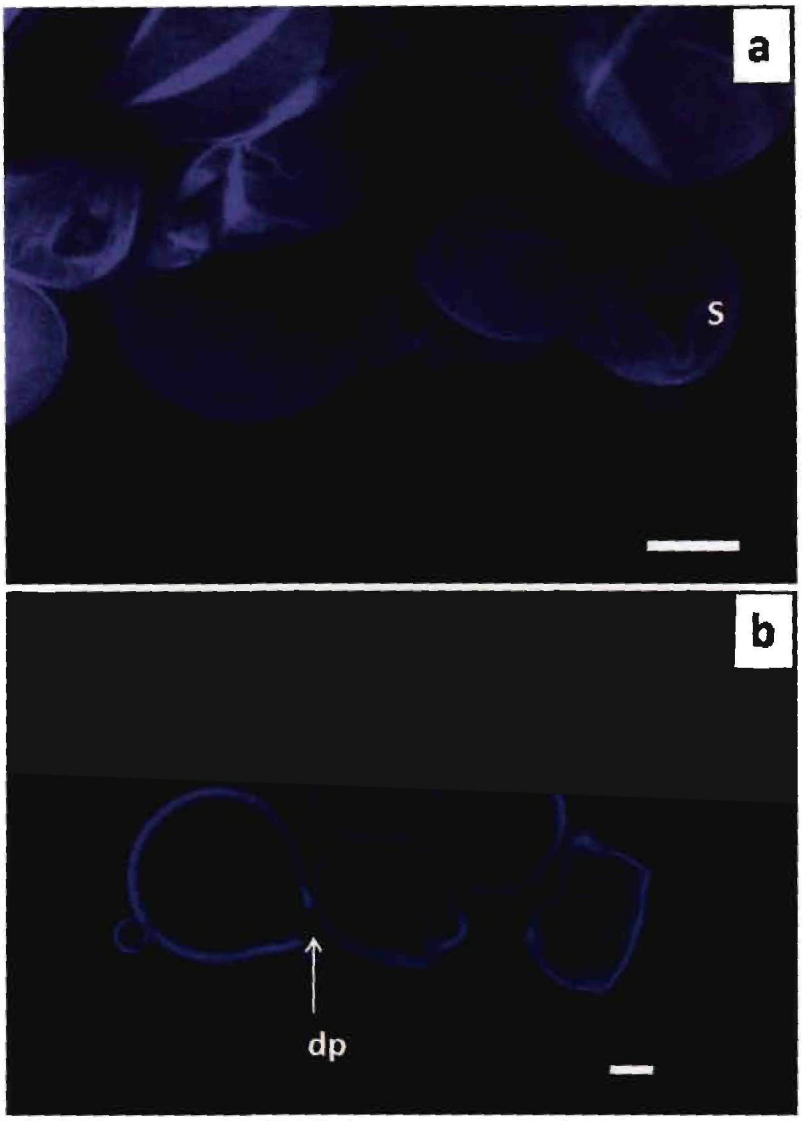


**Figure 3.9 *Batrachochytrium dendrobatidis* sporangia in culture are stained with Calcofluor White M2R. A septum is very clearly visible in the right lower corner in (a). In (b) an open discharge papilla is visible and indicated by an arrow. Abbreviations: s, septum; dp, discharge papilla (Scale bar for (a): 10 µm, for (b): 4 µm)**

When staining Bd culture with both Calcofluor White M2R and Nile Red the following results were obtained: Nile Red stained the lipid globules red in sporangia and the developing zoospores. Sporangia were surrounded by the blue cell wall stained by Calcofluor. The following images are from sporangia of different developmental phases: In Figure 3.10 (a) a developing sporangium can be seen where the different septa divide the zoosporangium into a colony. The bright blue colour achieved by Calcofluor staining

indicates that the septa consist of chitin. The plug blocking the opening in the sporangia can be seen in (b). This plug closes the opening of the discharge papilla and will dissolve to release the zoospores to the environment (Berger *et al.,* 2002). Since the chitin in the cell wall is stained a brighter blue, the diffuse blue of the plug is not considered to be chitin. In (c) the plug blocking the discharge papilla is dissolved and an open discharge tube, from which the cell content has escaped, can be seen from above. The red structures are the lipids within the sporangia. Note the bright red lipid globules present in the developing mass within the sporangium. The darker circular areas within the sporangia are most likely vacuoles.

In Figure 3.11 different stages of development can be identified. The zoospore is thought to not have a cell wall (James *et al.,* 2000) as all members of the Chytridiomycota have unwalled, flagellated zoospores. An encysted zoospore, however, can be seen as a small round structure, with a cell wall already present. It is not known when the cell wall develops. Due to the endogenous nature of the development of Bd, the encysted zoospore enlarges to form a reproductive structure – the sporangium (James *et al.,* 2000). Young developing sporangia have more complex, multinucleated contents with large dark structures. The dark structures are vacuoles according to Longcore *et al.* (1999). Mature sporangia, of which in this case only one is visible, contain zoospores which can be expelled through the discharge papillae. Bright red lipid globules can be seen in the mature zoospores as well as in the multinucleate mass of developing sporangia.

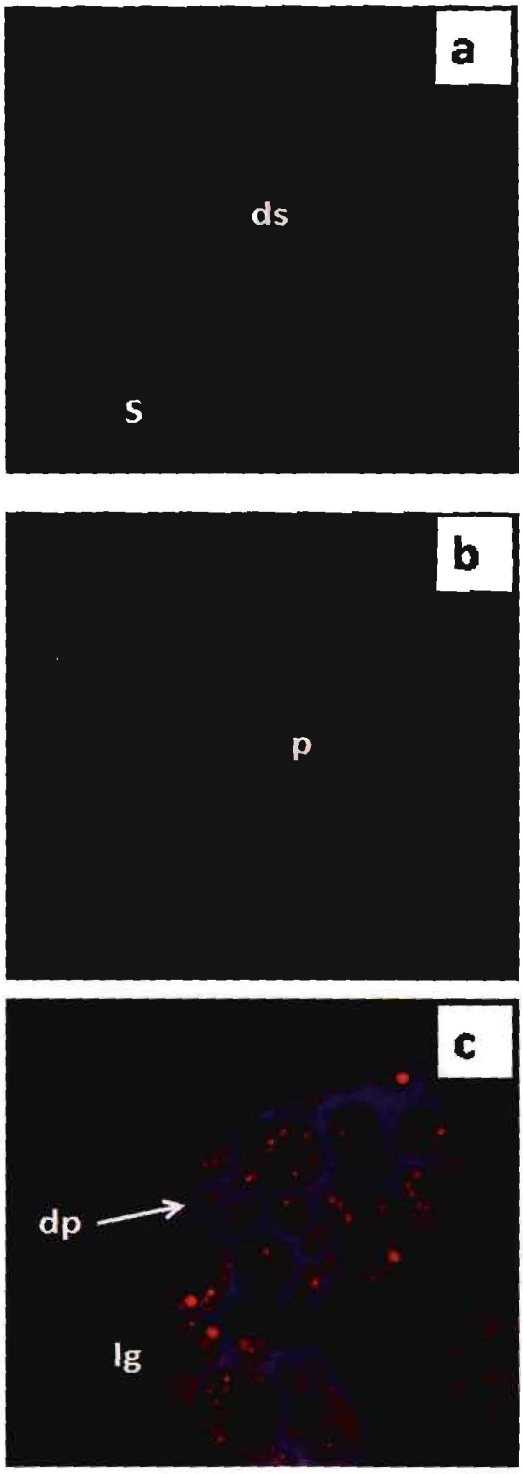


**Figure 3.10 Culture stained with Calcofluor White M2R and Nile Red. Abbreviations: s, septum; ds, developing sporangium; p, plug; dp, discharge papilla; lg, lipid globules**

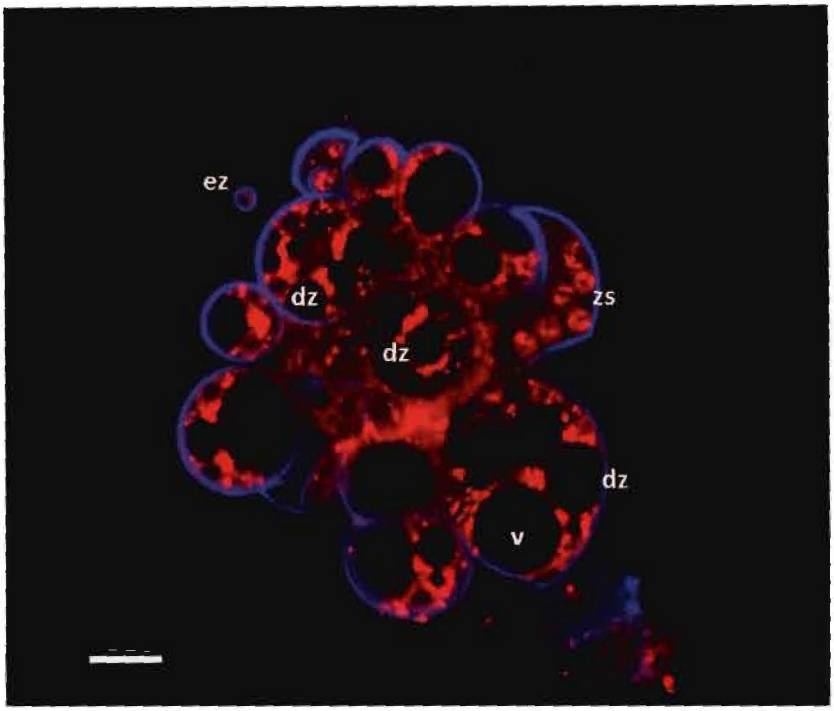


**Figure 3.11 Different developmental stages can be identified when Bd in culture is stained with Nile Red and Calcofluor White M2R. Abbreviations: ez, encysted zoospore; dz, developing sporangium; zs, zoospore; v, vacuole (Scale bar: 10 μm)**

When the Bd in culture was stained by both FUN1 and Calcofluor White M2R (part of Yeast Viability Kit by Invitrogen), different shades of yellow and blue resulted. FUN1 stains exploit normal endogenous biochemical processing mechanisms and are used to determine the metabolic activity of the cells. Only metabolically active cells are marked with intravacuolar structures while dead cells exhibit bright, diffuse green-yellow fluorescence. The intravacuolar structures can be orange-red or yellow-orange.

In Figure 3.12 different ages and stages of sporangia can be seen. One observation, which was seen with all Bd culture stained with Calcofluor White M2R, was that mature zoospores within sporangia seem to have cell walls around them. This phenomenon can be explained as a reflection off the cell wall, apparently colouring the plasma membrane around the zoospore blue. The diffused yellow structures are dead cells. Bright yellow

cells, like those in the zoospores, indicate living cells. Dead sporangia were clearly distinguishable from the living cells by their diffused yellow colour. The large numbers of dead cells were expected since the culture used was passaged six times, and are therefore not young. This "grouping effect" of growth, with the colonies grouping together during growth, does not occur in other chytrids, but has been observed in Bd (Berger *et al.*, 2005a). The red structures within the sporangia are intravacuolar structures and are only present in living cells (Molecular Probes).

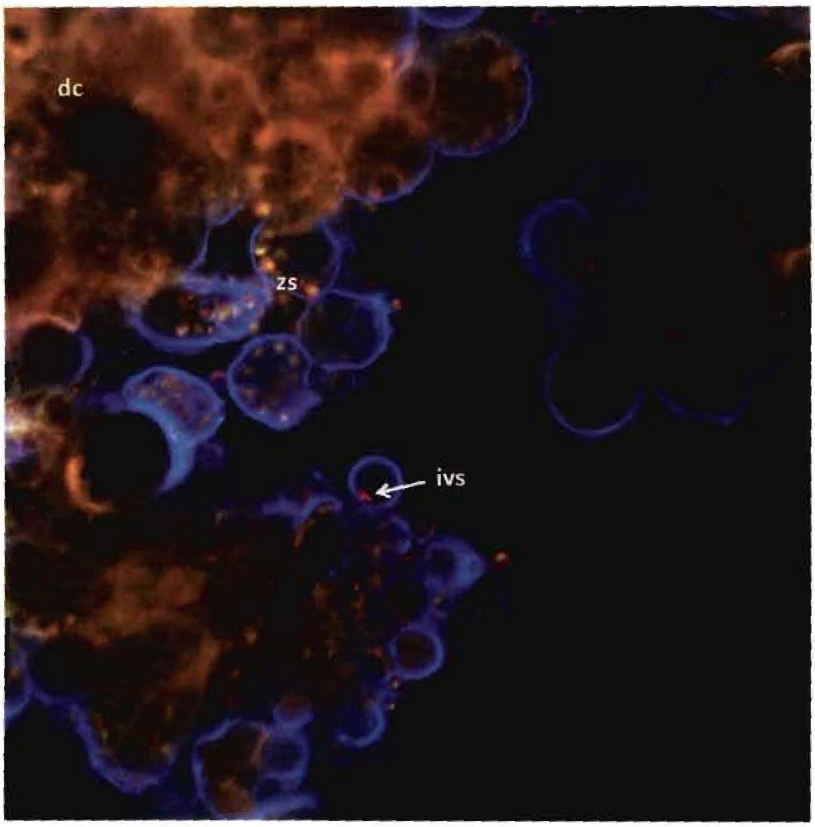


**Figure 3.12 Bd culture stained with Calcofluor White M2R and FUN1 showing diffused yellow, dead cells and bright yellow, living cells. Abbreviations: zs, zoospores; ivs, intravacuolar structures; dc, dead cells**

The combination of Calcofluor, LysoTracker Green and MitoTracker Red was expected to result in blue due to Calcofluor, red due to MitoTracker Red and green structures due to LysoTracker Green, but during our experiments this was not observed. Instead oranges, yellows and pinks were predominant colours, with the blue cell wall surrounding the confusing colours. The first two images, Figures 3.13 (a & b), resulted in bright,

colourful images. In (c) the same combination was used but different colours resulted, with a more whitish quality to the images and very little differentiation between the red and green stains. Due to the inconsistent colour results, the interpretation of the images posed to be a problem. Therefore, a grey-scale image, (d), of image (b) may give a better view of the colony. Developing encysted zoospores are visible on the periphery of the cluster. The darker colour of these areas may indicate the more complex multinucleated contents of the sporangia. Developing zoosporangia had vacuoles which were clearly visible as dark areas within the sporangia. Mature zoospores had already formed within mature sporangia and are ready to be released. The diffuse, grey areas with no distinctive structures are dead cells.

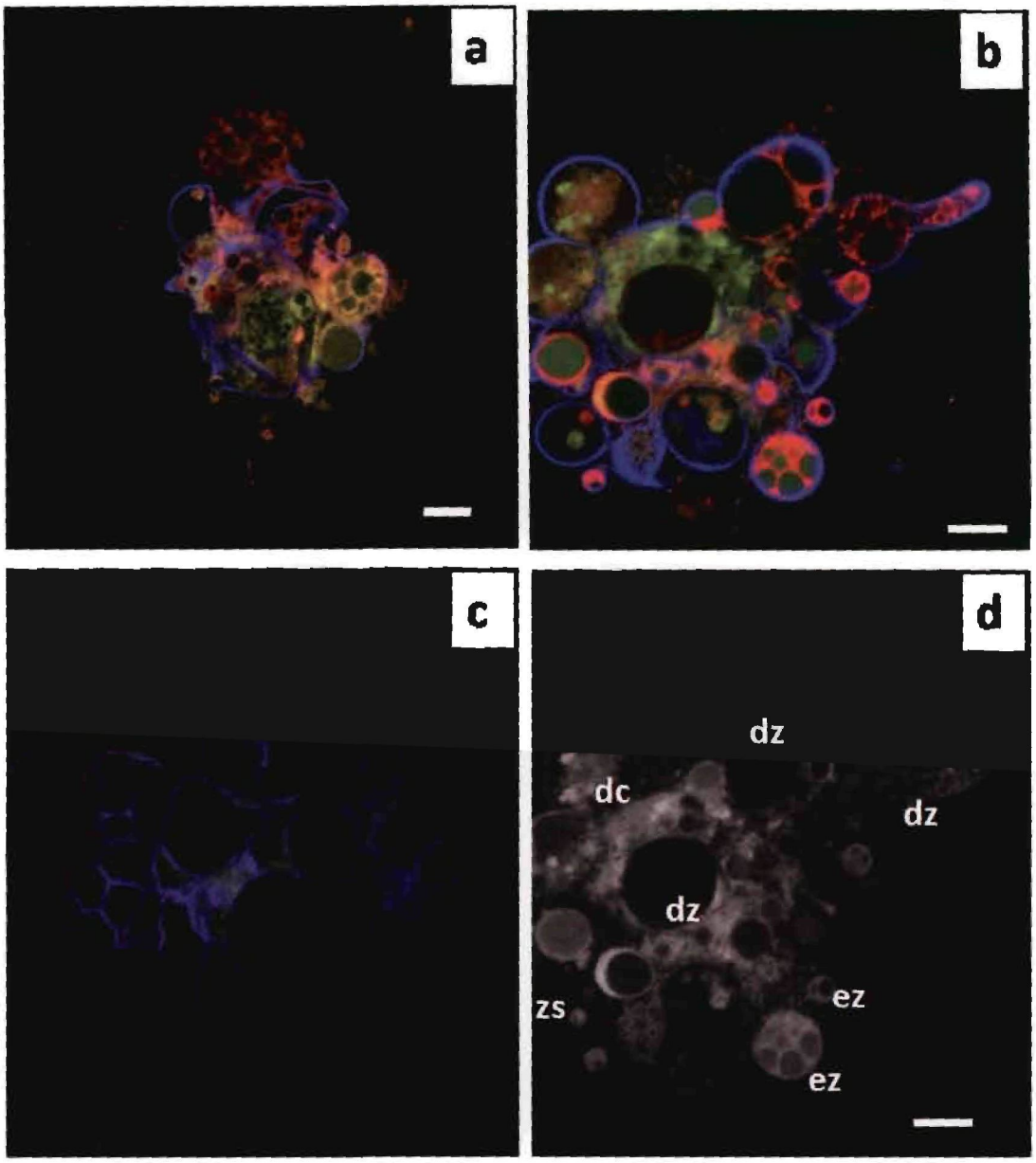


**Figure 3.13 Images acquired using a combination of LysoTracker Green, MitoTracker Red and Calcofluor White M2R showing different colour results. Abbreviations: ez, encysted zoospores; dz, developing sporangia; zs, zoospores; dc, dead cells (Scale bar: 10 μm)**

When only MitoTracker Red and LysoTracker Green were used (Fig. 3.14), without Calcofluor White M2R, red and green structures were achieved, leading to the assumption that the combination of MitoTracker Red, LysoTracker Green and Calcofluor White M2R is not preferred.

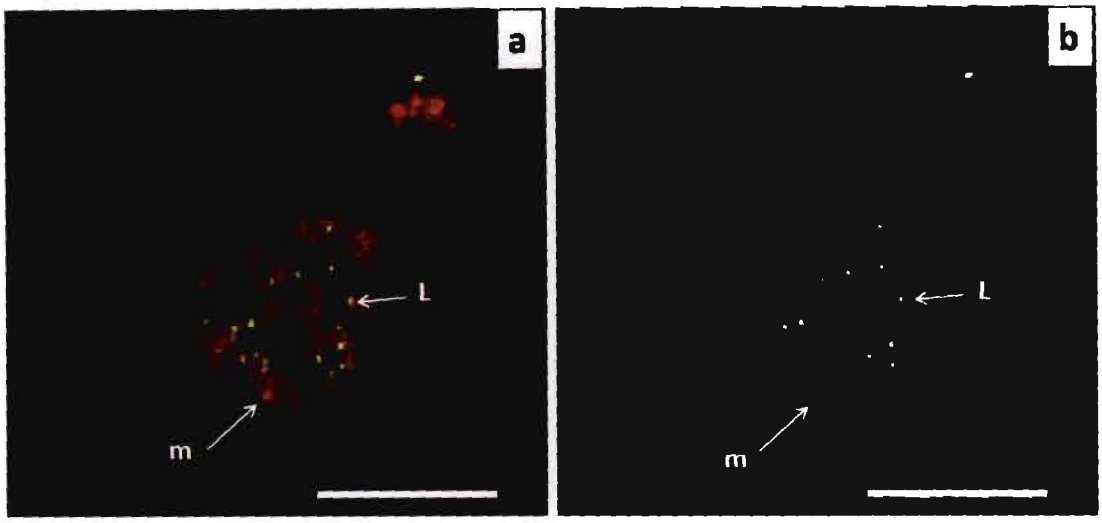


**Figure 3.14 Sporangia stained with both MitoTracker Red and LysoTracker Green resulted in red and green structures within the sporangia. Abbreviations: m, mitochondrion; L, lysosomes (Scale bar: 10 µm)**

Different results were also obtained during applications with MitoTracker Red and LysoTracker Green. Only lysosomes were identified within the sporangia during the first application depicted in Figure 3.14 (a). It is not completely clear why the sporangia may have lysosomes within the stroma. The green structures may also be acidic organelles such as vacuoles. MitoTracker Red did not provide good results on the whole, offering only a few images in which the presence of a structure with a membrane potential can be identified. When the image is shown in gray-scale (b), the bright structures of the lysosomes are still clearly visible.

The appearance of yellow pixels in the green pigmented areas may mean that the mitochondria and lysosomes are co-localised. Co-localisation is defined as the presence of two of more different molecules situated on the same location within a structure (Casavan *et al.*, 2008). This may lead to the presence of both a red and green colour in a pixel, resulting in orange to yellow colours. It is important to remember that the image achieved by confocal microscopy is not a photograph of the specimen, but rather a digital image composed of a multi-dimensional array with many voxels (detection volumes) that

represent pixels (Casavan *et al.,* 2008). This may lead to the change in colour where green and red stains overlap to form yellow or orange colours within an image.

During the second application of Bd in culture stained with LysoTracker Green and MitoTracker Red, different results were obtained from those in the first application. During the second application of the combination (Fig. 3.15 a & b) no distinct organelles could be discerned other than the mature zoospores in (b). Mitochondria seemed to be situated at the periphery of young and developing zoospores. This can also be mitochondria surrounding the nucleus, according to Berger *et al.* (2005a). Larger, mature zoospores, however, seem to lose their mitochondria since they stained green, indicating possible lysosome activity. It is impossible to state with accuracy that zoospores possess lysosomes, since no clear, bright green structures could be identified.

When cultures were stained with LysoTracker Green and Nile Red, the clear presence of lipid droplets within sporangia was however confirmed (Fig 3.16.a). In (a), only the red filter is used, giving a clear depiction of the lipid content of the sporangia, seen as bright red areas. When both the green and red filters were used, as seen in (b), lipid globules were clearly visible but no clear areas with lysosomes could be identified. The rhizoids, however, seem to possess lysosomes, resulting in their bright green colour. This is possible since the rhizoids can be used to attach sporangia to other sporangia and substrate or absorbing nutrients (Berger *et al.*, 2002). It may also help the fungus to penetrate deeper into the cell layers of host tissue. In (c) the shaft of the discharge papillae clearly has some lysosome activity, seen as a green area in the tube. Also note the lipid droplets in the sporangium in this image.

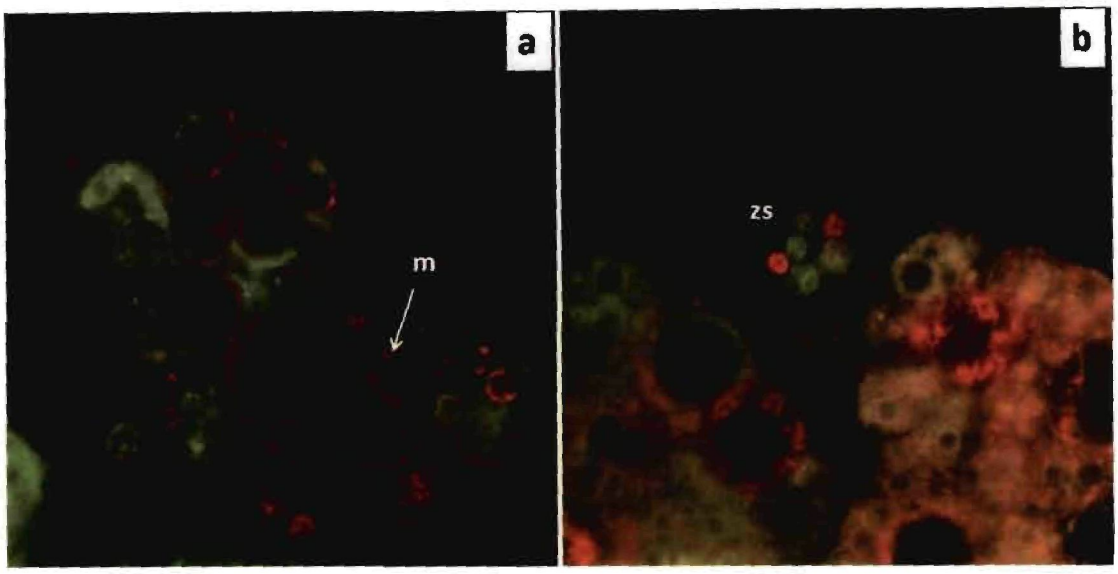


**Figure 3.15** ***Batrachochytrium dendrobatidis*** **in culture stained with both LysoTracker Green and MitoTracker Red during the second application showed different results from those from the first application. Abbreviations: m, mitochondrion; zs, zoospores (Scale bar: 7 μm)**

Alexa Fluor 488 Phalloidon (referred to as Phalloidon) did not yield any significant results with Bd. This stain is commonly used to stain actin filaments green. The stain was used because the use of uncommon stains may have a serendipitous effect, having unexpected results.

Unidentifiable structures were observed within sporangia (Fig. 3.17.a). These can either be indicative of the formation of new septa or the cleavage of the multinucleated sporangia to form zoospores. No other work has been done on fungi using Phalloidon. In (b) the rhizoids seem to have a beaded appearance. In another confocal study on Bd conducted by Weldon (2005) the same effect was found when sporangia were stained with acridine orange. According to Weldon (2005) these structures are RNA situated within mitochondria on the rhizoid. It may be the case here but remains unconfirmed.

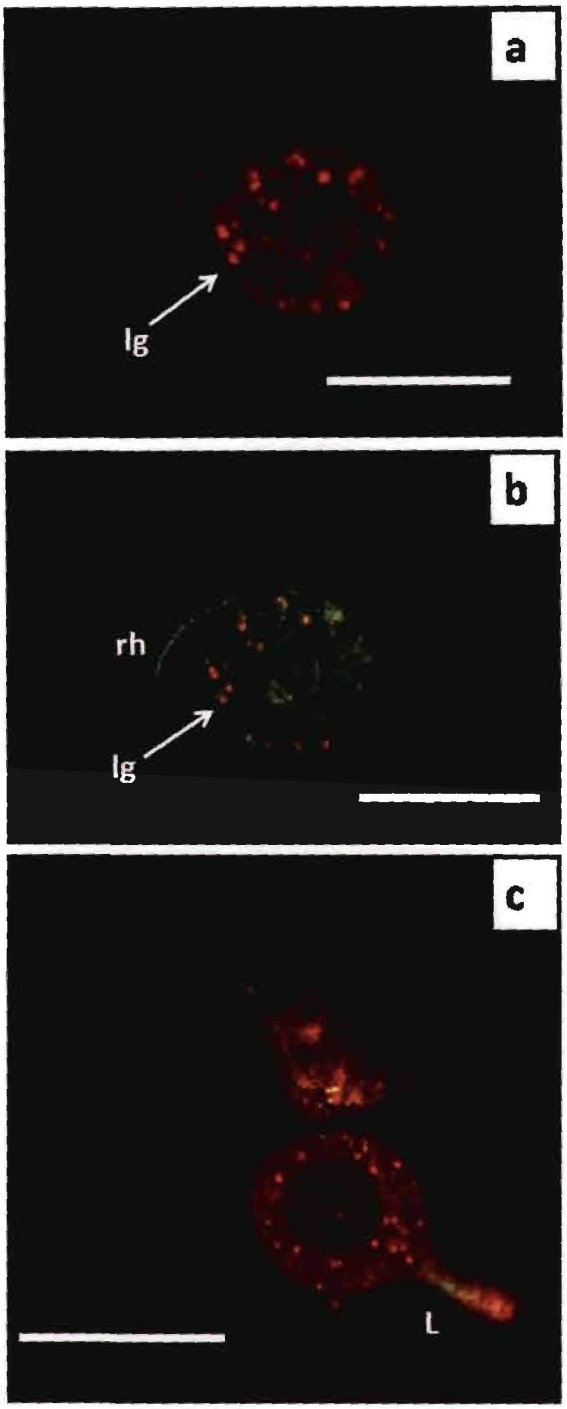


**Figure 3.16 Culture stained with Nile Red and LysoTracker Green. Abbreviations: lg – lipid globules, rh – rhizoids, L – lysosomes (Scale bars for (a) and (b): 10 µm; for (c): 7 µm)**

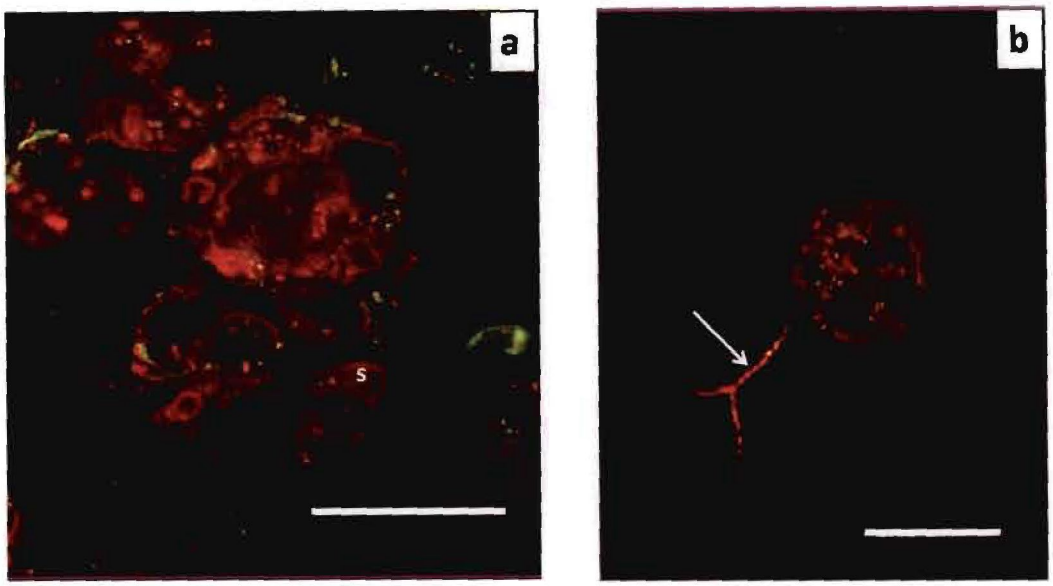


**Figure 3.17 Culture stained with Alexa Fluor 488 Phalloidon. Abbreviations: s, septate structures (Scale bar for (a): 8μm; for (b): 7 μm)**

Culture stained with acridine orange showed some clear red structures within the colony. Acridine orange stains DNA green and RNA red. In Fig. 3.18(a), RNA activity can be seen in the multinucleated sporangium. This is an indication that transcription is taking place within the sporangium. DNA is visible as bright green. The same situation can be seen in (b). During a different application of the stain, darker, orange structures were formed (c), but some clear activity can be seen in the central sporangium. Genetic material within this sporangium seems to be going through the anaphase of mitosis during which each of the chromatids move to opposite poles of the nuclear spindle. Zoospores seem to possess both DNA and RNA. A discharged zoospore (indicated) possessed a large RNA area, with a smaller DNA area (green) associated with it.

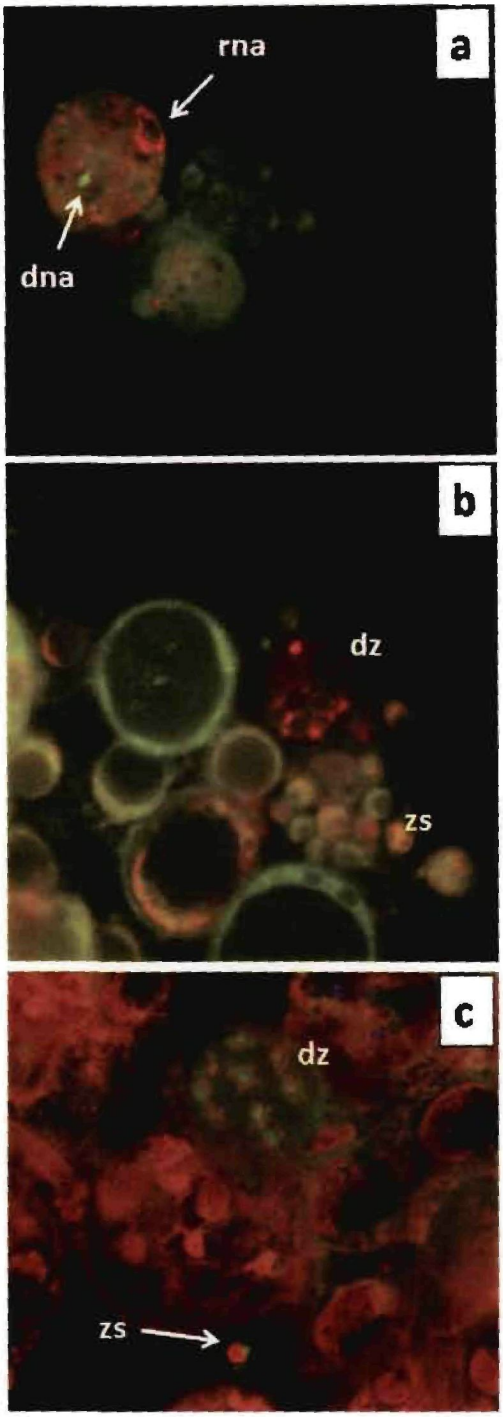


**Figure 3.18 Sporangia stained with acridine orange showed clear evidence of DNA (green) and RNA (red) within them. Abbreviations: dna, DNA; rna, RNA; dz, developing sporangia; zs, zoospore**

***Batrachochytrium dendrobatidis* in amphibian skin**

Of all the stains used in this study, Nile Red and Calcofluor White M2R yielded the best results.

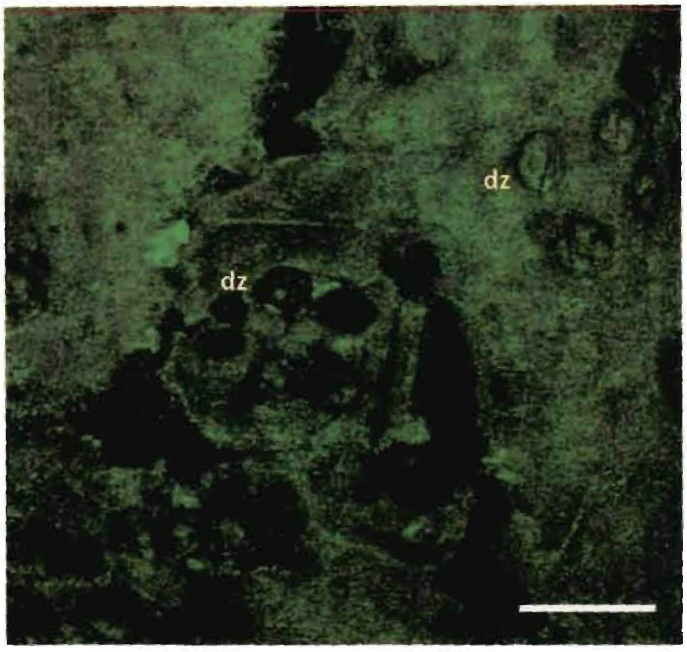


**Figure 3.19 The mouthparts of an infected *Hadromophryne natalensis* tadpole stained with Alexa Fluor 488 Phalloidon. Infected cells can be identified due to their seemingly empty contents. Abbreviations: dz, developing sporangia (Scale bar: 9 μm)**

When Alexa Fluor 488 Phalloidon was used (Fig. 3.19) to stain the epidermis of infected mouthparts, indistinct structures were seen. Sporangia could only be identified as darker round areas. Septa were visible in some of the sporangia.

The same results were achieved when the infected areas were stained with LysoTracker Green and MitoTracker Red (Fig. 3.20). Imbedded sporangia appeared to be large dark areas within the tissue and no distinct structures such as septa or zoospores could be seen.

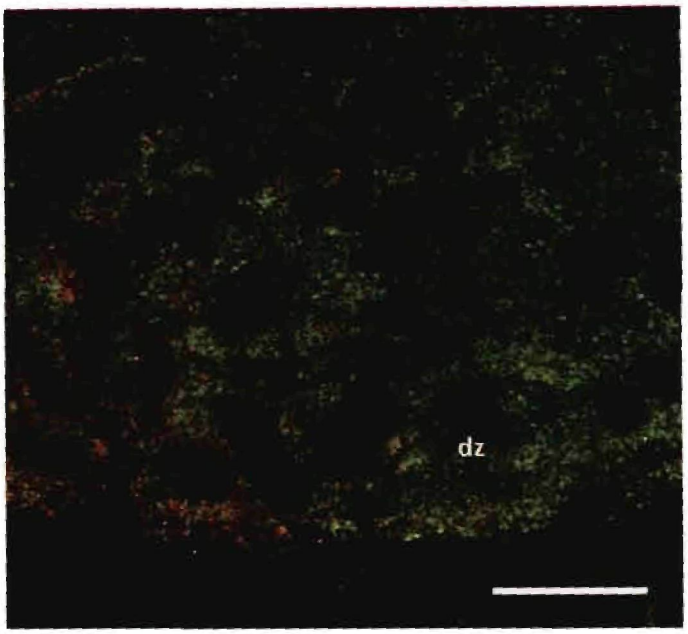


**Figure 3.20 Infected mouthparts stained with LysoTracker Green and MitoTracker Red only showed the infection as dark "holes" in the tissue. No distinct fungal structures can be identified. Abbreviations: dz, developing sporangium (Scale bar: 14 μm)**

When tissue was stained with Nile Red, structures could be identified. In Figure 3.21 the lipid droplets within the sporangia and zoospores are clearly visible within the spherical sporangia in the skin. The plasma membrane, which is made up of lipids, can also be seen in some empty sporangia. Darker areas within the sporangia can be seen as vacuoles (compare with sporangia in culture stained with Nile Red), thus identifying most of the sporangia as developing sporangia.

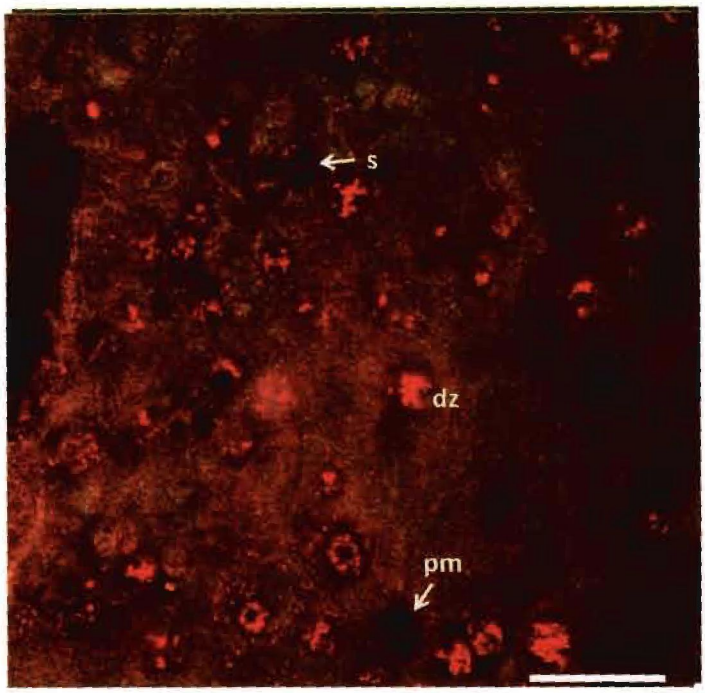


**Figure 3.21 Skin stained with Nile Red showed clear clusters of sporangia with bright red lipid droplets situated within the sporangia. The greenish hue of the surrounding tissue is due to autofluorescence from the host tissue. Abbreviations: dz, developing sporangium; pm, plasma membrane; s, septum (Scale bar: 15 μm)**

The best results obtained were from infected skin stained with Calcofluor White M2R. The fungal specificity of the stain made it easy to work with and to interpret the images. Because host cells do not contain cell walls, only the cell walls of the fungus were stained bright blue by the Calcofluor. In Figure 3.22 different structures could be identified, including septa and discharge papillae protruding from the skin epidermis. The reason why all the sporangia are approximately the same size is because the younger stages are situated in deeper cell layers, pushing older, more developed sporangia to the cell surface. This is an adaptation by Bd that allows the fungus to complete its life-cycle before being sloughed (Berger *et al.*, 2005). Mature sporangia are then able to release mature zoospores from the skin surface through the discharge papillae (a). Colonial and monocentric developmental growth can also be identified within the images. Colonial sporangia can be identified by the presence of septa dividing the contents of the zoosporangia into different chambers. Monocentric growth in sporangia consists of

undivided sporangia. Note the nuclei of the epithelial cells seen as the big green structures on the left of (b).

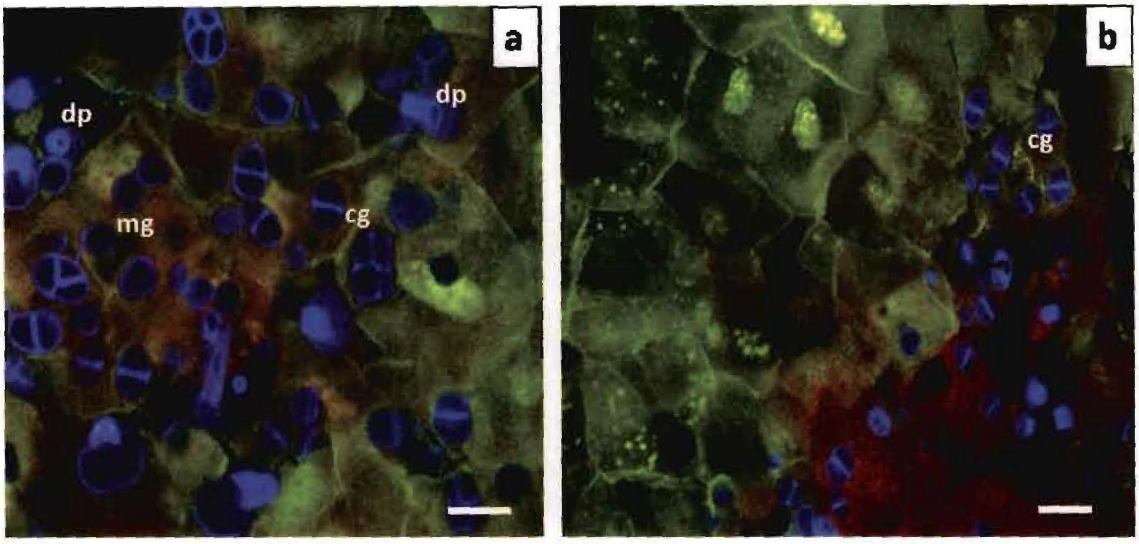


**Figure 3.22 Infected skin of the mouthpart of a tadpole was stained with Calcofluor White M2R. Abbreviations: mg, monocentric growth; cg, colonial growth; dp, discharge papilla (Scale bar for (a): 10 μm; for (b) 15μm)**

## 3.4 Skin peptides associated with *Amietia vertebralis* and *Hadromophryne natalensis*

### 3.4.1 Peptide collection

As seen in Figure 3.23, the amount of peptides eluted from the Sep-Pak cartridges from each species differs significantly in concentration. The average concentration of peptides in micrograms per gram body weight for *H. natalensis* is 2951.42 μg/g, in comparison with the meager 93.26 μg/g for *A. vertebralis.*

### 3.4.2 MALDI-TOF MS analysis

This analysis provides a rapid method to assess the relative complexity of a skin peptide mixture and also provide an estimate of the relative abundance of each peptide within the mixture. As seen in Figure 3.24, a complex pattern of peptides was found for both species of frog. The y-axis shows the percentage intensity of the isolated peptides. The higher peaks represent peptides with a relatively high abundance (percentage intensity).

The x-axis is the mass of the peptide, varying between 599 and 3000, showing the average size and complexity of the peptide.

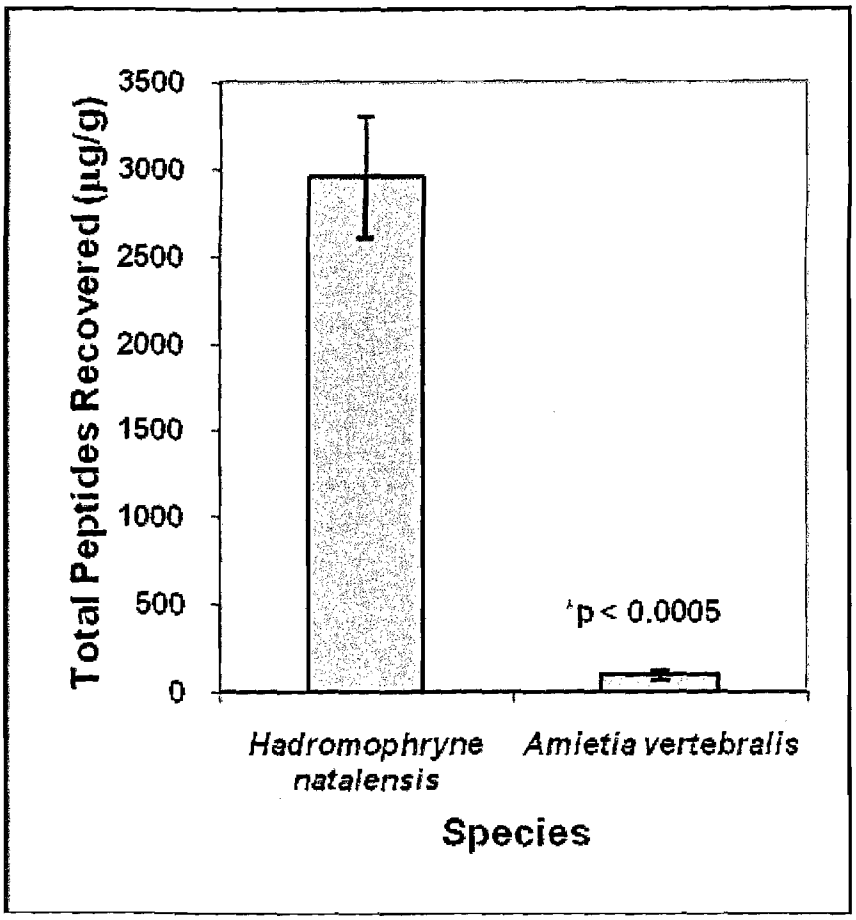


**Figure 3.23 A graph representing the average concentration of hydrophobic antimicrobial peptides isolated from *Hadromophryne natalensis* and *Amietia vertebralis* following a norepinephrine injection.**

Several peaks can be seen in each of the species' peptide composition. None of the peptides have been identified thus far. The next step will be fractionation during which the peptides will be separated by HPLC and selected peaks identified and tested for anti-bacterial activity. This procedure will be conducted by J. Michael Conlon[5].

[5] J.Michael Conlon – Department of Biochemistry, Faculty of Medicine and Health Sciences, United Arab Emirates University, 17666 Al-Ain, United Arab Emirates

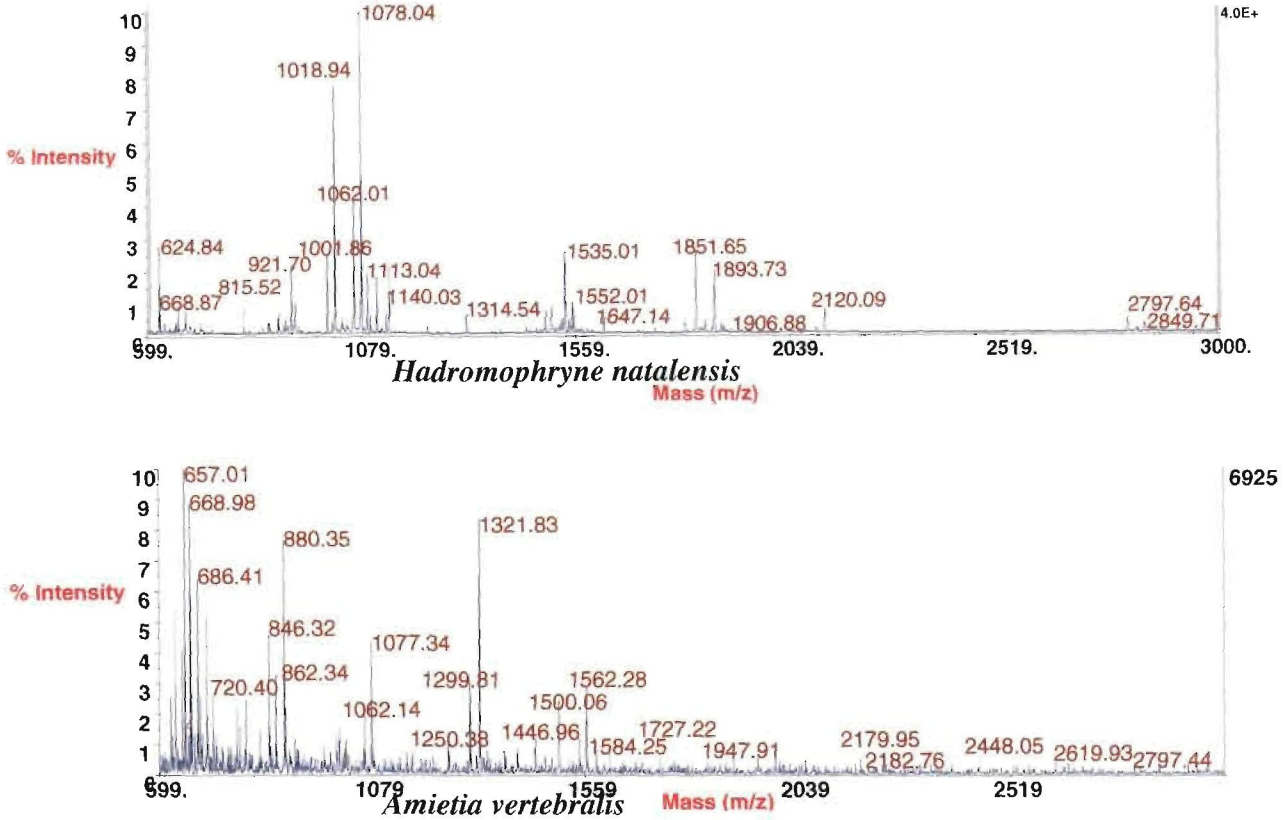


**Figure 3.24 MALDI-TOF mass spectrum of skin peptides recovered from *Hadromophryne natalensis* (top graph) and *Amietia vertebralis* (lower graph) after administration of norepinephrine. Several peptides show a high relative abundance, but have not been identified as yet.**

### 3.4.3 Bd anti-growth inhibition tests

When the relative effectiveness of the peptides against Bd was tested, clear differences between the peptides isolated from the species were seen. In Figure 3.25 the growth inhibition of the peptides isolated from *H. natalensis* is depicted. As seen from the graph, the y-axis represents the cell growth measured at an optical density of 490 nm ($OD_{490}$). The positive control was made up from culture to which no peptides were added, while the negative control was culture exposed to 60°C for 10 minutes, thereby killing the cells. The growth of Bd declined when higher concentrations of peptides from *H. natalensis* were used. The same inhibitory pattern of Bd at higher peptide concentrations was achieved from peptides isolated from other *H. natalensis* specimens (H2 and H5). The

minimal inhibitory concentration (MIC) is the lowest concentration of peptides at which no significant fungal cell growth due to Bd is detected. The MIC of peptides isolated from *H. natalensis* seemed to be about 2000 µg/mL according to the graph. Approximately 1000 mg/mL of the mixed peptides was required to completely inhibit growth of Bd, based on a micro BCA assay using a bradykinin standard.

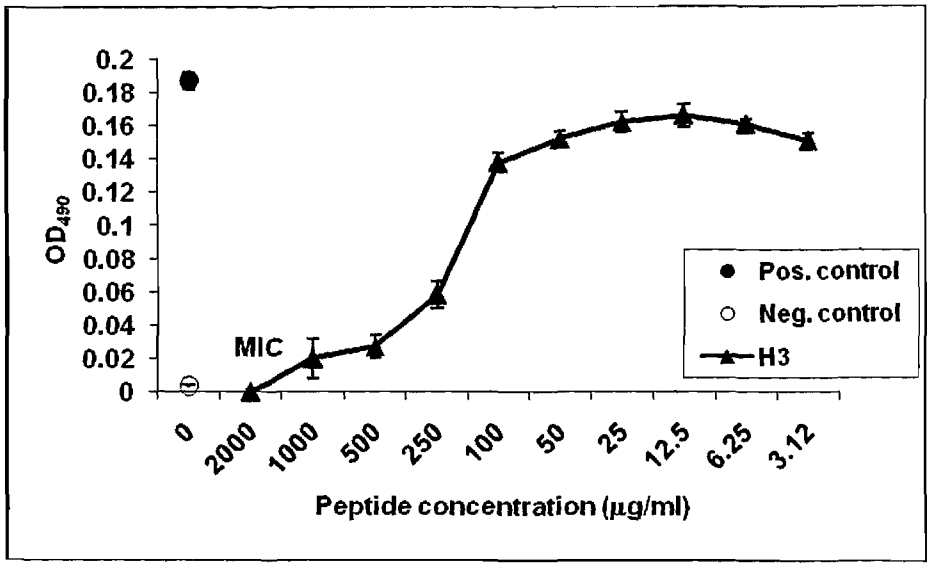


**Figure 3.25 Growth inhibition of *Batrachochytrium dendrobatidis* by the mixture of peptides isolated from *Hadromophryne natalensis* (H3).**

The same cannot be said about the mixture of peptides obtained from *A. vertebralis*. In Figure 3.26 the inhibition of Bd seems to remain the same, only increasing slightly when higher concentrations of peptides are used. Because the graph never intercepted the x-axis, the MIC was not determined. The reason for this was the low concentration of peptides isolated from *A. vertebralis* (Fig. 3.23), which meant that not enough peptides could be collected to successfully complete the experiment. For *A. vertebralis*, higher concentrations than we have used thus far, are required to determine the MIC of the peptide mixture from this frog.

From the data the relative effectiveness of the isolated peptide mixture of the two frogs was compared (Fig. 3.27). Relative effectiveness can be described as the percentage inhibition per 50 µL/mL multiplied by the amount of peptides extracted per gram body

weight. Peptides from *H. natalensis* were clearly more effective against Bd than those from *A. vertebralis,* as indicated by the p-value of less than 0.05 (95% certainty). These observations may explain the differences in the susceptibility of these frogs to the amphibian pathogen.

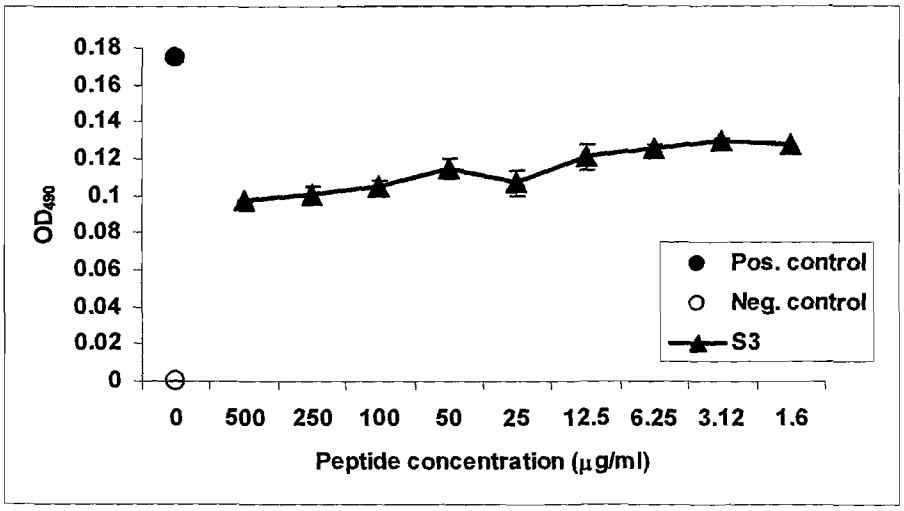


**Figure 3.26 Growth inhibition of *Batrachochytrium dendrobatidis* by the mixture peptides isolated from *Amietia vertebralis* (S3).**

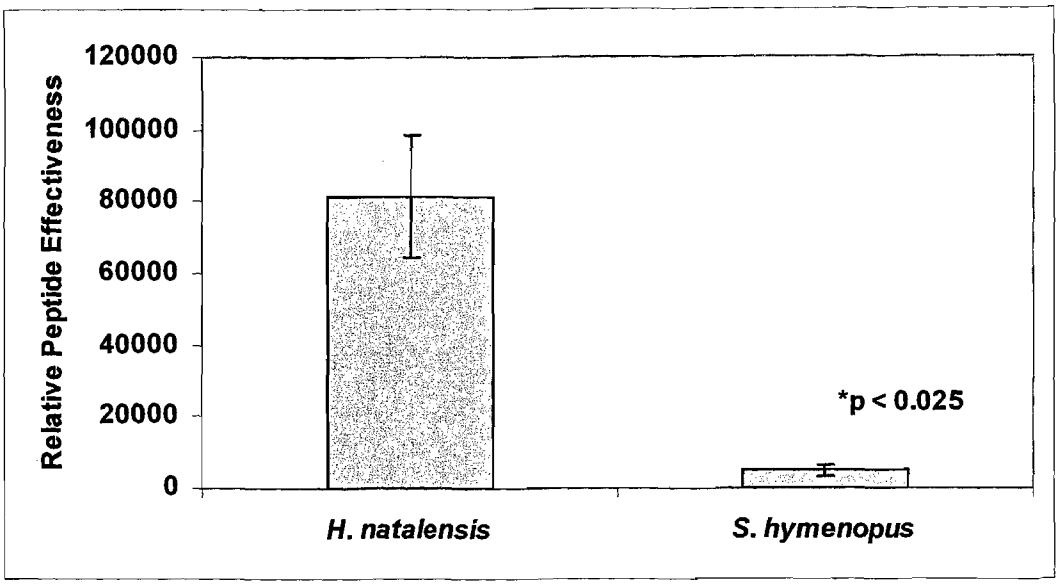


**Figure 3.27 Relative growth inhibition effectiveness* against Bd of peptides isolated from *Hadromophryne natalensis* and *Amietia vertebralis*. It is clear that there is a significant difference between the peptides of the species as indicated by the p-value. (* % inhibition at 50 µg/mL X total peptides per gram body weight)**

## 3.5 The effectiveness of F10 veterinary disinfectant as a disinfectant for frogs against *Batrachochytrium dendrobatidis*

Frogs were exposed to a 1:3 500 dilution of F10SC for 30 minutes at a time, in just enough dilution to cover the animal. After about ten minutes the *A. vertebralis* frogs started to move around in the dilution in such a manner as if they were irritated by the liquid. They also tried to escape from the containers. After the first exposure, all frogs were still alive and returned to their housing with fresh water and shelter directly after exposure.

While preparing for a second treatment, eight of the 20 *A. vertebralis* were dead. Excessive skin sloughing was evident. Upon closer examination, redness was observed around the eyes, on the feet (Fig. 3.28 a) and on the hind legs (Fig. 3.28 d) of the dead frogs. The webbing between the toes were swollen and filled with a bloody fluid (Fig 3.28 b). Frogs that were still alive at this point moved uncoordinatedly and were sluggish, and also struggled to right themselves after being turned over. Frogs seemed to

sit with one leg straightened and held away from the body. Redness was also observed on their hind legs.

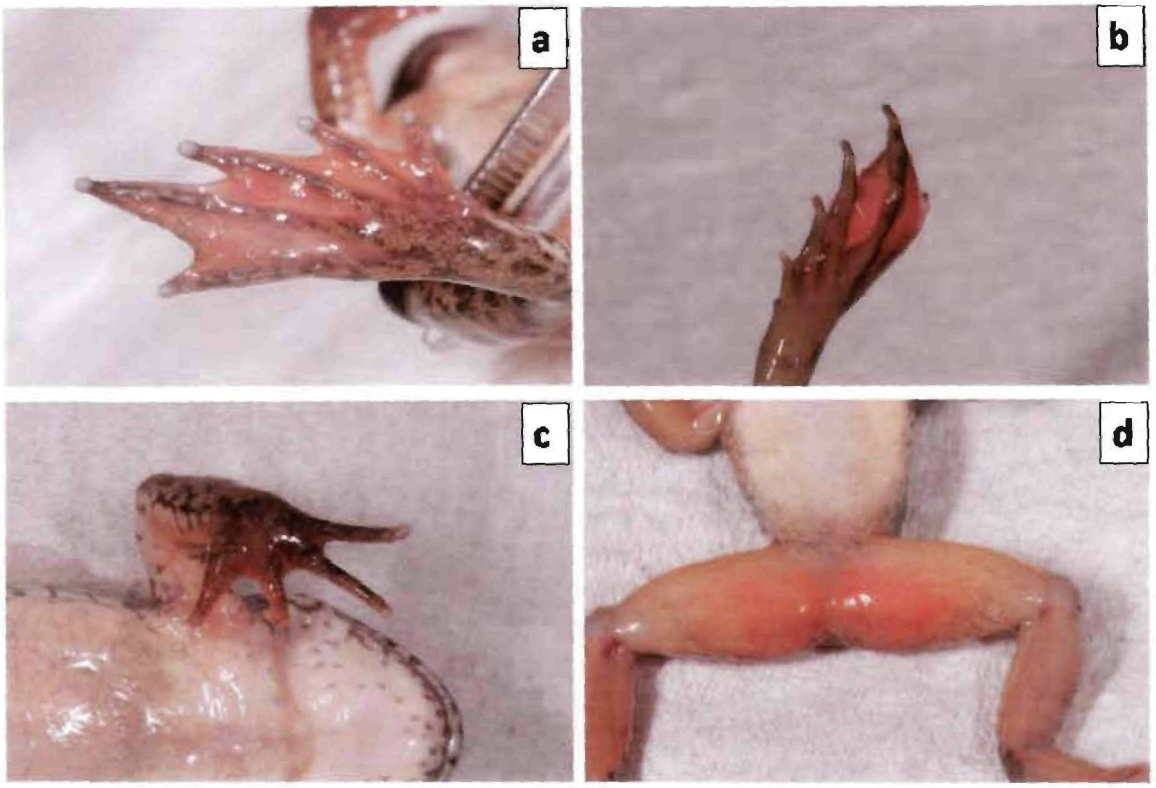


**Figure 3.28 The adverse effects experienced by *Amietia vertebralis* after exposure to a dilution of 1:3500 F10SC. Note the reddening of the webbing in (a), as well as the bloody, fluid-filled blister-like webbing in (b). The hands of the front legs also seemed swollen as seen in (c). The redness on the hind legs is pronounced (d).**

**Figure 3.29 *Amietia vertebralis* in water without any F10SC. Note the large piece of shedding situated at the head of the frog.**

*Hadromophryne natalensis* specimens did not have such a severe reaction to the F10SC. At first they fully submerged themselves in the dilution and only started to move about more after about 20 minutes, trying to escape. Some of the more visible effects of the disinfectant, like the swollen, bloody webbing and reddening of hind legs, were also not pronounced in them. They did, however, experience excessive skin shedding such as that of *A. vertebralis* (Fig. 3.29) to the extent that complete intact skins were shed. Reddening of the feet and uncoordinated movements were noted. If prodded with tweezers, the frogs felt hard, swollen and rubbery, not moving normally. When returned to the enclosures, the frogs seemed to prefer not to sit in the water. They also walked in an unnatural way, with their bodies held high above the ground, as if they tried to minimise contact between themselves and the substrate.

Sheddings from both frog species were collected and screened under a light microscope for Bd. Frogs exposed twice to F10 had no visible sporangia in the shedding. Those only exposed once to F10 still had visible, viable sporangia present in the skin.

At the end of the first experiment, 16 of the 20 *A. vertebralis* died as well as two of the six *H. natalensis*. As we were not certain what the reason for the deaths were, the

experiment was repeated eight weeks later with the same frogs, under the same conditions but in different containers to eliminate the possibility of contamination. The use of an experimental group, exposed to F10, and a control group not exposed to F10, was the only variation on the first experiment. The same results were obtained.

# Chapter 4

# Discussion

## 4.1 Localities and species sampled for *Batrachochytrium dendrobatidis*

Several new localities have been sampled during the course of this study, contributing to the knowledge we have on the distribution of Bd in South Africa. Other studies that reported on Bd in South Africa include those by Hopkins and Channing (2003), Lane *et al.* (2003) and Weldon (2005). Information on the distribution of Bd in South Africa will be incorporated into the African Bd Database (ongoing) as well as the Global Bd Mapping Project organized by staff of the Imperial College, London.

Some of the localities, for instance Memel, have special significance in this regard. Seekoeivlei near Memel is a recognised Ramsar wetland area with a rare diversity of animals. Another noteworthy locality is Jan Kempdorp. The dams from which Bd infected frogs were collected in Jan Kempdorp receive water from the Vaalharts water scheme. This makes tracing the source of the fungus and the monitoring of it much more complicated.

As demonstrated in the results, the predominant habitat sampled and positive for Bd comprises of streams. This same finding was also made by Kriger & Hero (2007). The significance of this observation is that the surveillance for Bd infected areas can be simplified, with the search for Bd in permanent waterbodies such as streams being a more accurate description of the condition of the area in terms of Bd infections. Because stream-breeding frogs are more exposed to the fungus, it can also help to identify species negatively affected by Bd since the habitat in which Bd is predominantly found is known (Kriger & Hero, 2007). The reasons for this are the aquatic nature of Bd, as well as the fact that the zoospore needs an aquatic environment in order to spread the pathogen. The presence of the fungus in an aquatic environment also ensures its optimal survival as a large part of its host's life-cycle occurs in water. Research into this phenomenon may shed some light on the interaction between the pathogen and the environment and may help to control the spread and presence of the fungus.

The African Bd Database is a new initiative started by Dr Ché Weldon (AACRG[6]) in 2006. The localities for both presence and absence data are incorporated into the database. These data will be analysed with a GIS program to see whether Bd has any environmental preferences. Aspects included in this database will be altitude, biome, rainfall and minimum and maximum temperatures. The advantage of this approach will be that localities with a higher probability for Bd infections can be identified. This will assist with the monitoring and surveillance of Bd infections. Currently data from Tanzania, Nigeria, South Africa, Botswana and Madagascar are being incorporated into the database.

Although a large part of the country has been sampled during the last few years, a number of gaps in the distribution of Bd must still be addressed. Areas in need of surveys include the North-West and Northern Cape Provinces. Additional surveys must also be conducted in areas previously declared free of Bd. A larger sample size in these areas may increase the probability of detecting Bd and therefore provide a clearer picture of the Bd distribution in South Africa.

## 4.2 Application of isolated *B. dendrobatidis*

### 4.2.1 Cultivation of *B. dendrobatidis*

The successful isolation and culturing of Bd in the laboratory is of the utmost importance for the research focus of the AACRG research group and forms a cornerstone of various research questions and focuses. The origin of Bd is still controversial and this will only be resolved once we have isolated many more strains from different geographical areas.

Research for which viable chytrid cultures will be needed includes various challenging experiments, including experiments in which Madagascan frogs[7] will be exposed to Bd in order to extrapolate the effect that the pathogen might have on the country's frog

[6] AACRG – African Amphibian Conservation Research Group situated at the North-West University
[7] April 2009 – in collaboration with Miguel Vendez and the Université d' Antanarivo in Madagascar

biodiversity should the pathogen be introduced. Thus far, Madagascar is Bd free (Weldon *et al.*, 2008).

The peptide study, initiated in this research project, will also rely on the availability of Bd cultures for exposure experiments. Frogs will be rid of their antimicrobial peptides using the protocol described under Section 2.4, followed by exposure to Bd in order to determine the efficiency of the peptides in protecting the animals against fungal infection.

The effect of Bd infections on tadpole development or the differences in virulence between developmental stages can also be researched, as well as the environmental factors driving the pathogenicity of Bd.

For this study the cultivation of Bd was especially important in order to supply the molecular laboratory at the National Zoological Gardens, Pretoria (NZG) with a positive standard to use in real time PCR and to extract DNA from South African strains for sequencing.

### 4.2.2 Extraction of DNA from Bd cultures

One of the objectives of the current research was to extract DNA from successfully maintained cultures. The relevance of this technique for the study was, firstly, to supply the laboratory at NZG with a positive standard (Fig. 4.1) for analysis of skin swabs by real time PCR. NZG is currently the only facility in South Africa that routinely performs these tests. MCT15, isolated from a *Hadromophryne natalensis* from Magoebaskloof (Limpopo Province), is currently being used with great success as a Bd-positive standard.

Secondly, the extraction of DNA from the isolates is necessary for genotyping the strains by multilocus sequence typing (MLST). The MLST data will be incorporated into the Global Bd Mapping Project, and will also shed light on the diversification and origin of Bd. MLST provides accurate, portable data which can be used in the epidemiological and population investigation of numerous pathogens, fungal, viral or bacterial, in order to reflect their evolutionary and population biology (Maiden *et al.*, 1998; Urwin & Maiden,

2003). In the case of Bd, the technique has special epidemiological value since it can assist in the identification of localised disease outbreaks and also with the monitoring of the national and global trends of the disease. The advantage of the method is that the results can be compared with one another, allowing for the development of one expanding global database, thus enabling the exchange of molecular typing data for global epidemiology through the internet (Maiden *et al.*, 1998), which is the aim of the Bd Mapping Project (http://spatialepidemiology.net/bd/information/#PD).

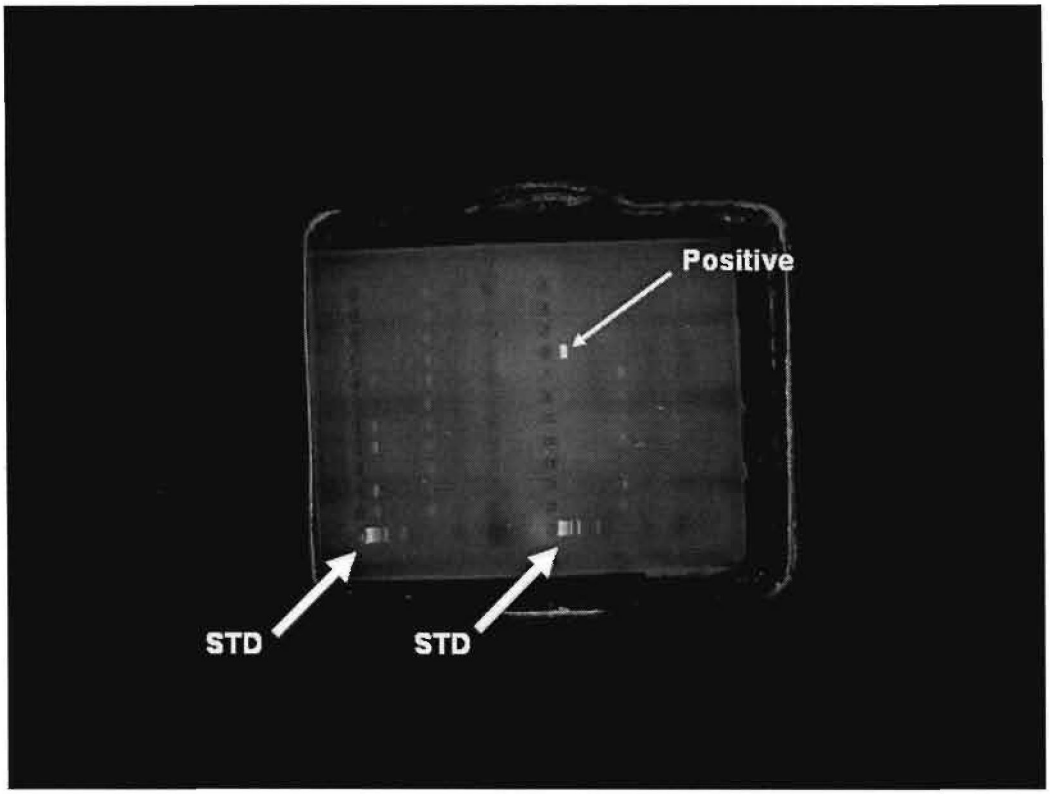


**Figure 4.1 The DNA from the MCT15 isolate is currently being used as a standard for detecting *Batrachochytrium dendrobatidis* positive samples analysed by real time PCR at the molecular laboratory at the National Zoological Gardens. The bottom wells are filled with the standard (STD) and positive samples can then be identified under UV light due to the similarity between the bands of the sample and those exhibited by the standard. A definite positive sample is indicated.**

MLST was first developed for prokaryotic organisms, but recently it has been used to analyse the genome of eukaryotes such as *Candida albicans* and *Batrachochytrium*

*dendrobatidis* (Urwin & Maiden, 2003). During MLST, all unique sequences for a given locus are assigned an allele number. All alleles present at each of the loci are then combined into an allelic profile and assigned a sequence type (ST). The STs of different isolates are then compared. STs that exhibit similarities are thought to be closely related and vice versa. The results and resolution between isolates increase when more loci are used in the procedure.

Only five loci have been used to determine the ST of South African strains (Weldon, 2005) thus far. During that analysis, two major clusters were identified: those of North America and then a second cluster including Africa, Panama, Australia and some North American strains. The same results were obtained by Morehouse *et al.* (2003). Locally, the isolates from South Africa also clustered according to the geographical distribution of the isolates. Strains from the Northern Cape were distinct from those isolated from frogs in the Western Cape, showing a strong geographical relatedness between the strains of South Africa (Weldon, 2005). The DNA isolated from the cultures cultivated during this study will be analysed at the Imperial College, London, using 17 loci. The increased number of loci analysed has the potential to increase the resolution between the different global strains. More related strains will also be identified. Though the "Out of Africa" hypothesis (Weldon *et al.*, 2004) has been partially proved incorrect with the help of MLST due to the apparent origin of Bd in North America, it has not been completely discarded. With the help of the DNA from the South African cultures, further resolution and genetic relationships may become apparent since previous sampling has been biased towards North American strains (Morehouse *et al.*, 2003).

### 4.2.3 Cryopreservation of cultures

Cryopreservation plays an important role in the cultivation process of Bd. Due to the short life-cycle of chytrids, the culture must be passaged frequently to reduce the rate at which nutrients are depleted within the growth medium. Not only is it a laborious process to maintain cultures, but passaging in itself is detrimental to the genetic makeup of the fungus (Boyle *et al.*, 2003). Genetic drift due to the selection pressures to survive in abnormal conditions such as cultures results in changes in the morphology and

physiology of the Bd over a long period (Gleason *et al.*, 2007). This also affects the virulence of cultures in the long term, as seen in other pathogens (Fromtling *et al.*, 1982; Ulett *et al.*, 2001). This phenomenon is called subculturing degeneracy (Arora *et al.*, 1992). If cultures are kept too long without transferring them, their growth slows down and they eventually die (Gleason *et al.*, 2007). By implementing a preservation technique such as cryo-preservation, the maintenance of the integrity of the genome as well as the biological activity of cultures can be ensured without the need to continuously monitor and transfer the cultures (Boyle *et al.*, 2003).

Cryopreservation by slow-freezing using the protocol suggested by Boyle *et al.* (2003) (Section 2.2.3) and modified by Johnson (2003) is very simple, with the only requirement being that the correct cryoprotectants (foetal calf serum (FCS) and dimethylsulfoxide (DMSO) are used, and the availability of either liquid nitrogen or an ultra freezer (-80°C). Due to the 100% success rate of the protocol by Boyle *et al.* (2003) it is a good procedure to implement in our laboratory. There are other methods of preserving chytrids, as evaluated in Boyle *et al.* (2003) and Gleason *et al.* (2007). This includes plunge-freezing the cultures in liquid nitrogen before storage in a -70°C freezer. While relatively effective, this method did not result in the revival of all Bd cultures (Boyle *et al.*, 2003). Other cryoprotectants, possible more inexpensive than DMSO and FCS, include skimmed milk and glycerol, and can be used during slow-freezing.

## 4.3 Microscopical examination of *Batrachochytrium dendrobatidis*

### 4.3.1 Scanning electron microscopy

Differences in the appearances of Bd infections on the keratinised mouthparts between various species of tadpoles can be visible to the naked eye or with the use of microscopes only. With the use of a 20X hand lens clearly defined areas of brown pigmentation can be seen between labial tooth rows of *Hadromophryne natalensis* (Fig. 4.2). This pigmentation is a highly reliable indicator of Bd infection in this species (Smith *et al.*, 2007). The examination of the mouthparts of *Amietia vertebralis* was conducted using a

light microscope and concentrating the search for Bd on the jaw sheaths and in the corners of the mouth opening.

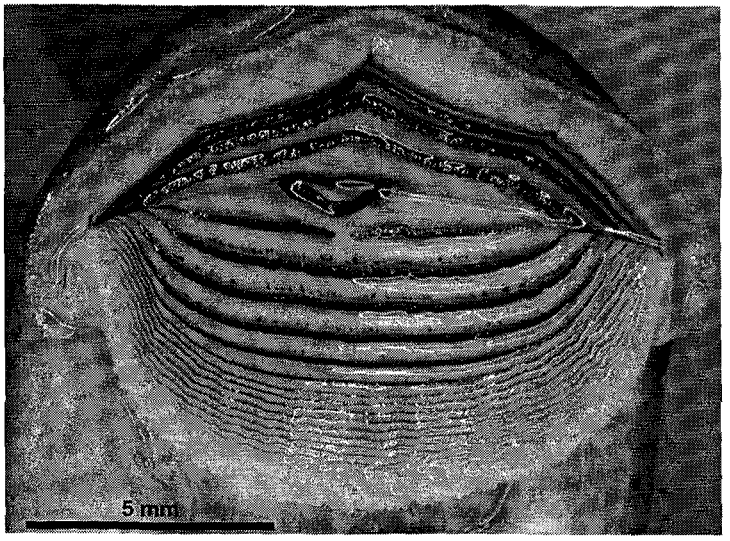


**Fig. 4.2 The pigmented areas between the labial tooth rows of *Hadromophryne natalensis* are a very accurate indication of Bd infections and can be used as a screening tool. (Picture from Smith *et al.*, 2007)**

During this study the differences in the appearance of Bd infections on the mouthparts of *H. natalensis* and *A. vertebralis* were inspected with the help of SEM. The location where Bd infections were most often found was along the jaw sheaths and in the first to third labial tooth rows from the rostrodont. This result can be compared with those found in other studies (Marantelli *et al.,* 2004) where heavier infections were also found associated with the jaw sheaths. Although both species experienced degradation of the skin in areas where Bd was present, the more severe damage was seen in the mouthparts of *A. vertebralis* in the form of missing teeth. The same high incidence of oral disfigurement in *A. vertebralis* was found by Smith *et al.* (2007). Other anomalies associated with Bd infections include oral disc abnormalities such as missing and damaged tooth rows, depigmentation and flattening of epithelial cells (Fellers *et al.*, 2001). Drake *et al.* (2007) found that abnormalities in the form of missing teeth and disfigured labial tooth rows were associated with the presence of Bd. They also found that the type of abnormalities also differed regarding their location on the mouthparts and that these abnormalities were not always due to Bd infection, thus complicating the

conclusion that all damage seen on the mouths of the tadpoles is due to Bd. Gross lesions and the depigmentation of mouthparts of tadpoles, however, are not always an indication of Bd infections (Drake *et al.*, 2007; Padgett-Flohr & Goble, 2007).

During the shift of the keratin from the mouthparts to the rest of the body (Marantelli *et al.*, 2004), the more adverse reaction of *A. vertebralis* tadpoles to Bd, due to interspecific variations in susceptibility (Berger *et al.*, 2005; Retallick & Miera, 2007), can mean that the postmetamorphic animals will also have a more dramatic reaction to Bd infections than the reaction observed in *H. natalensis*, causing the higher mortality in the species.

### 4.3.2 Laser scanning confocal microscopy

Bd has been extensively described by light microscopy, using stains such as hemotoxylin and eosin or periodic acid-Schiff (PAS) during histology (Gaffney, 1994; Berger *et al.*, 1998; Puschendorf & Bolanos, 2006). To increase differentiation between the fungus and skin tissue, fresh tissue can also be stained with cotton blue and 10% aqueous potassium hydroxide (Mazzoni *et al.*, 2003) or Congo red (Briggs & Burgin, 2003). The external ultrastructure of chytrids was also previously examined using scanning electron microscopy (SEM), while the internal ultrastructure were examined through transmission electron microscopy (Berger *et al.*, 1998; Longcore *et al.*,1999). The first immunoperoxidase test using polyclonal antibodies to identify Bd in tissue was developed by Berger *et al.* (2002). Rabbit antiserum reacts with Bd to form dark brown to red structures that contrast strongly with the blue host tissue. Thus far, little to no research has been conducted involving laser scanning confocal microscopy (LSCM) as a descriptive and diagnostic method for Bd.

During LSCM specimens are illuminated with a focused scanning laser beam and by placing a pinhole aperture in the image plane in front of an electronic photon detector (Murphy, 2001). The laser beam is then reflected by a dichromic mirror and scanned across the specimen. Secondary fluorescent emittance is emitted from points within the specimen from the plane on which the laser was concentrated and passed through the

dichromatic mirror which is then again detected at the detector pinhole aperture. Emission passing through the pinhole aperture is converted into an analog electical signal and converted to pixels. The image seen on the monitor is thus not a real image, but rather a reconstruction of emission photon signals by the photomultiplier which are temporarily stored in an image frame buffer card in the computer (Claxton *et al.*, 2006). Confocal microscopy is often capable of revealing the presence of a single molecule (Peterman *et al.*, 2004), and with the use of multiple-labeled specimens, different probes can simultaneously identify several molecules, both in fixed and living cells and tissue (Goldman & Spector, 2005). Probes or fluorophores are important due to the high degree of sensitivity of the probes to target specific structures and processes. Many of these fluorescent probes are formed around synthetic aromatic organic chemicals which bind to certain biological macromolecules, such as the proteins of nucleic acid. They can also be localised within specific organelles or structures, including the cytoskeleton, mitochondria, lysosomes, Golgi apparatus, endoplasmic reticulum and nucleus (Haugland, 2005). Fluorescent dyes are also used in monitoring cellular integrity (live versus dead cells), endocytosis, exocytosis, membrane fluidity, protein trafficking, signal transduction and enzymatic activity (Johnson, 1998). Confocal microscopy has become increasingly popular in recent years due to the relative ease with which extremely high-quality images can be obtained from specimens prepared for conventional fluorescence microscopy (Matsumoto, 2002; Muller, 2002).

The ultrastructure of the zoospore was used to group *Batrachochytrium dendrobatidis* in the order Chytridiales in Chytridiomycota (Longcore *et al.*, 1999), emphasising the importance of the examination of this small structure in taxonomy. The ultrastructure of the zoospore is therefore thoroughly studied but some of the more complex associations concerning the zoospore are still unknown, such as the infection process of Bd. Berger (2001) speculated that during the infection process the zoospore encyst on the surface of the host and injects the nucleus and cell contents through a germ tube into the host's epidermal cells. This is a characteristic of exogenous monocentric development, and since the development of Bd is endogenous - with the nucleus staying within the

zoospore cyst (James *et al.,* 2000) - it is most likely that a different method of infection is involved in this instance.

One observation that is not yet supported by any literature on the morphology of the zoospore, is the apparent presence of lysosomes within the structure. During transmission electron microscopy studies on Bd (Longcore *et al.,* 1999), the presence of mitochondria adjacent to the ribosomal mass was observed, but no lysosomes were reported. Lysosomal structures or acidic vacuoles, however, were clearly seen during the confocal microscopy examination of zoospores by Weldon (2005). During the examination of the fungus with LysoTracker Green and MitoTracker Red, two distinct green areas were seen, supporting the idea. These could either have been lysosomes or vacuoles. Due to the short life span of zoospores (spanning from 18 to 24 hours – see Piotrowski *et al.,* 2004), the zoospore is thought only to be a vessel for transmitting the genetic material of the fungus. The presence of lysosomes or acidic structures within the zoospore, however, may support the idea that the zoospore might have a function in the penetration of host tissue. Rosenblum *et al.* (2008) found a single fungalysin within zoospores. This discovery may lead to answers regarding the questions as how zoospores are able to colonise amphibian skin and gain entrance to host cells (Fisher, 2008).

Lysosomes have been identified in the rhizoids of sporangia (Weldon, 2005), but when Bd culture was stained by LysoTracker and Nile Red, there seemed to be some lysosome activity within the discharge tube. This may prove to be logical since the tube of the discharge papillae must be open in order for zoospore to pass through it. Lysosome activity within this structure can therefore help to digest lipid material and other cell content in order to form a passage for the release of zoospores. No other novel observations were made regarding the morphology of Bd.

Few of the stains used in this study showed consistent results. In the combination between LysoTracker Green and MitoTracker Red, LysoTracker Green was more reliable for indicating acidic organelles than MitoTracker Red in indicating mitochondria. A more effective stain for the examination of mitochondria, such as TMRM (Weldon, 2005)

could therefore be recommended. During a second repetition with this stain combination, non-specific structures stained, with no clearly indicated mitochondria or lysosomes. While this combination can work together, it is problematic since there is no predictability within the repetitions even though the same protocol was used in subsequent experiments. The same problem arose when acridine orange was used. Some sporangia showed clear areas where either RNA or DNA is situated, but not always in the expected pattern as described in the literature. An observation made during this study, as well as with studies conducted by Rosenblum *et al.* (2008), is the clear RNA abundance in zoospores.

Certain combinations of stains did not yield results that could be easily interpreted. The relative effectiveness of stains in combination is due to the differences between their excitation and emission spectra. The larger this difference, called the Stokes Shift, the better the probes will work together by not influencing the fluorescent signal produced by each. If the Stokes Shift is too small, bleed-through or cross-talk between the probes will occur, producing confusing and problematic colours (North, 2006). The combination of MitoTracker Red, LysoTracker Green and Calcofluor White M2R resulted in different shades of colours, with the shades differing between experimental repetitions. As mentioned in the results, the expected colours were red-stained mitochondria, green acidic organelles and blue cell walls. The resulting different colours may be caused by bleed-through. By selecting stains with a larger difference between their excitation and emission wavelengths, this problem can be reduced, but never entirely excluded.

Due to the lack of differentiation between structures in culture or of Bd in tissue and its non-specific nature, the use of Alexa Fluor 488 Phalloidon as a diagnostic or screening tool is not recommended. The same is true for LysoTracker Green and MitoTracker Red in tissue. Sporangia were only identified as large dark "holes" in the tissue. Because both the pathogen and the host cells possess mitochondria and lysosomes, background noise due to the staining of host tissue can be observed.

Nile Red indicated the phospholipids within the membranes and the lipid globules in developing and mature zoospores. The specificity and simplicity in the use of this stain make it one of most effective stains used, also in terms of repeatability and consistency in the results. Another reliable stain was Calcofluor White M2R. Due to its specificity for chitin in cell walls, it can be used as an effective screening tool for Bd in tissue, but not as a diagnostic method for the detection of Bd. Calcofluor will stain any fungi or yeast with a cell wall. It is therefore important for the operator to be familiar with the structure and morphology of Bd in order to diagnose it correctly in tissue.

Laser scanning confocal microscopy can therefore be used to study Bd in terms of cell dynamics, morphogenesis as well as organelle development. It can also be used to study the relationship between the fungus and its substratum (Spear *et al.,* 1999). Different methods exist in presenting the data acquired through LSCM, including:

- Composite or projection views where all the information in a series of images is taken at various focal planes along the z-axis and projected into one single focused image. With this method all planes can be seen simultaneously, giving the image depth and a three-dimensional quality.
- During a z-stack the x, y and z-axes can be displayed at different angular perspectives. The process results in a three-dimensional image of the studied organism which can be rotated and incorporated into presentations in order to show the three-dimensional structure of the object in question.
- During transverse xz or yz cross-sectional views, the images appear as if they have been cut transversely, in a plane oriented parallel to the optic axis.
- With the help of a time sequence, processes occurring within the fungi can be displayed as a movie in either real time or as a time-lapse sequence.

(Murphy, 2001)

The relative ease of using these different functions of the LSCM, however, was found to be entirely another matter. It is important to know the confocal software of the computer and therefore it is necessary for a trained technician to assist during the image acquisition process. Quality images could only be acquired when different parameters are set as

optimal for the objectives of the study. Such parameters included the objective used; fluorochrome specifications in combination with the lasers used; pinhole aperture; the gain (brightness) and offset (by which pixels below a certain threshold are defined as being black) and the exposure time of the living culture to the lasers (North, 2006). The selection of the correct objective with the right numerical aperture (light-gathering ability) ensures high resolution, which is especially important if co-localisation of structures is studied (Murphy, 2001; North, 2006). The pinhole aperture will also help to regulate the amount of light reaching the object, increasing the resolution of images. As said before, stains or fluorochromes with a large Stokes Shift intended to decrease bleed-through, as seen in the LysoTracker Green, MitoTracker Red and Calcofluor White M2R combination, must be selected and used in conjunction with lasers with the correct wavelength to excite the photons in the fluorochromes (Murphy, 2001; Claxton *et al.,* 2006; Murphy *et al.,* 2008). The gain can be used to increase or decrease the brightness of the image and also to decrease background noise. The offset also dims background noise and colours, but by increasing the offset too much, data is clipped at the minimum end of the dynamic range which can influence quantitative measurements. Background noise can also hide low levels of fluorescence from the organelle or protein studied. During live imaging, the time of exposure was very important since prolonged exposure times led to photobleaching of the image (Murphy, 2001; North, 2006) or the combination of the laser and the stain used could be toxic to the living organism, leading to abnormal processes or the cessation of all processes (Murphy, 2001). Autofluorescence, seen as the red colouring in Figure 3.22.b, can also prove to be problematic in the interpretation of images (Claxton *et al.,* 2006).

Not only do the above-mentioned technical aspects pose a number of problems, but incorrect sample preparation can also give rise to problems in the interpretation of the images. LSCM is therefore a good method of depicting processes and structures within the experimental organism, but due to all the technicalities, professional help is definitely needed in acquiring and interpreting images in order to present a true picture of the events within the cell.

## 4.4 Skin peptides associated with *Amietia vertebralis* and *Hadromophryne natalensis*

After this preliminary investigation, it is safe to say that the peptides from *H. natalensis* are more effective against Bd than those of *A. vertebralis.* This result supports the observations that *A. vertebralis* has experienced die-offs in the Drakensberg Mountains, while *H. natalensis* is not dying off. Differences in the efficiency of the crude peptide mixtures of the different species were observed. Approximately 2000 µg/mL of the antimicrobial peptides isolated from *H. natalensis* is needed to completely inhibit Bd growth, where as *A. vertebralis* needs a much larger concentration in order to achieve the minimal inhibitory concentration (MIC) for Bd. The slope of Figure 3.26 is almost parallel with the x-axis, showing that with increased concentrations of peptides almost no inhibition of Bd occurs. Therefore samples had to be pooled and concentrated in order to determine the MIC.

Even though the relative effectiveness of *H. natalensis* peptides against Bd is higher than that of *A. vertebralis*, compared to the minimal inhibitory concentration of other peptides (Conlon *et al.,* 2007; Rollins-Smith *et al.,* 2002c; Rollins-Smith *et al.,* 2003) the concentration needed to inhibit Bd is much higher. An average concentration of 500 µg/mL of individual peptides (temporins, dermaseptin, phylloseptin, brevinin-1TRa and Ranateurin-2TRa) was needed in studies to inhibit Bd while about 2000 µg/mL of the crude peptides mixture of *H. natalensis* was needed to inhibit it. The reason for this can be ascribed to the possibility that the different peptides may have an antagonistic effect on each other, decreasing their efficiency in mixture. Individual peptides may have a higher efficiency against Bd. Further research into the peptides isolated in this study will be conducted in 2009. The peptides associated with each species, seen as the peaks in Figure 3.24, must first be separated and purified before they can be identified. Individual peptides will then be tested for their efficiency against Bd.

*Hadromophryne natalensis* is an evolutionarily distinct species since Heleophrynidae formed part of the first anurans to form the Neobatrachia. *H. natalensis* occurred on the southern part of the Gondwanan unit which broke off the major plate along with the

sooglossides to form the first amphibians isolated from the Antarctic remnant some 100 million year ago, which explains why the most closely related frogs to *H. natalensis* are the Leptodactylidae and the Australian Myobatrachinae (Littlejohn *et al.,* 1993). *H. natalensis* may have more experience and evolution on their side in terms of host-pathogen interactions. Pathogens have the ability to exert selective pressures on a host (Zasloff, 2002). According to the hologenome model (Rosenberg, 2007) the hologenome (combined host and symbiont genomes) is thought to change with environmental conditions even though the host genome alone would have changed slower. Environmental changes such as increased pathogens and changing climate might have caused rapid evolutionary responses to these effects, increasing the survival rate of *H. natalensis.* Novel pathogens in the environment may also disrupt the host-pathogen balance, causing declines even in the presence of an effective immune response (Kurtz & Scharsack, 2007). New evidence concerning the molecular makeup of Bd has shown that Bd possesses a large serine-type peptidase gene family (Rosenblum *et al.,* 2008; Fisher, 2008), especially in its sporangia. The hypothesis exists that these peptidases are used to overcome antimicrobial peptides secreted by the host. The ability of the host to degrade these peptidases of the fungus will therefore determine the resistance of the frog against Bd. The presence of genes related to vertebrate clathrin, ITAM and Interleukin 1, with the ability to mimic vertebrate host proteins (Rosenblum *et al.,* 2008) has also led to an hypothesis that fungi have evolved these genes to "hide" from the frog's immune system within the skin cells (Fisher, 2008). Knowledge of the microbial interactions, host immune defenses, mechanisms of skin peptide induction and conditions under which stable associations between Bd and the host exist is therefore important (Woodhams *et al.,* 2007a).

Although peptides of frogs can inhibit Bd *in vitro*, other factors probably contribute to induction of skin defense and species-specific survival rates (Woodhams *et al.* 2007a). Frogs with apparent Bd inhibiting peptides are also experiencing die-offs, leading to several hypotheses. An example of this is *Rana pipiens*, possessing brevinin, esculentin-2, ranatuerin-2 and temporin, all effective against Bd. Although this is the case, this frog species still experiences population declines due to Bd (Rollins-Smith *et al.,* 2002b).

Likewise, *R. pipiens* also appears to be susceptible to Bd at high altitudes (Corn & Fogelman, 1984). Another species, *R. sylvatica*, though possessing adequate peptide defenses, experiences increased die-offs at low temperatures (Matutte *et al.*, 2000). A combination of these causal factors in the above mentioned species could explain the observed die-offs in *Amietia vertebralis*. *A. vertebralis* occurs in cool, high-altitude streams (1800 to >3000m), whereas *H. natalensis* occurs at a lower altitude of above 580 m in fast-flowing streams in forested habitats or rocky ravines (Minter *et al.*, 2004). The increased UV-B exposure at these high altitudes at which *A. vertebralis* is found, could also contribute to the decrease in peptide production (Rollins-Smith *et al.*, 2002b). Resting frogs, in the absence of substances that stimulate peptide excretion, may have less peptides available to ward off zoospores. The effect of the environment on peptide production may also cause a differential effect on the inhibition of Bd. Glucocorticoids, produced during stress (Simmaco *et al.*, 1997), and thus also decrease peptide production. Low levels of Bd infection may be tolerable under normal conditions, but stressful times such as the breeding season may prove to be too stressful, breaking through a threshold after which resistance against Bd is futile. The encounter rate of the pathogen and host will also determine the infection intensity and thus the immune response (Kurtz & Scharsack, 2007). Tree frogs, for example, are less likely to be exposed to the waterborne zoospores. Other possibilities are that because Bd mainly attaches to the ventral surface of the animal, peptides are not able to effectively reach and control the infection in this area (Apponyi *et al.*, 2004). Alternatively, it is possible that the frog's immune system does not recognise Bd as a possible threat and therefore does not launch any protective measures. The fungus itself may have adapted to the immune responses of its host and might have developed a mechanism to protect itself against peptides, for instance a protease which deactivates the peptide (Apponyi *et al.*, 2004). Research into these aspects is very important in order to determine with greater clarity what the role of the innate immune system is in the protection of the animals against specifically Bd.

Little is known about the mucosal antibodies (Kurtz & Scharsack, 2007) and whether repeated exposure to Bd will result in a lower susceptibility to Bd. Kamysz *et al.* (2003) found that the innate immune response recruits immune cells from the adaptive immune

system to the sites of microbial infection. These cells will again be activated during a later exposure, possibly forming memory cells for Bd (Råberg *et al.*, 2002). This will be advantageous, for the energy needed to launch an adaptive immune response is less than that needed for innate responses. Adaptive immune responses are also more specific than the innate immune system. Research into the role of the adaptive immune system in the protection against Bd must therefore be undertaken.

The relevance of the antimicrobial peptides in Bd research is in terms of conservation actions in response to the emergence of Bd (Garner, 2007). Species lacking peptides effective against Bd will be more susceptible to Bd, needing more urgent attention. If peptides really are a measure of susceptibility to infection, the peptide profiles of species will guide the dynamics modelling of Bd in amphibian communities (Woodhams *et al.*, 2006a; Garner, 2007). Because the peptide profiles of different amphibian species differ, these can also be used to indicate evolutionary relationships as in the case of the parotoid gland toxins, alkaloids and globin polypeptides of *Bufo* (Apponyi *et al.*, 2004).

The study of amphibian antimicrobial skin peptides not only has relevance for the field of amphibian research, but also for human immunological research. Caerin 1.1, caerin 1.9 and maculatin 1.1 are peptides able to inhibit HIV from infecting T-cells by disrupting the viral envelope integrity and preventing entry of the virus into target cells at concentrations which are not toxic to the host (VanCompernolle *et al.*, 2005). Magainins can be used against malaria (Apponyi *et al.*, 2004; VanCompernolle *et al.*, 2005) and against tumors, leukemia and ovarian tumour cells (Kamysz *et al.*, 2003). Antimicrobial skin peptides can also be used as mitogens to stimulate the growth of cells like fibroblasts and epithelial cells (Kamysz *et al.*, 2003). The importance and functions of these peptides are thus numerous for both amphibians and man.

## 4.5 Effectiveness of F10 veterinary disinfectant as a disinfectant for frogs against Bd

Animals are disinfected for the following reasons: biosecurity during laboratory research and captive husbandry, the reduction of cross-contamination and as a treatment against pathogens (Parker *et al.,* 2002) as well for the disinfection of instruments to be used on frogs (Webb *et al.,* 2007). The disinfectant used must therefore not be detrimental to the animal or to the environment.

The effectiveness of F10SC against Bd *in vivo* has been the subject of speculation in an article by Webb *et al.* (2007). The disinfectant was tested against Bd *in vitro* and proved to be effective against Bd at dilutions up to 1:3 500. As no exposures have been done on living animals before, we decided to use the disinfectant to rid *Amietia vertebralis* of and *Hadromophryne natalensis* frogs of Bd and other pathogens before an exposure experiment with Bd. We contacted the veterinarian at Johannesburg Zoo, Dr Michelle Burrows, who uses the product to treat animals with fungal infection such as aspergillosis and ringworm in reptiles, birds and mammals, as well as for disinfecting equipment and shelters. She has also used the disinfectant in a dilution of 1:3 000 to bathe a painted reed frog with cutaneous phaehyphomycosis daily for five minutes without any adverse effects (Burrows: personal communication). She suggested using a bath with a dilution of 1:2 500 for 30 minutes daily for aquatic frogs. We tried the lowest effective concentration (1:3 500) against Bd *in vitro,* as reported in Webb *et al.* (2005), first, as the product had not yet been tested on frogs for the treatment of Bd before.

The increased mortality directly after the first exposure and following repeated exposures was obviously due to F10SC. Clinical signs associated with chemical exposure can include increased mucus production, cutaneous erythema, petechiation and ulceration as well as postural and behavioral changes in order to escape the chemicals (Pessier, 2002). The postural and behavioral changes were especially visible during this experiment. *A. vertebralis* is an aquatic frog with the alarm response of diving under water in reaction to threats. The escape behavior exhibited by this species is therefore abnormal. Postural changes occurred in *H. natalensis. H. natalensis* escaping from the container during

exposures moved sluggishly and tried to keep contact with the bench to a minimum by crouching along on the tips of their hind- and forelegs.

Different disinfectants for *in vitro* use against Bd have been identified. These include 70% ethanol, Virkon, benzalkonium chloride (Johnson *et al.,* 2003), betadine, TriGene (Webb *et al.,* 2007). Frogs with Bd have been treated with chloramphenicol (Poulter *et al.,* poster presentation) and with commercial formalin/malachite green solution (Parker *et al.,* 2002). The use of table salt as a disinfectant against Bd has been speculated upon (White, 2006), but no clear results on the efficiency of the treatment have been achieved. A successful method to rid frogs of Bd has been developed by Woodhams *et al.* (2003) and involves raising the body temperature of frogs in order to form an environment not suitable for Bd. The question of whether F10SC can be used as a disinfectant for frogs against Bd is still unanswered. Shed skin examined after two exposures did not contain any sporangia or any sign of Bd. An explanation for this can be that the increased shedding rate, although a response to the irritation caused by F10SC, enables the frog to shed infected skin layers more quickly, killing the Bd in the process. F10SC thus has the potential to be used as a disinfectant against Bd, but more experiments are needed to determine the most effective method of administration: either using a F10SC bath or nebulising the frogs with a light mist of F10SC. The correct dilution and exposure time at which the host is not adversely affected must also be determined.

# Chapter 5

# Conclusion

Amphibian chytrid (*Batrachochytrium dendrobatidis*) occurs widely throughout South Africa and in various amphibian species with no apparent mass extinctions and major species declines. This situation provided an ideal opportunity to study *B. dendrobatidis* (Bd) infections under natural conditions. This study focused on the distribution of Bd in South Africa, and specifically also on the isolation and cultivation of the fungus, the preservation of cultures, microscopy examination of Bd infection on tadpoles and morphology of the fungus itself, the innate immunity of amphibians and the treatment of amphibians infected with Bd.

Bd infection is widely distributed in South Africa throughout several biomes and different environments. During this study, additional points were added to the African Bd Database as well as the Bd Mapping Project. The aim of these databases is to develop a better understanding of Bd and its the environmental preferences, contributing to the knowledge about the fungus. This will help in the monitoring, surveillance and, ultimately, the control of Bd infections in South Africa and globally.

Cultures were obtained from different localities in South Africa and these might prove to be of different strains. Through multilocus sequence typing these strains can be analysed and compared to other known strains. Since the "Out of Africa" Hypothesis (Weldon *et al.*, 2004) has not been proven be to right or wrong, the information on the isolates in this study will shed additional light on the subject. Knowledge of the origin and emergence of any pathogen is very important in the control and understanding of the disease. Cultures are not only important for epidemiological studies, but also for experimental work on Bd. An important consideration in any work involving pathogens in culture is the effect of subculturing on the pathogen. For this reason, certain preservation measures are very important in order to ensure the virulence and genetic integrity of the strains. Cryopreservation with foetal calf serum and DMSO as cryoprotectants was used to

cryopreserve the different cultures for future use. The advantage of this method is that the cultures can be preserved for up to a year without being passaged.

In tadpoles, Bd is associated with the oral region and it is believed that Bd lives off the keratinised mouthparts. We examined the tadpole mouthpart morphology of the Natal cascade frog (*Hadromophryne natalensis*) as well as the Phophung river frog (*Amietia vertebralis*). Tadpoles of *Amietia vertebralis*, a species experiencing die-offs due to Bd infection, showed more damage to their labial toothrows due to Bd infection than observed for *H. natalensis* tadpoles where hyperpigmentation was observed in areas of Bd infection. The adverse effects of Bd on *A. vertebralis* were quite severe with complete toothrows missing, even though the tadpoles do not experience mortalities due to the infection. The severity of damage occurring during the larval stage can therefore be indicative of the expected reaction of subadult and adult frogs.

Antimicrobial skin peptides have also been linked to resistance against Bd as well as other pathogens. We found that *H. natalensis* have significantly more peptides in their skin than *A. vertebralis*. This might explain the observed sensitivity and mortality in Bd infected *A. vertebralis*. The identification of the individual peptides extracted from both species is therefore very important in order to determine the relative efficiency of the peptides against Bd. If *H. natalensis* possesses specific peptides that provide better protection against Bd, it may explain why, in spite of heavy infections, they do not experience die-offs. Further research into the role of the adaptive immunity in the defense against Bd is also needed in order to form a complete picture of the role of immunity in the resistance against Bd. Co-evolution of Bd itself in the form of gene regulating for fungalysins and genes mimicking vertebrate host proteins should be investigated in more depth. Knowledge of the microbial interactions, host immune defenses, mechanisms of skin peptide induction and conditions under which stable associations between Bd and the host exist are therefore important.

Laser scanning confocal microscopy is a novel method for examining Bd in cultures and also Bd infections on the mouthparts of tadpoles. This study showed that this technique

could be valuable for clarifying aspects of the morphology of the fungus. However, since this is a novel field, much more research must be conducted into the specificity of stains for specific fungal structures. One stain, Calcofluor White M2R, showed especially promising results due to its specificity to the chitin in fungal cell walls. The use of this stain as a screening tool for Bd in tissue is therefore possible. Its use as a diagnostic tool will, however, depend largely on the knowledge of the microscope operator since the identification of Bd in tissue will be based on the morphology of Bd, as well as its relative position within the epithelial layers, since all fungi and yeast present in the skin will be stained. Confocal microscopy has the ability of showing different processes and structures within sporangia and zoospores, and can be used to increase our understanding of Bd. One specific observation includes the apparent presence of lysosomes within the zoospore. We observed concentrations thereof in developing discharge papillae and speculated that the lysosomes might play a role in preparing the discharge papilla for the release of zoospores. The relevance of these structures in the infection process of Bd must be considered and further researched.

Disinfection of frogs is important in order to determine the real effect of pathogens on the host and to reduce the risk of cross-contamination during laboratory experiments and captive husbandry. The prerequisite for an effective disinfectant however, is that it should not harm the animal or the environment. F10SC, as used in this study, does not comply with this criterion. Additional experiments into the dilution and exposure time most suited for *in vivo* experiments are urgently needed as it seems that F10SC is effective in killing Bd in shed skin layers.

It is clear from this study that a multidisciplinary approach is needed in order to understand *B. dendrobatidis* infections in South Africa and in the rest of the world. Currently, mycologists, pathologists, geneticists, immunologists and epidemiologists are working together in order to form a holistic picture of interactions between the amphibian host and the pathogenic fungus. It was a privilege to form part of this process.

# Bibliography

AANENSEN, D., FISHER, M. 2008. Spatialepidemiology.net/*Batrachochytrium dendrobatidis.* Available from: http://www.spatialepidemiology.net/bd/ Accessed on 20 May 2008

ALTIG, R. 2007. Comments on the description and evaluations of tadpole mouthpart anomalies. *Herpetological Conservation and Biology* **2(1):** 1-4

APPONYI, M.A., PUKALA, T.L., BRINKWORTH, C.S., MASELLI, V.M., BOWIE, J.H., TYLER, M.J., BOOKER, G.W., WALLACE, J.C., CARVER, J.A., SEPAROVIC, F., DOYLE, J. & LLEWELLYN, L.E. 2004. Host-defence peptides of Australian anurans: structure, mechanisms of action and evolutionary significance. *Peptides* **25**: 1035-1054

ARORA, D.K., BHATNAGAR, D., AJELLO, L., MUKERJI, K.G., MARTH, E.H., LILLEHOJ, E.B. & ELANDER, R.P. 1992. Handbook of applied mycology. Volume 5. CRC Press.

BEARD, K.H. & O'NEILL, E.M. 2005. Infection of an invasive frog *Eleutherodactylus cocqui* by the chytrid fungus *Batrachochytrium dendrobatidis* in Hawaii. *Biological Conservation* **126:** 591-595

BELDEN, L.K. & HARRIS, R.N. 2007. Infectious disease in wildlife: the community ecology context. *Frontiers of Ecological Environment* **5(10):** 533-539

BERGER, L., SPEARE, R., DASZAK, P.I., GREEN, D.E., CUNNINGHAM, A.A., GOGGIN, C.L., SLOCOMBE, R., RAGAN, M.A., HYATT, A.D., MCDONALD, K.R., HINES, H.B., LIPS, K.R., MARANTELLI, G. & PARKES, H. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rainforests of Australia and Central America. *Proceedings of the National Academy of Science* **95:** 9031-9036

BERGER, L., SPEARE, R. & HYATT, A.D. 1999. Chytrid fungi and amphibian declines: Overview, implications and future directions. *Declines and Disappearances of Australian Frogs*: 23-34

BERGER, L. 2001. Diseases in Australian frogs. Thesis submitted for the degree Doctor of Philosophy at James Cook University, Townsville

BERGER, L., MENDEZ, D. & SPEARE, R. 2001. Histological diagnosis of chytridiomycosis. In: Developing management strategies to control amphibian diseases: Decreasing the risks due to communicable diseases. Rick SPEARE and Steering Committee of Getting the Jump on Amphibian Disease. School of Public Health and Tropical Medicine, James Cook University: Townsville

BERGER, L., HYATT, A.D., OLSEN, V., HENGSTBERGER, S.G., BOYLE, D., MARANTELLI, G., HUMPHREYS, K. & LONGCORE, J.E. 2002. Production of polyclonal antibodies to *Batrachochytrium dendrobatidis* and their use in an immunoperoxidase test for Chytridiomycosis in amphibians. *Diseases of Aquatic Organisms* **48(3):** 213-220

BERGER, L., SPEARE, R., HINES, H.B., MARANTELLI, G., HYATT, A.D., MCDONALD, K.R., SKERRATT, L.F., OLSEN, V., CLARKE, J.M., GILLESPIE, G., MAHONY, M., SHEPPARD, N., WILLIAMS, C. & TYLER, M.J. 2004. Effect of season and temperature on mortality in amphibians due to chytridiomycosis. *Australian Veterinary Journal* **82(7):** 434-439

BERGER, L., HYATT, A.D., SPEARE, R. & LONGCORE, J.E. 2005a. Life cycle stages of *Batrachochytrium dendrobatidis* Longcore *et al.* 1999, the amphibian chytrid. *Diseases of Aquatic Organisms* **68:**51-63.

BERGER, L., SPEARE, R. & SKERRATT, L. 2005b. Distribution of *Batrachochytrium dendrobatidis* and pathology in the skin of green tree frogs (*Litoria caerulea*) with severe chytridiomycosis. *Diseases of Aquatic Organisms* **68**: 65-70

BERGER, L., MARANTELLI, G., SKERRATT, L.F. & SPEARE, R. 2005c. Virulence of the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, varies with the strain. *Diseases of Aquatic Organisms* **68**:47-50.

BLAUSTEIN, A.R., WAKE, B.D. & WAYNE, P.S. 1994. Amphibian declines: Judging stability, persistence and susceptibility of populations to local and global extinctions. *Conservation Biology* **8**: 60-71.

BLAUSTEIN, A.R., ROMANSIC, J.M., SCHEESSELE, E.A., HAN, B.A., PESSIER, A.P. & LONGCORE, J.E. 2005. Interspecific variation in susceptibility of frog tadpoles to the pathogenic fungus *Batrachochytrium dendrobatidis*. *Conservation Biology* **19(5):**1460-1468.

BOSCH, J., MARTINEZ-SOLANO, I. & GARCIA-PARIS, M. 2001. Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biological Conservation* **97**: 331-337

BOSCH, J., CARRASCAL, L.M., DURAN, L., WALKER, S. & FISHER, M.C. 2007. Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain: is there a link? *Proceedings of the Royal Society* **274:** 253-260

BOYLE, D.G., HYATT, A.D., DASZAK, P., BERGER, L., LONGCORE, J.E., PORTER, D., HENGSTBERGER, S.G. & Olsen, V. 2003. Cryo-archiving of *Batrachochytrium dendrobatidis* and other chytridiomycetes. *Diseases of Aquatic Organisms* **56**: 59-64

BRIGGS, C. & BURGIN, S. 2003. A rapid technique to detect chytrid infection in adult frogs. *Herpetological Review* **34(2):**124-126.

BRIGGS, C.J., VREDENBURG, V.T., KNAPP, R.A. & RACHOWICZ, L.J. 2005. Investigating the population-level effects of chytridiomycosis: An emerging infectious disease of amphibians. *Ecology* **86(12):** 3149-3159.

BURGER, J. & SNODDGRASS, J.W. 2000. Oral deformities in several species of frogs from the Savannah River site, USA. *Environmental Toxicology and Chemistry* **19:** 2519-2524

BURGIN, S., SCHELL, C.B. & BRIGGS, C. 2005. Is *Batrachochytrium dendrobatidis* really the proximate cause of frog decline? *Acta Zoologica Sinica* **51(2):** 344-348

CANONICO, P.G. & BIRD, J.W.C. 1969. The use of acridine orange as a lysosomal marker in rat skeletal muscle. *Journal of Cell Biology* **43(2):** 367-371

CAREY, C., COHEN, N. & ROLLINS-SMITH, L. 1999. Amphibian declines: an immunological perspective. *Developmental and Comparative Immunology* **23(6):** 459-472

CAREY, C., BRADFORD, D.F., BRUNNER, J.L., COLLINS, J.P., DAVIDSON, E.W., LONGCORE, J.E., OULLET, M., PESSIER, A.P. & SCHOCK, D.M. 2003. Biotic factors in amphibian population declines. Pages 153-208 in G. Linder, S.K. Krest & D.W. Sparling, editors. Amphibian decline: an integrated analysis of multiple stressor effects. Society of Environmental Toxicology and Chemistry, Pensacola, Florida.

CAREY, C., BRUZGUL, J.E., LIVO, L.J., WALLING, M.L., KUEHL, K.A., DIXON, B.F., PESSIER, A.P., ALFORD, R.A. & ROGERS, K.B. 2006. Experimental Exposures of Boreal Toads (*Bufo boreas*) to a Pathogenic Chytrid Fungus (*Batrachochytrium dendrobatidis*). *EcoHealth* **3**: 5-21.

CASAVAN, W., GAIDOUKEVITCH, Y., MURPHY, D.B., CLAXTON, N.S. & DAVIDSON, M.W. 2008. Colocalization of fluorophores in confocal microscopy. Olympus Corporation. Available from: www.olympusconfocal.com/applications/colocalization.html. Accessed August 26, 2008

CHANNING, A., FINLOW-BATES, K.S., HAARKLAU, S.E. & HAWKES, P.G. 2006. The biology and recent history of the critically endangered kihansi spray toad *Nectophrynoides asperginis* in Tanzania. *Journal of East African Natural History* **95:** 117-138

CHINCHAR, V.G., BRYAN, L., SILPHADAUNG, U., NOGA, E., WADE, D. & ROLLINS-SMITH, L.A. 2004. Inactivation of viruses infecting ectothermic animals by amphibian and piscine antimicrobial peptides. *Virology* **323**: 268-275.

CLAXTON, N.S., FELLERS, T.J. & DAVIDSON, M.W. 2006. Laser scanning confocal microscopy. Olympus Available from: http://www.olympusconfocal.com/theory/LSCMIntro.pdf

COLLINS, J.P. & STORFER, A. 2003. Global amphibian declines: sorting the hypotheses. *Diversity and Distributions* **9:**89-98.

CONLON, J.M., WOODHAMS, D.C., RAZA, H., COQUET, L., LEPRINCE, J., JOUENNE, T., VAUDRY, H. & ROLLINS-SMITH, L.A. 2007. Peptides with differential cytolytic activity from skin secretions of the lemur leaf frog *Hylomantis lemur* (Hylidae: Phyllomedusinae) *Toxicon* DOI: 10.1016/j.toxicon.2007.04.017

COOPER, E.L., WRIGHT, R.K., KLEMPAU, A.E. & SMITH, C.T. 1992. Hibernation alters the frog's immune system. *Cryobiology* **29**: 616-631

CORN, P.S. & FOGELMAN, J.C. 1984. Extinction of montane population of the northern leopard frog (*Rana pipiens*) in Colorado USA. *Journal of Herpetology* **18**: 147-152

CUNNINGHAM, A.A., GARNER, T.W.J., AGUILAR-SANCHEZ, V., BANKS, B., FOSTER, J., SAINSBURY, A.W., PERKINS, M., WALKER, S.F., HYATT, A.D. & FISHER, M.C. 2005. Emergence of amphibian chytridiomycosis in Britain. *Veterinary Record* **157:** 386-387

DASZAK, P., BERGER, L., CUNNINGHAM, A.A., HYATT, A.D., GREEN, D.E. & SPEARE R. 1999. *Emerging Infectious Diseases* and amphibian population declines. *Emergent Infectious Diseases* **5**: 735–48.

DASZAK, P. & CUNNINGHAM, A.A. 2003. Anthropogenic change, biodiversity loss and a new agenda for emerging diseases. *Journal of Parasitology* **89:** 837-841

DASZAK, P., CUNNINGHAM, A.A. & HYATT, A.D. 2003. Infectious disease and amphibian population declines. *Diversity and Distributions* **9:** 141-150

DASZAK, P., STRIEBY, A., CUNNINGHAM, A.A., LONGCORE, J.E., BROWN, C.C. & PORTER, D. 2004. Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. *Herpetological Journal* **14(4):** 201-207

DASZAK, P., TABOR, G.M., KILPATRICK, A.M., EPSTEIN, J. & PLOWRIGHT, R. 2004. Conservation medicine and a new agenda for emerging diseases. *Annual New York Academy of Science* **1024:** 1-11

DAVIDSON, E.W., PARRIS, M., COLLINS, J.P., LONGCORE, J.E., PESSIER, A.P. & BRUNNER, J. 2003. Pathogenicity and transmission of Chytridiomycosis in tiger salamander (*Ambystoma tigrinum*). *Copeia* **3**: 601-607.

DE CASTRO, F. & BOLKER, B. 2005. Mechanisms of disease-induced extinction. *Ecological Letters* **8:** 117-126

DECLINING AMPHIBIAN POPULATIONS TASK FORCE. 2004. About amphibian declines. Available from: http://www.open.ac.uk/daptf/declines/decl0.htm

DI ROSA, I., SIMONCELLI, F., FAGOTTI, A. & PASCOLINI, R. 2007. The proximate cause of frog declines? *Nature* **447:** E4-E5

DRAKE, D.L., ALTIG, R., GRACE, J.B. & WALLS, S.C. 2007. Occurrence of oral deformities in larval anurans. *Copeia* **2:** 449-458

DREW, A., ALLEN, E.J. & ALLEN, L.J. 2006. Analysis of climatic and geographic factors affecting the presence of chytridiomycosis in Australia. *Diseases of Aquatic Organisms* **68(3):** 245-250.

FELLERS, G.M., GREEN, D.E. & LONGCORE, J.E. 2001. Oral chytridiomycosis in the mountain Yellow-legged frog (*Rana muscosa*). *Copeia* **4:** 945-953

FISHER, M.C. & GARNER, T.W.J. 2007. The relationship between the emergence of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduced amphibian species. *Fungal Biology Reviews* **21**: 2-9

FISHER, M.C. 2008. Molecular toolkit unlocks life cycle of the panzootic amphibian pathogen *Batrachochytrium dendrobatidis. PNAS* **105(45):** 17209-17210

FROMTLING, R.A., SHADOMY, H.J. & JACOBSON, E.S. 1982. Decreased virulence in stable, acapsular mutants of *Cryptococcus neoformans. Mycopathologia* **79(1):** 23-29

GAFFNEY, E. 1994. Carbon hydrates. *In* Laboratory methods in histotechnology, E.B. Prophet, B. Mills, J.B. Arrington, and L.H. Sobin (eds.) *American Registry of Pathology.* Washington, D.C: 149-174

GARNER, T.W.J., WALKER, S., BOSCH, J., HYATT, A.D., CUNNINGHAM, A.A. & FISHER, M.C. 2005. Chytrid fungus in Europe. *Emerging Infectious Diseases* **11(10):** 1639-1641.

GARNER, T.W.J., PERKINS, M.W., GOVINDARAJULU, P., SEGLIE, D., WALKER, S., CUNNINGHAM, A.A. & FISHER, M.C. 2006. The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana. Biology Letters*: 1-5

GARNER, T.W.J. 2007. Experimental evidence of innate immunity: a matter of design, convenience or constraint. *Animal Conservation* **10**: 418-419

GLEASON, F.H., MOZLEY-STANDRIDGE, S.E., PORTER, D., BOYLE, D.G. & HYATT, A.D. 2007. Preservation of Chytridiomycota in culture collections. *Mycological Research* **111**: 129-136

GOLDMAN, R.D. & SPECTOR, D.L. 2005. Live Cell Imaging: A Laboratory Manual. New York: Cold Spring Harbor Press.

GREEN, D.E., CONVERSE, K.A. & SCHRADER, A.K. 2002. Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996-2001. *New York Academy of Sciences* **969:** 323-339

GREENBAUM, E., KUSAMBA, C., ARISTOTE, M.M. & REED, K. 2008. Amphibian chytrid fungus infections in *Hyperolius* (Anura: Hyperoliidae) from Eastern Democratic Republic of Congo. *Herpetological Review* **39(1):** 70-73

HALE, S.F., ROSEN, P.C., JARCHOW, J.L. & BRADLEY, G.A. 2005. Effects of the chytrid fungus on the Tarahymara frog (*Rana tarahumarae*) in Arizona and Sonora, Mexico. USDA Forest Service Proceedings RMRS-P 36

HANSELMANN, R., RODRIGUEZ, A., LAMPO, M., FAJARDO-RAMOS, L., AGUIRRE, A.A., KILPATRICK, A.M., RODRIGUEZ, J.P. & DASZAK, P. 2004. Presence of an

emerging pathogen of amphibians in introduced bullfrogs *Rana catesbeiana* in Venezuela. *Biological Conservation* **120:** 115-119

HARRIS, R.N., JAMES, T.Y., LAUER, A., SIMON, M.A. & PATEL, A. 2006. Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *EcoHealth* **3:** 53-56.

HARVELL, C.D., MITCHELL, C.E., WARD, J.R., ALTIZER, S. & DOBSON, A.P. 2002. Climate warming and disease risks for terrestrial and marine biota. *Science* **296**: 2158-2162

HAUGLAND, R.P. 2005. The Handbook: A Guide to Fluorescent Probes and Labeling Technologies. Chicago: Invitrogen Molecular Probes.

HERO, J.M., WILLIAMS, S.E. & MAGNUSSON, W.E. 2005. Ecological traits of declining amphibians in upland areas of eastern Australia. *Journal of Zoology* **267:** 221-232

HOPKINS, S. & CHANNING, A. 2003. Chytrid fungus in northern and western cape frog populations, South Africa. *Herpetological Review* **34**: 334-336

IUCN, CONSERVATION INTERNATIONAL AND NATURE SERVE. 2006. Global Amphibian Assessment. Available: http://www.globalamphibian.org. Accessed on 29 October 2006.

JAMES, T.Y., PORTER, D., LEANDER, C.A., VILGALYS, R. & LONGCORE, J.E. 2000. Molecular phylogenetics of the Chytridiomycota supports the utility of ultrastructural data in chytrid systematics. *Canadian Journal of Botany* **78(3):**336-350

JOHNSON, I. 1998. Fluorescent Probes for Living Cells. *Histochemistry Journal* **30:** 123-140

JOHNSON, M.L. & SPEARE, R. 2003. Survival of *Batrachochytrium dendrobatidis* in water: quarantine and disease control implications. Available from www.cdc.gov/ncidod/EID/vol9no8/03-0145.htm Accessed on 12 June 2007

JOHNSON, M. 2003. Cryopreservation Protocol for *Batrachochytrium dendrobatidis*. Available at: http://www.jcu.edu.au/school/phtm/PHTM/frogs/protocols/bd-cryo-protocol.pdf Accessed on 21 November 2007

JOHNSON, M., BERGER, L., PHILIPS, L. & SPEARE, R. 2003. *In vitro* evaluation of chemical disinfectants, UV light, desiccation and heat on the amphibian chytrid *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms* **57:** 255-260

JOHNSON, M.L. & SPEARE, R. 2005. Possible modes of dissemination of the amphibian chytrid *Batrachochytrium dendrobatidis* in the environment. *Diseases of Aquatic Organisms* **65:** 181-186

JOHNSON, P.T.J. 2006. Amphibian diversity: Decimation by disease. *National Academy of Sciences* **103(9):** 3011-3012

KAMYSZ, W., OKROJ, M. & LUKASIAK, J. 2003. Novel properties of antimicrobial peptides. *Acta Biochimica Polonica* **50(2):** 461-469

KARLING, J.S. 1977. Chytridiomycetarum iconographia: an illustrated and brief descriptive guide to the Chytridiomycetous genera with a supplement of the Hyphochytriomycetes. Monticello: Lubrecht and Cramer.

KNAPP, R.A. & MORGAN, J.A.T. 2006. Tadpole mouthpart depigmentation as an accurate indicator of chytridiomycosis, an emerging disease of amphibians. *Copeia* **2:** 188-197

KRIGER, K.M. & HERO, J.M. 2006a. Survivorship in wild frogs infected with chytridiomycosis. *EcoHealth* **3:** 171-177

KRIGER, K.M. & HERO, J.M. 2006b. Large scale seasonal variation in the prevalence and severity of chytridiomycosis. *Journal of Zoology* DOI: 10.1111/j.1469-7998.2006.00220.x

KRIGER, K.M., ASHTON, K.J., HINES, H.B. & HERO, J.M. 2007. On the biological relevance of a single *Batrachochytrium dendrobatidis* zoospore: a reply to Smith (2007). *Diseases of Aquatic Organisms* **73:** 257-260

KRIGER, K.M. & HERO, J.M. 2007. The chytrid fungus *Batrachochytrium dendrobatidis* is non-randomly distributed across amphibian breeding habitats. *Diversity and Distributions* DOI: 10.0000/j.1472-4642.2007.00394.x

KURTZ, J. & SCHARSACK, J.P. 2007. Resistance is skin-deep: innate immunity may help amphibians to survive a deadly fungus. *Animal Conservation* **10**: 422-424

LAMIRANDE, E.W. & NICHOLS, D.K. 2002. Effects of host age on susceptibility to cutaneous chytridiomycosis in blue-and-yellow poison dart frogs (*Dendrobates tinctorius*). In: McKinnell, R.G., Carlson, D.L. *Proceedings of the Sixth International Symposium on the Pathology of Reptiles and Amphibians. Saint Paul, Minnesota, USA*: 3-13

LANE, E.P., WELDON, C. & BINGHAM, J. 2003. Histological evidence of chytridiomycete fungal infection in a free-ranging amphibian, *Afrana fuscigula* (Anura: Ranidae) in South Africa. *Tydskrif van die Suid-Afrikaanse Veterinêre Vereniging* **74(1):** 20-21

LAURENCE, W.F., MCDONALD, K.R. & SPEARE, R. 1996. Epidemic disease and the catastrophic decline of Australian rain forest frogs. *Conservation Biology* **10(2):** 406-413

LAURENCE, W.F., MCDONALD, K.R. & SPEARE, R. 1997. In defense of the Epidemic Disease Hypothesis. *Conservation Biology* **11(4):** 1030-1034

LIPS, K.R., MENDELSON III, J.R., MUNOZ-ALONSO, A., CANSECO-MARQUEZ, L. & MULCAHY, D.G. 2004. Amphibian population declines in montane southern Mexico: resurveys of historical localities. *Biological Conservation* DOI: 10.1016/j.biocon.2004.01.017v

LIPS, K.R., BURROWS, P.A., MENDELSON III, J.R. & PARRA-OLSON, G. 2005. Amphibian population declines in Latin America: a synthesis. *Biotropica* **37(2):** 222-226

LIPS, K.R., BREM, F., BRENES, R., REEVE, J.D., ALFORD, A.A., VOYLES, J., CAREY, C., LIVO, L, PESSIER, A.P. & COLLINS, J.P. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings of the National Academy of Sciences of the United States of America* **102:** 3165-3170

LIPS, K.R., DIFFENDORFER, J., MENDELSON III, J.R. & SEARS, M.W. 2008. Riding the wave: Reconciling the roles of disease and climate change in amphibian declines. *PloS Biology* **6(3):** e72

LITTLEJOHN, M.J., ROBERTS, J.D., WATSON, G.F. & DAVIES, M. 1993. 7. Family Myobatrachidae", Fauna of Australia 2A. Australian Government Publishing Service. Available from: http://www.environment.gov.au/biodiversity/abrs/publications/fauna-of-australia/pubs/ volume2a/07-fauna-2a-amphibia-myobatrachidae.pdf Accessed on: 21 October 2008.

LONGCORE, J.E., PESSIER, A.P. & NICHOLS, D.K. 1999. *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia* **91(2):** 219-227

MAIDEN, M.C.J., BYGRAVES, J.A., FEIL, E., MORELLI, G., RUSSELL, J.E., URWIN, R., ZHANG, Q., ZHOU, J., ZURTH, K., CAUGANT, D.A., FEAVERS, I.M., ACHTMAN, M. & SPRATT, B.G. 1998. Multilocus sequence typing: a portable approach to the

dentification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Science* **95:** 3140-3145

MANIERO, G.D. & CAREY, C. 1997. Changes in selected aspects of immune function in the leopard frog, *Rana pipiens*, associated with exposure to cold. *Journal of Comparative Physiology* **167**: 256-263

MARANTELLI, G., BERGER, L., SPEARE, R. & KEEGAN, L. 2004. Distribution of the amphibian chytrid *Batrachochytrium dendrobatidis* and keratin during tadpole development. Pacific *Conservation Biology* **10:** 173-179

MATSUMOTO, B. 2002. Cell biological applications of confocal microscopy. Methods in Cell Biology, volume 70, New York: Academic Press

MATUTTE, B., STOREY, K.B., KNOOP, F.C. & CONLON, J.M. 2000. Induction of synthesis of an antimicrobial peptide in the skin of the freeze-tolerant frog, *Rana sylvatica* in response to environmental stimuli. *FEBS Letters* **483:** 135-138

MAZZONI, R., CUNNINGHAM, A.A., DASZAK, P., APOLO, A., PERDOMO, E. & SPERANZA, G. 2003. Emerging pathogen of wild amphibians in frogs (*Rana catesbeiana*) farmed for international trade. *Emerging Infectious Diseases* **9(8):** 995-998

MCCALLUM, H. 2005. Inconclusiveness of chytridiomycosis as the agent of widespread frog declines. *Conservation Biology* **19(5):** 1421-1430

MCCALLUM, M.L. 2007. Amphibian Decline or Extinction? Current Declines Dwarf Background Extinction Rate. *Journal of Herpetology* **(41)3:** 483-491

MCDONALD, K. & ALFORD, R.A. 1999. A review of declining frogs in northern Queensland. Campbell A (ed) Declines and disappearances of Australian frogs. *Environment Australia*: 14-22

MCDONALD, K.R., MENDEZ, D., MULLER, R., FREEMAN, A.B. & SPEARE, R. 2005. Decline in the prevalence of chytridiomycosis in frog populations in North Queensland, Australia. *Pacific Conservation Biology* **11**: 114-120

MCLEOD, D.S., SHERIDAN, J.A., JIRAUNGKOORSKUL, W. & KHONSUE, W. 2008. A survey for chytrid fungus in Thai amphibians. *The Raffles Bulletin of Zoology* **56(1):** 199-204

MENDELSON III, J.R., LIPS, K.R., GAGLIARDO, R.W., RABB, G.B., COLLINS, J.P., DIFFENDORFER, J.E., DASZAK, P., IBANEZ, R., ZIPPEL, K.C., LAWSON, D.P., WRIGHT, K.M., STUART, S.N., GASCON, C., DA SILVA, H.R., BURROWS, P.A., JOGLAR, R.L., LA MARCA, E., LOTTORS, S., DU PREEZ, L., WELDON, C., HYATT, A., RODRIGUEZ-MAHECHA, J.V., HUNT, S., ROBERTSON, H., LOCK, B., RAXWORTHY, C.J., FROST, D.R., LACY, R.C., ALFORD, R.A., CAMPBELL, J.A., PARRA-MITCHELL, K.M., CHURCHER, T.S., GARNER, T.W.J. & FISHER, M.C. 2006. Confronting amphibian declines and extinctions. *Science* **313:** 48

MINTER, L.R., BURGER, M., HARRISON, J.A., BRAACK, H.H., BISHOP, P.J. & KLOEPFER, D. 2004. Atlas and red data book of the frogs of South Africa, Lesotho and Swaziland, SI/MAB series No. 9. Smithsonian Institution/Avian Demography Unit, Rondebosch

MITCHELL, K.M., CHURCHER, T.S., GARNER, T.W.J. & FISHER, M.C. 2008. Persistence of the emerging pathogen *Batrachochytrium dendrobatidis* outside the amphibian host greatly increase the probability of host extinction. *Proceedings of the Royal Society Britain* **275:** 329-334

MOREHOUSE, E.A., JAMES, T.Y., GANLEY, A.R., VILGALYS, R., BERGER, L., MURPHY, P.J. & LONGCORE, J.E. 2003. Multilocus sequence typing suggests the chytrid pathogen of amphibians is a recently emerged clone. *Molecular Ecology* **12:** 395-403

MORGAN, J.A.T., VREDENBURG, V.T., RACHOWICZ, L.J., KNAPP, R.A., STICE, M.J., TUNSTALL, T., BINGHAM, R.E., PARKER, J.M., LONGCORE, J.E., MORITZ, C., BRIGGS, C.J. & TAYLOR, J.W. 2007. Population genetics of the frog-killing fungus *Batrachochytrium dendrobatidis*. *PNAS* **104(34):** 13845-13850

MULLER, M. 2002. Introduction to confocal fluorescence microscopy. Maastricht, Netherlands: Shaker

MURPHY, D.B. 2001. Fundamentals of light microscopy and electronic imaging. Wiley-Liss Publication. ISBN 0-471-25391-X

MURPHY, D.B., PISTON, D.W., SHAND, S.H., WATKINS, S.C. & DAVIDSON, M.W. 2008. Theory of confocal microscopy: Spectral bleed-through artifacts in confocal microscopy. Olympus. Available from: http://www.olympusconfocal.com/theory/bleedthrough.html Accessed on 15 May 2008

MUTSCHMANN, F., BERGER, L., ZWART, P. & GAEDICKE, C. 2000. Chytridiomykose bei Amphibien – erstmaliger Nachweis fur Europa. *Berliner und Munchener Tierarzliche Wochenschrift* **113:** 380-383

MUTHS, E., CORN, P.S., PESSIER, A.P. & GREEN, D.E. 2003. Evidence for disease-related amphibian decline in Colorado. *Biological Conservation* **110:** 357-365

NICHOLS, D.K., LAMIRANDE, E.W., PESSIER, A.P. & LONGCORE, J.E. 2001. Experimental transmission of cutaneous chytridiomycosis in dendrobatid frogs. *Journal of Wildlife Diseases* **37(1):** 1-11

NORTH, A.J. 2006. Seeing is believing? A beginner's guide to practical pitfalls in image acquisition. *The Rockefeller University Press, JCB* **172(1):** 9-18. ]

OULLET, M., MIKAELIAN, I., PAULI, B.D., RODRIQUE, J. & GREEN, D.M. 2004. Historical Evidence of Widespread Chytrid Infection in North American Amphibian Population. *Conservation Biology*: 1431-1440.

PADGETT-FLOHR, G.E. & GOBLE, M.E. 2007. Evaluation of tadpole mouthpart depigmentation as a diagnostic test for infection by *Batrachochytrium dendrobatidis* for four California anurans. *Journal of Wildlife Diseases* **43(4):** 690-699

PARKER, J.M., MIKAELIAN, D.V.M., HAHN, N. & DIGGS, H.E. 2002. Clinical diagnosis and treatment of epidermal chytridiomycosis in African Clawed Frogs (*Xenopus tropicalis*). *Comparative Medicine* **52(3):** 266-268

PARRIS, M. J. & CORNELIUS, T.O. 2004. Fungal pathogen causes competitive and developmental stress in larval amphibian communities. *Ecology* **85:** 3385-3395.

PAUZA, M. & DRIESSEN, M. 2008. Distribution and potential spread of amphibian chytrid fungus *Batrachochytrium dendrobatidis* in the Tasmanian Wilderness World Heritage Area. Available from: http://www.dpiw.tas.gov.au/inter.nsf/Attachments/LJEM-7BY3CR/$FILE/Chytrid%20Report%20Feb%202008.pdf Accessed: 14 September 2008

PESSIER, A.P., NICHOLS, D.K., LONGCORE, J.E. & FULLER, M.S. 1999. Cutaneous chytridiomycosis in poison dart frog (*Dendrobates spp.*) and White's tree frogs (*Litoria caerulea). Journal of Veterinary Diagnostic Investigation* **11**:194-1999

PESSIER, A.P. 2002. An overview of amphibian skin disease. *Seminars in Avian and Exotic Pet Medicine* **11(3):** 162-174

PETERMAN, E.J.G., SOSA, H. & MOERNER, W.E. 2004. Single-molecule fluorescence spectroscopy and microscopy of biomolecular motors. *Annual Review of Physical Chemistry* **55:** 79-96

PIOTROWSKI, J.S., ANNIS, S.L. & LONGCORE, J.E. 2004. Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia* **96(1):** 9-15

POULTER, R.T.M., BUSBY, J.N., BISHOP, P.J., BUTLER, M.I. & SPEARE, R. Chloramphenicol cures chytridiomycosis. Available from: http://www.nzfrogs.org/site/nzfrog/files/poulter.pdf. Accessed on 17 November 2008

POUNDS, J.A., FOGDEN, M.P.L. & CAMPBELL, J.H. 1999. Biological response to climate change on a tropical mountain. *Nature* **398**: 611-615

POUNDS, J.A., BUSTAMANTE, M.R., COLOMA, L.A., CONSUEGRA, J.A., FOGDEN, M.P.L., FOSTER, P.N., LA MARCA, E., MASTERS, K.L., MERINO-VITERI, A., PUSCHENDORF, R., RON, S.R., SANCHEZ-AZOFEIFA, G.A., STILL, C.J. & YOUNG, B.E. 2006. Widespread amphibian extinctions form epidemic disease driven by global warming. *Nature* **439(12):** 161-167

PUSCHENDORF, R. & BOLANOS, F. 2006. Detection of *Batrachochytrium dendrobatidis* in *Eleutherodactylus fitzingeri*: effects of skin sample location and histological stain. *Journal of Wildlife Diseases* **42(2):** 301-306

PUSCHENDORF, R., CASTENEDA, F. & MCCRANIE, J.R. 2006a. Chytridiomycosis in wild frogs from Pico Bonito National Park, Honduras. *EcoHealth*: 1-4

PUSCHENDORF, R., BOLANOS, F. & CHAVES, G. 2006b. The amphibian chytrid fungus along an altitudinal transect before the first reported declines in Costa Rica. *Biological Conservation* **132:** 136-142

RåBERG, L., VESTBERG, M., HASSELQUIST, D., HOLMDAHL, R., SVENSSON, E. & NILSSON, J. 2002. Basal metabolic rate and the evolution of the adaptive immune system. *Proceedings of the Royal Society London* **269:** 871-821

RACHOWICZ, L.R. 2002. Mouthpart depigmentation on *Rana muscosa* tadpoles: seasonal changes without chytridiomycosis. *Herpetological Review* **33:** 263-265

RACHOWICZ, L.R. & VREDENBURG, V.T. 2004. Transmission of *Batrachochytrium dendrobatidis* within and between amphibian life stages. *Diseases of Aquatic Organisms* **61**: 75-83

RACHOWICZ, L.R., HERO, J.M., ALFORD, R.A., TAYLOR, J.W., MORGAN, J.A.T., VREDENBURG, V.T., COLLINS, J.P. & BRIGGS, C.J. 2005. The novel and endemic pathogen hypotheses: explanations for the origin of emerging infectious diseases in wildlife. *Conservation Biology*: 1441-1448.

RACHOWICZ, L.J., KNAPP, R.A., MORGAN, J.A.T., STICE, M.J., VREDENBURG, V.T., PARKER, J.M. & BRIGGS, C.J. 2006. Emerging infectious disease as a proximate cause of amphibian mass mortality. *Ecology* **87(7):** 1671-1683

RETALLICK, R.W.R., MCCALLUM, H. & SPEARE, R. 2004. Endemic infection of the amphibian chytrid fungus in a frog community post-decline. *Public Library of Science Biology* **2:** e351

RETALLICK, R.W.R. & MIERA, V. 2007. Strain differences in the amphibian chtrid *Batrachochytrium dendrobatidis* and non-permanent, sub-lethal effects of infection. *Diseases of Aquatic Organisms* **75:** 201-207

ROLLINS-SMITH, L.A. 1998. Metamorphosis and the amphibian immune system. *Immunological Reviews* **166:** 221-230

ROLLINS-SMITH, L.A. 2001. Neuroendocrine-immune system interactions in amphibians – implications for understanding global amphibian declines. *Immunologic Research* **23-2/3**: 273-280

ROLLINS-SMITH, L.A., DOERSAM, J.K., LONGCORE, J.E., TAYLOR, S.K., SHAMBLIN, J.C., CAREY, C. & ZASLOFF, M.A. 2002a. Antimicrobial peptide defenses against pathogens associated with global amphibian declines. *Development and Comparative Immunology* **26(1):**63-72

ROLLINS-SMITH, L.A., CAREY, C., LONGCORE, J., DOERSAM, J.K., BOUTTE, J.E., BRUZGAL, J.E. & CONLON, J.M. 2002b. Activity of antimicrobial skin peptides from ranid frogs against *Batrachochytrium dendrobatidis*, the chytrid fungus associated with global amphibian declines. *Developmental and Comparative Immunology* **26:** 471-479

ROLLINS-SMITH, L.A., REINERT, L.K., MIERA, V. & CONLON, J.M. 2002c. Antimicrobial peptide defences of the Tarahumara frog, *Rana tarahumarae*. *Biochemical and Biophysical Research Communications* **297:** 361-367

ROLLINS-SMITH, L.A., CAREY, C., CONLON, J.M., REINERT, L.K., DOERSAM, J.K., BERGMAN, T., SILBERRING, J., LANKINEN, H. & WADE, D. 2003. Activities of Temporin family peptides against the chytrid fungus (*Batrachochytrium dendrobatidis*) associated with global amphibian declines. *Antimicrobial Agents and Chemotherapy* **47(3):** 1157-1160

ROLLINS-SMITH, L.A., REINERT, L.K., O'LEARY, C.J., HOUSTON, L.E. & WOODHAMS, D.C. 2005. Antimicrobial peptide defences in Amphibian Skin. *Integrated Competitive Biology* **45:** 137-142

RON, S.R. 2005. Predicting the distribution of the amphibian pathogen *Batrachochytrium dendrobatidis* in the New World. *Biotropica* **37(2):** 209-221

ROSENBERG, E., KOREN, O., RESHEF, L., EFRONY, R. & ZILBER-ROSENBERG, I. 2007. The role of microorganisms in coral health, disease and evolution. *Natural Review of Microbiology* **5:** 355-362

ROSENBLUM, E.B., STAJICH, J.E., MADDOX, N. & EISEN, M.B. 2008. Global gene expression profiles for life stages of the deadly amphibian pathogen *Batrachochytrium dendrobatidis*. *PNAS* **105(44):** 17034-17039

ROWLEY, J.J.L. & ALFORD, R.A. 2007. Behaviour of Australian rainforest stream frogs may affect the transmission of chytridiomycosis. *Diseases of Aquatic Organisms* **77:** 1-9

SCHOEGEL, L.M., HERO, J.M., BERGER, L., SPEARE, R., MCDONALD, K. & DASZAK, P. 2006. The decline of the sharp-snouted day frog (*Taudactylus acutirostris*): the first documented case of extinction by infection in a free-ranging wildlife species. *EcoHealth* **3:** 35-40

SIMMACO, M., BOMAN, A., MANGONI, M.L., MIGNOGNA, G., MIELE, R., BARRA, D. & BOMAN, H.G. 1997. Effect of glucocorticoids on the synthesis of antimicrobial peptides in amphibian skin. *FEBS Letters* **416:** 273-275

SKERRATT, L.F., BERGER, L., SPEARE, R., CASHINS, S., MCDONALD, K.R., PHILLOT, A.D., HINES, H.B. & KENYON, N. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth* DOI: 10.1007/s10393-007-0093-5

SMITH, K.F., SAX, D.F. & LAFFERTY, K.D. 2006. Evidence for the role of infectious disease in species extinction and endangerment. *Conservation Biology* **20(5):** 1349-1357

SMITH, K.G., WELDON, C., CONRADIE, W. & DU PREEZ, L.H. 2007. Relationship among size, development, and *Batrachochytrium dendrobatidis* infection in African tadpoles. *Diseases of Aquatic Organisms* **74(2):** 159-164

SPARROW, F.K. 1960. Aquatic phycomycetes. Ann Arbor: University of Michigan.

SPEAR, R.N., CULLEN, D. & ANDREWS, J.H. 1999. Fluorescent labels, confocal microscopy and quantitative image analysis in study of fungal biology. *Methods in Enzymology* **307:** 607-623

SPEARE, R., CORE WORKING GROUP OF GETTING THE JUMP ON AMPHIBIAN DISEASES. 2001. Developing management strategies to control amphibian diseases: decreasing the risks due to communicable diseases. Townsville, Australia: School of Public Health and Tropical Medicine, James Cook University, Australia. Available from www.jcu.edu.au/school/phtm/PHTM/frogs/ampdis.htm

SPEARE, R. 2006. Threat abatement plan – infection of amphibians with chytrid fungus resulting in chytridiomycosis – Background document. Department of Environment and Heritage

STAGNI, G., SCOCCIANTI, C. & FUSINI, R. 2002. Segnalazione di chytridiomicosi in popolazioni di *Bombina pachypus* (Anura, Bombinatoridae) dell'Appennino Tosco-emiliano. Proceedings of the Fourth Congress of Societas Herpetologica Italica. Ercolano, Italy, June 18 to 22

TOLEDO, L.F., BRITTO, F.B., ARAUJO, O.G.S., GIASSON, L.M.O. & HADDAN, C.F.B. 2006. The occurrence of *Batrachochytrium dendrobatidis* in Brazil and the inclusion of 17 new cases of infection. *South American Journal of Herpetology* **1(3):** 185-191

ULETT, G.C., CURRIE, B.J., CLAIR, T.W., MAYO, M., KETHEESAN, N., LABROOY, J., GAL, D., NORTON, R., ASHHURST SMITH, C., BARNES, J., WARNER, J. & HIRST, R.G. 2001. *Burkholderia pseudomallei* virulence: definition, stability and association with clonality. *Microbes and Infection* **3(8):** 621-631

URWIN, R. & MAIDEN, M.C.J. 2003. Multi-locus sequence typing: a tool for global epidemiology. *Trends in Microbiology* **11(10):** 479-487

VANCOMPERNOLLE, S.E., TAYLOR, R.J., OSWALD-RICHTER, K., JIANG, J., YOUREE, B.E., BOWIE, J.H., TYLER, M.J., CONLON, J.M., WADE, D., AIKEN, C., DERMODY, T.S., KEWALRAMINI, V.N., ROLLINS-SMITH, L.A. & UNUTMAZ, D. 2005. Antimicrobial peptides from amphibian skin potently inhibit human immunodeficiency virus infection and transfer of virus from dendritic cells to T cells. *Journal of Virology* **79(18):** 11598-11606

VOYLES, J., BERGER, L., YOUNG, S., SPEARE, R., WEBB, R., WARNER, J., RUDD, D., CAMPBELL, R. & SKERRATT, L.F. 2007. Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis. *Diseases of Aquatic Organisms* **77:** 113-118

VREDENBURG, V.T. & SUMMERS, A.P. 2001. Field identification of Chytridimycosis in *Rana muscosa*. *Herpetological Review* **32(3):** 151-152

WADE, D., SILVEIRA, A., ROLLINS-SMITH, L.A., BERGMAN, T., SILBERRING, J. & LANKINEN, H. 2001. Hematological and antifungal properties of temporin A and a cecropin A-temporin A hybrid. *Acta Biochemica Polonica* **48(4):** 1185-1189

WALDMAN, B. & VAN DE WOLFSHAAR, K.E. 2001. Chytridiomycosis in New Zealand frogs. *Surveillance* **28(3):** 9-11

WALKER, S.F., BALDI SALAS, M., JENKINS, D., GARNER, T.W.J., CUNNINGHAM, A.A., HYATT, A.D., BOSCH, J. & FISHER, M.C. 2007. Environmental detection of *Batrachochytrium dendrobatidis* in a temperate climate. *Diseases of Aquatic Organisms* **77:** 105-112

WEBB, R., BERGER, L., MENDEZ, D. & SPEARE, R. 2005. MS-222 (tricaine methane sulfonate) does not kill the amphibian chytrid fungus *Batrachochytrium dendrobatidis. Diseases of Aquatic Organisms* **68:** 89-90

WEBB, R., MENDEZ, D., BERGER, L. & SPEARE, R. 2007. Additional disinfectants effective against the amphibian chytrid fungus *Batrachochytrium dendrobatidis. Diseases of Aquatic Organisms* **74:** 13-16

WELDON, C. & DU PREEZ, L.H. 2004. Decline in the Kihansi spray toad, *Nectophrynoides asperginis,* from the Udzungwa Mountain, Tanzania. *Froglog* **62**: 2-3

WELDON, C., DU PREEZ, L.H., HYATT, A.D., MULLER, R. & SPEARE, R. 2004. Origin of the amphibian chytrid fungus. Available from: http://www.cdc.gov/ncidod/EID/vol10no12/03-0804.htm

WELDON, C. 2005. Chytridiomycosis, an emerging infectious disease of amphibians in South Africa. Thesis submitted for the degree Doctor of Philosophy in Zoology at the North-West University. Unpublished.

WELDON C. & DU PREEZ L.H. 2006. Quantitative measurement of *Batrachochytrium dendrobatidis* in amphibian skin. *Diseases of Aquatic Organisms* **72(2):** 153-161.

WELDON, C., DU PREEZ, L.H. & VENCES, M. 2008. Lack of detection of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) in Madagascar. *Monografie del Museo Rigionale di Scienze Naturali di Torino, XLV*: 95-106

WHITE, A.W. 2006. A trial using salt to protect green and golden bell frogs from chytrid infection. *Herpetofauna* **36(2):** 93-96

WILLIAMS, S.E. & HERO, J.M. 1998. Rainforest frogs of the Australian Wet Tropics: guild classification and the ecological similarity of declining species. *Proceedings of the Royal Society of London* **265:** 597-602

WOODHAMS, D.C., ALFORD, R.A. & MARANTELLI, G. 2003. Emerging disease of amphibians cured by elevated body temperature. *Diseases of Aquatic Organisms* **55**: 65-67

WOODHAMS, D.C. & ALFORD, R.A. 2005. Ecology of Chytridiomycosis in rainforest stream frog assemblages of Tropical Queensland. *Conservation Biology:* 1449-1459

WOODHAMS, D.C., VOYLES, J., LIPS, K.R., CAREY, C. & ROLLINS-SMITH, L.A. 2006a. Predicted disease susceptibility in a Panamanian amphibian assemblage based on skin peptide defenses. *Journal of Wildlife Diseases* **42(2):** 207-218

WOODHAMS, D.C., ROLLINS-SMITH, L.A., CAREY, C., REINERT, L., TYLER, M.J. & ALFORD, R.A. 2006b. Population trends associated with skin peptide defenses against chytridiomycosis in Australian frogs. *Oecologia* **146:** 531-540

WOODHAMS, D.C., ROLLINS-SMITH, L.A., ALFORD, R.A., SIMON, M.A. & HARRIS, R.N. 2007a. Innate immune defenses of amphibian skin: antimicrobial peptides and more. *Animal Conservation* **10:** 425-428

WOODHAMS, D.C., ARDIPRADJA, K., ALFORD, R.A., MARANTELLI, G., REINERT, L.K. & ROLLINS-SMITH, L.A. 2007b. Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defences. *Animal Conservation* **10:** 409-417

WOODHAMS, D.C., VREDENBURG, V.T., SIMON, M.A., BILLHEIMER, D., SHAKHTOUR, B., SHYR, Y., BRIGGS, C.J., ROLLINS-SMITH, L.A. & HARRIS,

R.N. 2007c. Symbiotic bacteria contribute to innate immune defences of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biological Conservation* **138:** 390-398

WOODHAMS, D.C., ALFORD, R.A., BRIGGS, C.J., JOHNSON, M. & ROLLINS-SMITH, L.A. 2008. Life-history trade-offs influence disease in changing climates: strategies of an amphibian pathogen. *Ecology* **9(6):** 1627-1639

ZASLOFF, M. 2002. Antimicrobial peptides of multicellular organisms. *Nature* 415: 389-395