Efficacy of F10 against amphibian chytrid fungus

MS de Jong
21108838

Dissertation submitted in fulfilment of the requirements for the degree *Magister Scientiae* in Environmental Sciences at the Potchefstroom Campus of the North-West University

Supervisor: Prof C Weldon

November 2014

It all starts here ™

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT

To admit that species generally become rare before they become extinct, to feel no surprise at the rarity of the species, and yet to marvel greatly when the species ceases to exist, is much the same as to admit that sickness in the individual is the forerunner of death – to feel no surprise at sickness, but, when the sick man dies, to wonder and to suspect he died of some deed of violence – **(Charles Darwin, The Origin of Species, Chapter 11)**

I would like to dedicate this dissertation to my loving and caring parents Braam and Miekie de Jong. You taught me the value of life and how to care not just for those around me, but for my surroundings as well. You bred a deep love and respect for nature in all of us and I thank you for that. You are my rock,
Now and Forever

ABSTRACT

Efficacy of F10 against amphibian chytrid fungus

Outbreaks of pathogens that threaten both human and nature have increased in recent years. Infectious and transmittable diseases, such as chytridiomycosis, which is caused by the emerging pathogen *Batrachochytrium dendrobatidis*, has been identified as one of the most important drivers of the current declines in amphibian numbers. This pathogen has spread globally and is not only responsible for the declines in amphibian population numbers, but also for the extinction of species in several countries. As part of the Amphibian Conservation Action Plan, the IUCN recommended *ex situ* breeding of amphibian species to try and stem the global loss of amphibian species. Due to chytridiomycosis being one of the most eminent threats for amphibians, it poses an additional threat for the *ex situ* breeding plan. There is thus a need for safe and effective measures to treat chytridiomycosis, especially in breeding programs for endangered species. F10 (Health and Hygiene) is a veterinary antiseptic that has shown to be 100% effective in killing *B. Dendrobatidis in vitro*. Before any chemical treatment can be applied the efficacy and toxicity of F10 has to be determined to establish if F10 can be effectively applied across different amphibian species and across different life stages. We propose to develop a treatment protocol for F10 for the effective treatment of amphibian chytridiomycosis by challenging juveniles of *Amietophrynus gutturalis* with *B. dendrobatidis* and subsequently treating the infection with a proposed concentration of F10. The survival of *B. dendrobatidis* zoospores was also determined in the presence of F10. The results obtained showed survival of tadpoles at a 1:10,000 concentration of F10 for 30min, and juveniles at a concentration of 1:2000 for 15 min. Furthermore the *in vitro* tests showed that the *B. dendrobatidis* zoospores died after 10 min at a 1:10,000 concentration and 30 min at a 1:15,000 concentration. The successful treatment of tadpoles as well as juveniles will increase any species chance for survival, especially when treating tadpoles as the pathogen will then be eradicated before the tadpole metamorphoses and reaches the disease-susceptible life stage. By establishing a partnership between the industry, academic and zoo/wildlife communities we hope to maximise the likelihood of implementing this program in the future and thus ensuring long term sustainability.

Keywords: Anticeptic treatment, chytrid fungus, F10, *Amietophrynus gutturalis*

OPSOMMING

Effektiwiteit van die produk F10 teen die amfibieër chytrid fungus

Uitbrake van patogene wat beide die mens en natuur bedreig het die afgelope paar jaar toegeneem. Oordraagbare en aansteeklike siektes, veral chytridiomikose, wat veroorsaak word deur die patogeen, *Batrachochytrium dendrobatidis*, is geïdentifiseer as een van die mees belangrikste drywers in die afname van amfibieër getalle. Die patogeen is regoor die wêreld versprei en nie net verantwoordelik vir die afname in amfibieër populasie getalle nie, maar ook die uitsterwing van spesies in verskeie lande. In 2005 het die IUCN *ex situ* teling van amfibieër spesies aanbeveel as deel van die "Amphibian Conservation Action Plan" om die globale verlies van amfibieër spesies te stuit. Weens die feit dat chytridiomikose een van die vooraanstaande bedreigings vir amfibieë wêreldwyd is, hou dit 'n bykomende uitdaging vir die *ex situ* bewarings plan in. Dus is daar 'n leemte vir doeltreffende en veilige behandeling van chytridiomikose in amfibieë, veral die in aanhoudingsprogramme vir bedreigde spesies. F10 (Health and Hygiene) is 'n veeartsenykundige antiseptiese middel wat 100% effektiwiteit toon in die behandeling van *in vitro* chytrid kulture. Voordat enige chemiese behandeling toegepas kan word, moet daar eers bepaal word wat F10 se doeltreffendheid is, aangesien daar tans geen behandeling is wat effektief aangewend kan word oor verskillende spesies en verskillende lewens stadiums nie. Ons beplan dus om 'n effektiewe behandelingsprotokol vir die toediening van F10 te ontwikkel deur metaworwe van *Amietophrynus gutturalis* te infekteer met *B. dendrobatidis* en gevolglik te behandel met 'n voorgestelde konsentrasie van F10. Die oorlewing van *B. dendrobatidis* zoospore in die teenwoordigheid van F10 is ook bepaal. Daar is bevind dat paddavisse oorleef by 'n 1:10,000 konsentrasie F10 vir 30 min, en die metamorwe by 'n konsentrasie van 1:2000 vir 15 min. Verder het die *in vitro* toetse getoon dat *B. dendrobatidis* zoospore by 'n 1:10 000 verdunning reeds sterf na 10 min en by 1:15,000 na 30 min. Die suksesvolle behandeling van paddavisse sowel as metamorwe sal dus die paddaspesie se kans op oorlewing aansienlik verhoog, veral in die geval by behandelde paddavisse aangesien die patogeen gedood sal wees voor die paddavis metamorfeer en in die siekte-vatbare lewensstadium in beweeg.Deur 'n vennootskap tussen die industrie, akademie en dieretuin gemeenskappe te vestig, beoog ons om die waarskynlikheid van die program

se implementering in die praktyk te maksimaliseer, en dus langtermyn volhoubaarheid te verseker.

Sleutelwoorde: Antiseptiese behandeling, chytrid fungus, F10, *Amietophrynus gutturalis*

ACKNOWLEDGEMENTS

A small ancient amphibious soul.
Webbed feet, slimy mucous skin,
Eyes sometimes red, green or even gold
A creature now endangered, or so I've been told
Without him, a world sterile, archaic and utterly cold
The frog: A small ancient amphibious soul
~Joseph Calamia

****

I would like to thank the following persons and institutions for their help during my research project:

The National Research Foundation (NRF) for the financial assistance supplied towards this research, grant number UID 89774. Thank you very much for your help. *Opinions expressed and conclusions arrived at, are those of me (the author) and are not necessarily to be attributed to the NRF.*

My wonderful parents for their continued support throughout my education. Thank you for always trusting me to make the right decisions for me, and for always believing in me no matter what.

I would also like to thank my supervisor, Prof. Ché Weldon for his continued support, patience, advice and long hours of hard work during this study. Thank you for always being available for any and all questions. Without your guidance this project would still only be an idea and not a reality. Thank you!

Then a special thanks to the following people:

- Dr. Trenton Garner, thank you for teaching me to "push the boundaries" and for the vote of confidence in times where I doubted myself. Thank you for trusting me to assist you in your research projects, I learned valuable skills during that time which was of great use during my own research.

- Dr Desire Dalton, Clearance and all the other people from the molecular laboratory at the National Zoological Gardens in Pretoria whom assisted in PCR training and DNA extraction.
- Dr Jaco Bezuidenhout for his expertise in Statistica.
- Health and Hygiene for providing all the necessary F10 to do this project.
- The following friends and colleagues:
  - Maxi Snyman
  - Roelien van Vuuren
  - Jessica de Bruyn-Smith
  - Jaco Odendaal
  - Leon Meyer

CONTENTS

LIST OF TABLES AND FIGURES

TABLES

FIGURES

CHAPTER OUTLAY

Chapter 1 contains an overview of some of the general reasons behind the recent amphibian declines. It focuses on the background and biology of the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, and assesses the strengths and limitations of current mitigation measures. Then the aim and objectives of this study follows along with the chapter layout.

Chapter 2 describes the materials and methods used during this study, including the culturing of *Batrachochytrium dendrobatidis,* husbandry of and infection of tadpoles and juveniles with *B. dendrobatidis*, how to prepare and obtain swabs, wet mounds and whole frog tissues and how to treat juveniles with F10.

Chapter 3 outlines the results obtained from the above mentioned experiments. The zoospore density and number of doses needed to successfully infect amphibians with *Batrachochytrium dendrobatidis* is indicated as well as the concentration of F10 needed to kill *B. dendrobatidis in vitro*. The qPCR results show the effectiveness of treating infected amphibians with F10.

Chapter 4 discusses the results and findings obtained from all the results as well as our recommendations on how to obtain the best results when treating juveniles with F10. We further discuss the need for an effective treatment against *Batrachochytrium dendrobatidis* and why we chose to test F10.

Chapter 5 contains a summary of the entire study along with the conclusions drawn from our results and suggestions regarding possible improvements in terms of this study for any future research.

CHAPTER 1

Introduction and Literature study

1.1 Importance of amphibians

Amphibians are most commonly known as ectothermic vertebrates that are divided into three Orders namely: Anura (frogs and toads), Caudata (salamanders and newts), and Gymnophiona (caecilians) (Duellman and Trueb, 1994). Amphibians owe their name to the Greek word “amphibious” which, when translated, means “double life”. This is due to the fact that most amphibians undergo metamorphosis, starting off as aquatic larva (tadpoles) and metamorphosing into terrestrial juveniles. Granting not all amphibian species abide entirely to this pattern, they still remain the lone class of vertebrates with this unique adaptation (Zug *et al.*, 2001; O'Rourke, 2007). According to the fossil record, the first evidence of amphibians appears from the Devonian period around 350 million years ago (Duellman and Trueb, 1994). Currently there are more than 6000 recognized species of amphibians living in almost all terrestrial and fresh water habitats, ranging from below sea level to altitudes above 5000m, which makes them fascinating subjects for scientific research (Blaustein and Wake, 1995; Stuart *et al.*, 2008).

Amphibians have always been a popular group to use in the study of developmental and physiological processes and naturally also as the primary subjects for herpetological studies. Much of what we know today about the basic knowledge of vertebrate embryology came from studying amphibian shell-less eggs. Knowledge about the actions of many hormones such as pituitary and thyroid hormones were obtained through endocrinological studies on amphibians (Duellman and Trueb, 1994; Blaustein and Wake, 1995).

Amphibians have become increasingly important role players in conservation ecology, partly because they are reliable indicators of ecosystem health in a world where global pollution increases on a daily basis. This is largely because the skin of amphibians differs radically from that of most vertebrates in the sense that amphibian skin is highly permeable and thus directly involved in a number of very important physiological processes such as osmoregulation and in some cases even helps with respiration (Blaustein and Wake, 1995). Their skin consists of a very thin layer of epithelial tissue

with minimum keratinisation and lacks hair or feathers for protection (Blaustein *et al.*, 1994), which makes them particularly sensitive to most environmental disturbances and toxins (Duellman and Trueb, 1994), and thus the perfect indicators for any environmental degradation and community instability (Warkentin *et al.*, 2009). Furthermore amphibians also form an integral part of any ecosystem as they serve as predators as well as prey for a number of animals both aquatic and terrestrial (Kats *et al*., 1988; Menin *et al*., 2005; Han *et al*., 2011).

1.2 Amphibian declines

The First World Congress of Herpetology in 1989 sparked the initial interest and concern in the possibility that amphibian declines could be a global phenomenon. According to Soulé (1986) “A good field biologist is often more concerned with what is missing in a situation than with what is present”, and presently a great deal of amphibian species are missing. When compared to the fossil record the global extinction rates for plants and animals are estimated to be over 100 to 1000 times higher than before humans became a part of the ecosystem and subsequently increased the amount of poisonous substances, pollution and toxins that are released into nature (Chapin III *et al.*, 1997; Vitousek *et al.*, 1997; Balmford *et al.*, 2003; Baillie *et al.*, 2004; Cushman, 2006). It is evident that Earth is indeed facing the biggest mass extinction recorded in over 65 million years (Vitousek *et al.*, 1997; Cushman, 2006). More concerning is the fact that of all the vertebrate taxa that are currently disappearing at alarmingly high rates, amphibians have been identified as the group facing the highest percentage of species threatened with extinction (Baillie *et al*., 2004; Stuart *et al.*, 2004; Beebee and Griffiths, 2005). Furthermore, the global decline of amphibian population numbers can be considered one of the most urgent and enigmatic environmental problems of the century (Daszak *et al.*, 1999). Amphibian species numbers are declining worldwide at a rate higher than that of birds and mammals with 32.5% of the world’s amphibian species already threatened, an additional 34 species extinct and 88 possibly extinct (Lips *et al.*, 2006; Gascon *et al.*, 2007). Amphibians are a very diverse class that had the ability to exist for many centuries, but are now teetering on the brink of extinction. Recent studies have shown that these declines can be attributed to a number of factors including loss of habitat, pollution, climate change, increased exposure to ultraviolet radiation and the introduction of pathogens (Alford and Richards, 1999; Stuart *et al.*, 2004; O'Rourke, 2007).

1.2.1. Habitat modification

With the start of intensive farming in the mid-20th century much of the natural habitat was destroyed, which led to the reduction of many species including amphibians (Kolozsvary and Swihart, 1999; Beebee and Griffiths, 2005). Consequently, habitat modification is one of the major and most well-known reasons for amphibian declines, which results in drastic reduction in amphibian diversity and abundance in areas that are directly affected (Hecnar and M'Closkey, 1996b, 1997). Deforestation, clear-cutting or even just the removal of some of the vegetation in a habitat can have a severe impact on the local amphibian population (Kolozsvary and Swihart, 1999). Reasons for this include desiccation due to exposure, soil compaction, reduction in habitat intricacy, drastically altered microclimates and amplified siltation for aquatic amphibians and life stages (Welsh, 1990; Ash, 1997).

The exclusion of breeding sites by draining wetlands directly influences amphibian population numbers (Elmberg, 1993; Semlitsch and Bodie, 1998) and thus increases the chances of population extinction in that region. Furthermore, urban development changes terrestrial and aquatic habitats and decreases or eliminates amphibian populations (Delis *et al.*, 1996). Another by-product of habitat destruction or modification is the change in vegetation structure, with the increase or decrease of shade, which could have detrimental effects on the local amphibian population.

Species that don't possess limited mobility, require large areas of forest or wetland or have less specific requirements for breeding are less likely to be affected by habitat destruction or habitat fragmentation, whereas species that do will likely lose huge numbers of their populations (Kolozsvary and Swihart, 1999). Public roads along with road traffic also have huge impacts on amphibian numbers especially during the breeding season when species have to migrate to their breeding sites. This can be because they actually get killed by traffic or due to their habitat being fragmented by either the traffic or the road (Hels and Buchwald, 2001).

1.2.2. Acidity and Toxicants

Amphibian reproduction, rate of death, dispersal and abundance as well as egg development and larval growth are hugely influenced by the acidity of their aquatic habitats (Freda and Dunson, 1986; Freda *et al.*, 1991; Alford and Richards, 1999). The level of influence is determined by the sensitivity of the species, which differs between and within each species (Freda *et al.*, 1991). The mortality rate depends on the number of deaths taking place in both the embryonic and larval stages of the amphibians due to incomplete/partial absorption of the yolk plug, failure of the vitelline membrane to expand, deformation of larvae and arrested development (Freda *et al.*, 1991; Beattie and Tyler-Jones, 1992; Horne and Dunson, 1994). Additional side effects of acidification include direct non-lethal effects such as: belated or premature hatching, (Bradford *et al.*, 1992; Horne and Dunson, 1994), abnormal swimming behaviour (Andrén *et al.*, 1988) and retarded growth rates due to reduced capturing of prey that results in reduced larval body size (Bradford *et al.*, 1992). The indirect non-lethal side effects include: alterations to tadpole food sources (e.g. algae populations) (Vertucci and Corn, 1996) as well as changes in the predator-prey relationships due to sudden deaths of certain invertebrates and predatory fish (Henrikson, 1990) or due to cations such as Sodium (Na), Magnesium (Mg), Aluminium (Al) and Calcium (Ca), and other unknown compounds (Freda and Dunson, 1986).

Amphibian diversity, distribution and density tends to be lower in acidic soil as well as in breeding sites with lower pH levels (Wyman and Hawksley-Lescault, 1987; Wyman and Jancola, 1992). In spite of all the well documented side effects of acidic environments on amphibians, these data still don't implicate acidification in the sudden unexplainable population declines of recent years (Harte and Hoffman, 1989; Bradford *et al.*, 1992). However, amphibians are still killed by environmental toxins, be it directly or indirectly by altering reproduction, growth rates or even by increasing their susceptibility to diseases (Alford and Richards, 1999).

1.2.3. Chemical pollution

With farming increasing due to human population growth, the amount of habitat obliteration along with the use of harmful and sometimes lethal chemicals and fertilizers also increased. But unlike habitat destruction, the full extent of the effects that chemicals and fertilizers have on amphibian species is not yet known (Collins and Storfer, 2003). However, pesticides have long been suspected as one of the reasons behind amphibian declines (Carey and Bryant, 1995; Lips, 1998), especially with the recent growing evidence that links the use of pesticides, herbicides and insecticides with negative side-effects/alterations in amphibians regarding growth, reproduction, development and behaviour (Carey and Bryant, 1995).

There is evidence that indicates how certain species of amphibians are declining due to windborne pesticides associated with upwind farming and agricultural land use (Davidson, 2004). Croplands and agricultural land receive large amounts of chemicals (pesticides and herbicides) intended to kill/control fungal infections, weeds and pests. However, these chemicals tend to have a negative effect on more than just weeds, pests and funguses (Mann *et al.*, 2009). One of the problems with agricultural land use is that farmers tend to use broad-based pesticides that affect a variety of organisms instead of just the intended culprit, thus leading to non-target effects such as killing amphibians and other unintended vertebrates.

Since amphibians possess an extremely permeable skin to maintain electrolyte balance they are more likely to be susceptible to pesticides, be it airborne or waterborne. Dermal uptake of chemicals is inevitable for amphibians especially since streams, ponds and temporary pools often receive pesticides as a result of accidental pollution due to agricultural run-off or spray drift (Relyea *et al.*, 2005) causing huge concern as amphibians spend a large percentage of their life-cycle in water. Furthermore their life cycle also includes a terrestrial phase which ultimately exposes them to toxicants in two environments (Todd *et al.*, 2011; Fryday and Thompson, 2012). Amphibians inhabiting these areas are in danger of suffering toxic side-effects due to herbicides and pesticides present in the water as well as those on land (Mann *et al.*, 2009). Another problem is that most amphibian species tend to migrate and breed in the summer, which coincides with agricultural pesticide applications, and thus get exposed to a number of pesticides intentionally applied to orchards or fields (Becker *et al.*, 2007).

Effects suffered by amphibians are not restricted to specific pesticides or only active substances, but also formulation additives in pesticides, herbicides, insecticides and fungicides. Granting that crops reduce exposure through interception, the recurrent application of pesticides during growing seasons causes amphibians to be exposed on more than one occasion to multiple products (Brühl *et al.*, 2013). Although some pesticides possess the ability to cause direct effects on amphibians by causing mortalities, most pesticides occur in nature at very diluted concentrations and that doesn't lead to immediate wide scale mortalities in amphibian populations (Davidson, 2004; Mann *et al.*, 2009). However, toxicities observed in amphibians for certain pesticides include manifestations such as abnormal sexual development, limb abnormalities, endocrine disruption, abnormal growth and abnormal time to metamorphosis which can ultimately lead to the indirect effect of population mortalities (Mann *et al.*, 2009). Numerous times direct lethal effects are only visible in certain species or certain life stages. For example, malathion has little direct effects on tadpoles (in the absence of predators) but shows more prominent direct lethal effects for adult toads. However, other pesticides have the opposite effect on amphibians by mostly effecting tadpoles (reducing survival as well as biomass) (Relyea *et al.*, 2005).

The toxicity of contaminants can increase or decrease when certain stressors, be it abiotic (temperature, ultraviolet radiation and pH) or biotic (chemical cues emitted by predators, increased competition, low food availability) are present (Relyea and Mills, 2001; Relyea, 2003, 2004; Relyea and Hoverman, 2006; Jones *et al.*, 2011). Commercial formulations of glyphosate have the potential to be moderately or very toxic to tadpoles under the right set of environmental conditions, especially when predators are present (Baylis, 2000; Relyea *et al.*, 2005; Bernal *et al.*, 2009; Relyea and Jones, 2009). Most of the glyphosate-based products available today contain a surfactant usually polyethoxylated tallowamine (POEA), which is lethal to amphibians and fish especially when the environmental pH is unstable or when chemical cues from predators are present (Jones *et al.*, 2011). However, the concentrations of herbicides present in nature and particularly in wetlands depends on whether the application was accidental such as forest application or as a result of run-off, spray-drift, plant wash off or soil run-off (Thompson *et al.*, 2004). The recent decline in amphibians is also thought to be due to a decrease in their immune response as a result of increased anthropogenic stressors (agrochemicals) in their environment, which makes them more susceptible to diseases and infections (Carey *et al.*, 1999; Forson and Storfer, 2006).

1.2.4. Climate change

Evidence shows that amphibian populations might be negatively impacted by the recent global climate changes. Over the past 100 years the mean global temperature has increased by approximately 0.6°C on average, with further evidence that this increase has numerous effects on wildlife and subsequently any and all ecosystems (Parmesan and Yohe, 2003). This is vividly illustrated by the fate of the golden toads, *Incilius periglenes*, of Costa Rica. Immediately following the lowest rainfall in 20 years, the golden toads disappeared at the end of the 1980's and have not been found since. This was as a result of the toads being forced to change their habitat usage due to the extremely low rainfall (Pounds and Crump, 1994). Furthermore the dry circumstances could have also triggered unknown interactions with unidentified factors such as diseases or contaminants in cloud water that eradicated toad populations (Pounds and Crump, 1994; Pounds *et al.*, 1997; Beebee and Griffiths, 2005).

Amphibian population dynamics are also changed by severe sudden climatic events such as erupting volcanoes, floods and violent storms. These altered environments can cause certain species to decrease in numbers while others increase (Woolbright, 1996). Predation rates may also be altered which could disadvantage certain species within a community (Parmesan and Yohe, 2003). Warmer sea surface temperatures have also led to a thermal uplift in the atmosphere resulting in higher cloud formation, which then leads to drier terrestrial habitats, causing amphibians to reduce their breeding activities (Pounds *et al.*, 1999).

Changes in weather patterns and conditions due to climate change also changes behavioural patterns of certain species of amphibians. Breeding behaviour as well as breeding sites are forced to change due to changing weather and shifts in air and water temperatures, thus phonological and distribution/abundance shifts may take place (Terhivuo, 1988; Parmesan and Yohe, 2003; Beebee and Griffiths, 2005). Breeding ponds may fill later or sooner and persist for shorter or longer periods of time, thus altering amphibian distribution as well as their population numbers. Amphibians exposed to these new stresses often become more vulnerable to parasites and diseases (Donnelly and Crump, 1998).

1.2.5. Ultraviolet radiation

The catalytic destruction and thinning of Earth's ozone layer (stratospheric ozone) due to increased and continued use of greenhouse gasses, such as chlorofluorocarbons, has led to an increase in ultraviolet B (UV-B) radiation (Kerr and McElroy, 1993), which in turn causes huge amounts of damage to amphibian embryos and results in population declines (Alford and Richards, 1999). The levels of the photo reactivating DNA repair enzyme (photolyase), which is responsible for repairing UV-B damage to DNA, differs between species of amphibians (Blaustein *et al.*, 1994). According to Blaustein *et al.* (1994), field experiments showed that species with higher levels of photolyase are less inclined to suffer from population declines than species with lower levels of photolyase. Photolyase can be found in many organisms as it is a very important enzyme for repairing cytogenic and mutagenic photoproducts in DNA. Other studies have also shown that embryo mortality or lower hatching success can be attributed to enhanced ambient or UV-B radiation (Blaustein *et al.*, 1998). Even if UV-B radiation has a negligible effect on embryo survival numbers the potential indirect effects such as, changes in food supplies, water chemistry and shifts in predator-prey relationships, will affect embryos as well as adult amphibian population numbers (Alford and Richards, 1999). Other negative side effects due to increased exposure to UV-B radiation in adult amphibians include an increase in tumours, cancers, damaged eyes and immunosuppressant, which ultimately leads to a reduction in their survival rates (Fite *et al.*, 1998)

1.2.6. Predation

The distribution and population dynamics of amphibians are greatly influenced by both abiotic and biotic factors which include interactions between amphibians and other organisms or even among amphibians themselves (Alford, 1999). Vertebrate and invertebrate predators (spiders, otters, birds, snakes etc.) pose a great threat to juveniles/larval amphibians and habitually influence the abundance and diversity of aquatic amphibian groupings along with community structures (Kats *et al*., 1988; Hecnar and M'Closkey, 1996a; Alford, 1999; Menin *et al*., 2005). Usually amphibians that coexist alongside predators have developed anti-predator mechanisms over the years such as: toxic or noxious secretions (chemical repellents); chemical cues to detect predators; ability to escape capture; cryptic coloration; size preference and an inherit avoidance response to certain predators (shift in diet activity response) (Formanowicz and Brodie, 1982; Brodie and Formanowicz, 1987; Kats *et al.*, 1988; Williams *et al.*, 2000). However,

when native populations of amphibians are suddenly introduced to new predators (especially predatory fish) they are unable to survive, because they have not yet developed the mechanisms to do so. This ultimately leads to an increase in mortality within populations or even local population extirpation (Gamradt and Kats, 1996; Kiesecker and Blaustein, 1997). More subtle effects such as preventing colonization of new ponds resulting from migration may also occur if surrounding ponds contain predatory fish (Bradford *et al.*, 1993). There is also the exploitation for human consumption which has decimated certain frog populations as a result of the frog-leg trade, especially over the past 20 years. Frog legs as a food source can be found from school cafeterias in France to cuisine restaurants and even dinner tables in Southeast Asia (Warkentin *et al.*, 2009).

1.2.7. Diseases

Evidence exists that diseases may have contributed significantly to mass mortalities in some declining amphibian species and regions to a degree never witnessed before (Cunningham *et al.*, 1996; Berger *et al.*, 1998; Daszak *et al.*, 2003).

Macro-parasites: Pathogens and parasites have the ability to alter the behavior, structure and even survival rate of their hosts, be it directly or indirectly (Parris *et al.*, 2004b). Changes in water quality due to human interference have led to altered community structures and predation patterns that affect host-pathogen and host-parasite systems (Johnson *et al.*, 2002; Beebee and Griffiths, 2005). These altered systems lead to an increase or decrease in certain parasite infections, e.g. the density, geographic range and pathogenesis of *Ribeiroia ondatrae* (trematode worm), that causes leg deformities in frogs, have changed in recent years (Johnson *et al.*, 2002; Johnson and Chase, 2004). However, there is still no substantial evidence that the amphibians with high incidences of parasite infection are causing the rapid decline in amphibian population numbers, but these subtle effects might be altering other important systems (Beebee and Griffiths, 2005).

Bacteria: Although bacterial infections in amphibians are considered secondary to viral and fungal infections they still contribute to huge numbers of mortalities in certain populations, especially in captive conditions (Carey *et al.*, 1999). Infection with bacteria could cause massive die-offs in amphibians because they seem to thrive during conditions of stress such as crowding, hibernation or exposure to toxicants (Hird *et al.*, 1981). Facultative pathogenic bacteria thrive under these circumstances and can be present on the skin, digestive tract as well as liver and kidneys of frogs without showing any signs of contamination or infection (Carr *et al.*, 1976; Hird *et al.*, 1981; Carey *et al.*, 1999). Scientists involved in using amphibians for research have long been struggling bacterial infections in the specimens, (Emerson and Norris, 1905; Hubbard, 1981; Carey *et al.*, 1999; Hill *et al.*, 2010) with *Mycobacterium* spp. being the most common culprit behind diseases in amphibians. *Mycobacterium* spp. is cause for concern since they are capable of being potential zoonotic pathogens. *Aeromonas hydrophila,* an opportunistic bacterium found on the skin and in the digestive tract of seemingly healthy amphibians (Hird *et al.*, 1981; Rollins-Smith *et al.*, 2002; Hill *et al.*, 2010), is capable of inducing disease in amphibians especially the African clawed frog (*Xenopus laevis*) (Hubbard, 1981) as well as the American toad (*Bufo americanus*) (Dusi, 1949). Historically, infection with *Aeromonas hydrophila* was called red-leg disease and was reported as being one of the most devastating infectious diseases in laboratory amphibians as well as wide-spread mortalities in wild amphibian populations (Hubbard, 1981; Hill *et al.*, 2010). However recent evidence implies that a number of other pathogens also present with similar signs of infection and as a result may have been misdiagnosed (Hill *et al.*, 2010). These pathogens include ranavirus and the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Densmore and Green, 2007).

Viruses: Wildlife has long been impacted by viruses. Iridoviruses form part of a family of DNA-based viruses which are able to infect invertebrates, amphibians, reptiles and even fish (Rollins-Smith, 2009). Ranaviruses are emerging infectious disease agents capable of affecting a wide array of poikilothermic as well as ectothermic vertebrates such as fish, reptiles and amphibians (Jancovich *et al.*, 1997; Jancovich *et al.*, 2001; Collins *et al.*, 2004; Chinchar *et al.*, 2009; Miller *et al.*, 2011). Very little consideration was given to ranaviruses when they first emerged, but this view point soon changed when ranaviruses were associated with the mass die-off events in ecological and economically important reptiles, fish and amphibians. The first emergence of this virus in the United Kingdom presented with adult mass morbidity and mortality events in the late 1980's and early 1990's (Cunningham *et al.*, 1996; Duffus *et al.*, 2013). Today ranaviruses have been identified in a large variety of habitats including amphibians and fish in aquaculture,

zoos and wild populations (Lesbarrères *et al.*, 2012). The spread and translocation of ranaviruses have been facilitated by the international and commercial trade of their infected vertebrate hosts (reptiles and amphibians) which get translocated for food, fish bait as well as for the pet industry. Their spread is also facilitated by the fact that they have a large host range and can therefore easily infect novel populations. Research has shown that isolated ranavirus from these types of translocations tend to be more virulent than those found in the wild (Miller *et al.*, 2011; Lesbarrères *et al.*, 2012).

Infection of amphibians with ranavirus differs from each class as well as within and among species and across phylogenetic lineages (Schock *et al*., 2009). Certain species may also be severely impacted while others show little to no symptoms at all. Signs of infection with ranavirus can include erratic swimming (in tadpoles), difficulty with buoyancy, lethargy, weight loss and in fatal cases swelling of the hind limbs and body, redness of the legs along with red blotches near the vent and urostyle and necrosis of organs (Bollinger *et al.*, 1999; Docherty *et al.*, 2003; Miller *et al.*, 2011; Duffus *et al.*, 2013).Throughout the changing developmental stages of amphibians, their susceptibility to ranavirus infection also changes e.g. (Haislip *et al.*, 2011; Warne *et al.*, 2011). Events of mass die-offs of populations have been observed and usually span over days or even months with later death being more likely due to secondary infection with bacteria or funguses (Jancovich *et al.*, 1997; Miller *et al.*, 2008; Warne *et al.*, 2011). The interaction between ranavirus and its host is impacted by the surrounding environment (temperature, stages of larval development, availability of resources, competition as well as virulence of the virus), because ranavirus infects ectothermic vertebrates. Other mechanisms such as presence/absence of predators can also influence the virus-host interaction along with anthropogenic stressors such as agrochemicals (Lesbarrères *et al.*, 2012). Transmission of ranavirus can occur through various routes, namely direct transmission between infected animals and through contaminated water/sediment as well as through ingestion by means of necrophagy or cannibalism (Jancovich *et al.*, 1997; Brunner *et al.*, 2004; Pearman *et al.*, 2004; Brunner *et al.*, 2005; Harp and Petranka, 2006; Gray *et al.*, 2009).

Fungi: One fungus in particular that has received a lot of attention the past three decades is the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* or better known as amphibian chytrid, and is responsible for the disease chytridiomycosis. According to Fisher *et al.* (2009) *B. dendrobatidis* is currently recognized as the proximate driver behind the global amphibian declines, mass mortality and even extinctions (Stuart *et al.*, 2004). This is because *B. dendrobatidis* appears able to infect most amphibian species around the world and is able to persist at low host densities, leading to devastating population declines and extinctions (Berger *et al.*, 1998; Fisher *et al.*, 2009; Voyles *et al.*, 2009). According to Gascon *et al.* (2007) *B. dendrobatidis* has been called "the worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and it's propensity to drive them to extinction". This dramatic effect is further amplified by the fact that recent molecular studies on the phylogeography of *B. dendrobatidis* have also shown that a virulent clone is spreading globally and wreaking havoc wherever it spreads (Morehouse *et al.*, 2003). These were just some of the reasons *B. dendrobatidis* was placed on the Office Internationale des Epizootes Wildlife Disease List in 2001, which made it the first ever amphibian disease, to make this list (Johnson and Speare, 2003). What makes this fungus even more unique is the fact that until very recently *B. dendrobatidis* was the only known member of its phylum that is able to cause disease in a vertebrate (Berger *et al.*, 2005), that was until the discovery of a second highly divergent chytrid pathogen, *Batrachochytrium salamandrivorans.* This newly discovered species is able to cause lethal skin infections in salamanders resulting in rapid population declines as well as providing an additional explanation for the sudden and problematic decline of amphibian biodiversity (Martel *et al*., 2013).

Although all of the above mentioned factors can lead to a decline in amphibian population numbers, most studies raise the question whether one of these factors alone can be blamed or whether interactions among these factors are necessary to cause a marked decline in amphibians (Alford and Richards, 1999)? For example an increase in UV-B radiation can lead to increased vulnerability to pathogens along with altering the natural pH of the environment, which would then have the domino effect of changing the diversity, distribution and density of amphibian populations (Wyman and Hawksley-Lescault, 1987; Wyman and Jancola, 1992). All local environmental factors could potentially interact and influence one another in complex effects which should be taken into consideration when planning any programs involving experimentation or observation of one of these factors (Alford and Richards, 1999). In this study however the focus was placed on disease being the proximate

driver behind the amphibian declines, particularly *Batrachochytrium dendrobatidis,* since *B. dendrobatidis* has the potential to act synergistically with some or all of the other anthropogenic drivers of amphibian declines and therefore could contribute in large to the ongoing 6th mass extinction event (Olson *et al.,* 2013).

1.3. The amphibian chytrid, *Batrachochytrium dendrobatidis*

In 1998 evidence for the presence of an unknown amphibian fungus was detected in dead and dying anurans, where after it was isolated from the blue poison dart frog (*Dendrobates auratus*), identified and described in 1999 and placed in a new genus namely *Batrachochytrium*; Phylum: Chytridiomycota, Class:Chytridiomycetes, Order: Chytridiales (Longcore *et al.*, 1999; Hyatt *et al.*, 2007). The phylum chytridiomycota represents a very large and diverse group that can be found in a variety of habitats including rainforests, deserts and even the arctic tundra (Powell, 1993). However, they are typically found in water habitats where they are responsible for the degradation of chitin (from dead insects), cellulose (from vegetable matter), pollen and keratin (from hair and skin). They therefore play a crucial role as biodegraders in ecosystems (Barr, 1990). Since the discovery of *B. dendrobatidis*, research has shown that this fungus has a near global distribution and can be found on all continents except Antarctica (Berger *et al.*, 1999; Lips, 1999; Longcore *et al.*, 1999; Bosch *et al.*, 2001; Weldon *et al.*, 2004) and thus mirrors the distribution of amphibians. In a recent study conducted by Huss *et al.* (2013), it was confirmed that the earliest recorded *B. dendrobatidis*-positive specimen is that of a North American bullfrog (*Lithobates catesbeianus)* that was collected in 1928 in California and after that a Frazer's clawed frog (*Xenopus fraseri)* collected in 1933 from Cameroon (Africa) (Soto-Azat *et al.*, 2010), and an African clawed frog (*Xenopus laevis)* from South-Africa collected in 1938 (Weldon *et al.*, 2004). *B. dendrobatidis* has been implicated in the decline, mass mortality and even extinction of numerous amphibian species throughout the world, and has been described in both wild (Berger *et al.*, 1999; Bosch *et al.*, 2001) and captive amphibians as being a cutaneous disease (Pessier *et al.*, 1999).

1.3.1 Biology and life cycle

Batrachochytrium dendrobatidis has a very simple life cycle consisting of two stages typical of chytrids, progressing from a single uniflagellated zoospore to a growing organism known as a thallus that produces a single cutaneous zoosporangium that in turn produces the zoospores (Longcore *et al.*, 1999; Johnson and Speare, 2003; Berger *et al.*, 2005; Kilpatrick *et al.*, 2009). Multiple zoospores are cleaved from the contents of the sporangium and then exit the sporangium through one or more discharge papillae (inoperculate openings) (Longcore *et al.*, 1999). The released zoospores can swim for up to 24 hours and cover a distance of approximately 2 cm in media (Kilpatrick *et al.*, 2009). The only known deviation from this simple life cycle is when colonies form (when a single zoospore forms more than one sporangium) and is known as asexual amplification (Longcore *et al.*, 1999; Berger *et al.*, 2005). The life cycle of *B. dendrobatidis* appears to be no different when it is in culture or skin and therefore it is assumed that the duration of the life cycle (4 to 5 days at 22°C) would also be the same under both conditions. In culture *B. dendrobatidis* can reproduce and grow in temperatures ranging from 4 - 28°C, although optimal growth temperature ranges between 17 - 25°C. *B. dendrobatidis* can even withstand freezing to some degree and survives cryopreservation (Kilpatrick *et al.*, 2009).

Due to *B. dendrobatidis* occurring in the superficial epidermal layer of amphibians, a loss of infection can occur when shedding takes place (Berger *et al.*, 2004; Weldon and du Preez, 2006). *B. dendrobatidis* has an optimal growth temperature range between 17 - 25°C. When environmental temperatures rise above 25°C it increases the rate of epidermal turnover while decreasing the growth of *B. dendrobatidis* (Piotrowski *et al.*, 2004). According to Berger *et al.* (2005) one of the reasons behind this can be that the fungus has insufficient time to complete its life cycle before the amphibian sheds its skin again.

It is important for *B. dendrobatidis* and other pathogens (especially with regards to transmission) to be able to survive outside their hosts, which makes it more probable that they would drive their host species to extinction (Mitchell *et al.*, 2008; Kilpatrick *et al.*, 2009). After vigorous testing it was determined that *B. dendrobatidis* is able to survive in sterile moist sand or lake water for a period of up to three months under laboratory conditions (Johnson and Speare, 2005). Furthermore, the fact that *B. dendrobatidis* can be cultured, implies that it can survive indefinitely outside of a host,

but it has not yet been established whether *B. dendrobatidis* is able to survive in the environment after host extinction has occurred (Morgan *et al.*, 2007; James *et al.*, 2009).

1.3.2 Transmission and infection

When amphibians are infected with *B. dendrobatidis* the sporangia infect the *stratum granulosum* and *stratum corneum* situated in the superficial epidermis. The younger, immature sporangia are typically found deeper in the more viable cells of the epidermis, moving to the outer more keratinized layers as they become more mature (Berger *et al.*, 2005). The zoospores are then released into the surrounding environment through the discharge papillae that are projected towards the skin surface. The zoospores are then able to spread via host contact with infected water bodies, instruments, equipment etc. or via host-to-host contact (Forzán *et al.*, 2008; Pessier, 2008; Olson *et al.*, 2013). Once infected the distribution of keratin in the epidermis influences the allocation of the sporangia in the tadpoles and post-metamorphic amphibians with infection leading to the formation of hyperkeratosis near the thalli (Marantelli *et al.*, 2004). Further evidence also shows that infection with *B. dendrobatidis* can spread directly from tadpoles to juveniles and ultimately to adult amphibians (Rachowicz and Vredenburg, 2004). Infection with *B. dendrobatidis* leads to certain changes in the amphibian host such as: epidermal cell layer disorder, irregular multifocal hyperplasia, spongiosis, and erosion/ulceration of the skin as well as thinning/thickening in certain areas of the epidermis. Sloughing also occurs as a result of vacuolated degenerate cells that combine into vesicles that ultimately lift the epidermis.

Techniques used to ultimately test for *B. dendrobatidis* infection include visual inspection where the pigmentation of tadpole jaw sheaths and tooth rows are examined with a x10 hand lens (Fellers *et al.*, 2001; Smith and Weldon, 2007) and histological diagnosis which requires identification of the intracellular sporangia within the epidermis, polymerase chain reaction (PCR) (Forzán *et al.*, 2008) and wet mounts where skin scrapings are investigated microscopically to verify the presence of spheroid, walled and occasionally septate sporangia (Pessier, 2008). The first attempt to quantify *B. dendrobatidis* infection by morphology made use of microscopic examination of wet mounts (Weldon and du Preez, 2006).

There were until recently two hypotheses as to why a fungus restricted to the epidermis can cause mass mortalities in amphibians (Berger *et al.*, 1998; Pessier *et al.*, 1999).

(1) Proteolytic enzymes/active compounds may be released by *B. dendrobatidis*, which are then absorbed through the very permeable skin of the host (Voyles *et al*., 2007).
(2) The damage that *B. dendrobatidis* causes to the skin can lead to malfunction/disturbance of the electrolyte balance, thus resulting in the death of the specimen (Berger *et al*., 1998; Voyles *et al*., 2007)

However, the first hypothesis has since been discarded as the second proved to be validated in a study by Voyles *et al.* (2009) in which it was proved that *B. dendrobatidis* compromises the epidermis of amphibians by impairing cutaneous osmoregulation (electrolyte balance) of the amphibian. Clusters are also known to form where the zoospores ultimately become encysted when infecting amphibians. Clustering can be due to the fact that the zoospores have limited time of mobility and thus infect the surrounding cells in the skin (Piotrowski *et al.*, 2004). The forming colonies tend to expand concentrically from the original point of infection and in doing so a core of hyperkeratotic tissue devoid of sporangia develops (Weldon and du Preez, 2006).

The rate of mortality and time it takes for the animal to eventually die from infection depends on many factors including fungal dose, temperature, age of the specimen and the host species (Berger *et al.*, 1999; Berger *et al.*, 2005). However, not all species infected with *B. dendrobatidis* become sick or die. Some species such as *Rana muscosa* show little to no effect in the tadpoles, but suffer huge mortalities in juveniles after metamorphosis takes place when more keratinized skin tissue is available for *B. dendrobatidis* to infect. Other species such as the American bullfrog (*Lithobates catesbeiana*), the African clawed frog (*Xenopus laevis*) and the Tiger salamander (*Ambystoma tigrinum*) among others, have been known to become sub-clinically infected, meaning they carry *B. dendrobatidis* but do not develop chytridiomycosis and subsequently show relative tolerance to *B. dendrobatidis* infection (Daszak *et al.*, 2003; Davidson *et al.*, 2003; Weldon *et al.*, 2004; Garner *et al.*, 2006; Fisher and Garner, 2007). This substantiates the possibility that the global distribution of this fungus can be attributed to the international trade of some of these amphibian species for laboratories (experimental animals), as pets and even as cuisine delicacies for fine dining (Weldon and Fisher, 2011). These exported and imported amphibians might be sub-clinically infected and can thus act as environmental reservoirs of *B. dendrobatidis* for novel or susceptible species prone to developing chytridiomycosis (Pessier, 2008).

1.3.3 Pathogenesis

After more than a decade of research regarding the life cycle, transmission etc. on *B. dendrobatidis*, its pathogenesis is still not completely understood. Disease and population dynamics might possibly be changed dramatically by understanding the pathogenesis of *B. dendrobatidis* and how the infection can cause alterations in the behavior of infected individuals, including basking and water seeking behavior (Parris *et al.*, 2004b).

Colonization of the pathogen is considered as the primary step and takes place in the cells of the epidermis (Longcore *et al.*, 1999; Berger *et al.*, 2005). Although it remains unclear exactly how cell entry is accomplished. Longcore *et al.* (1999) did hypothesize that zoospores could insert *B. dendrobatidis* nuclear material into the host via a germ tube after encysting on the outer surface of an epidermal cell. *B. dendrobatidis* subsequently develops sporangia which ultimately fills the epidermal cells (Berger *et al.*, 2005). The rate at which *B. dendrobatidis* then develops will only reach the final stages i.e. the formation of discharge papilla once the infected epidermal cells reach the upper most layer that takes place during directional movement of epidermal cells. Clinical signs of infection may occur two weeks after exposure to *B. dendrobatidis* (Forzán *et al.*, 2008), but varies greatly with species.

An exponential increase in pathogen load can be seen in hosts experiencing re-infection, which leads to an increase in the rate of mortality in populations (Briggs *et al.*, 2010; Vredenburg *et al.*, 2010). The growth rate and development of *B. dendrobatidis* is influenced by abiotic factors such as temperature. Higher temperatures tend to kill *B. dendrobatidis* while a lower temperature either kills *B. dendrobatidis* or inhibits growth resulting in longer time periods to encyst, mature and reproduce (Woodhams *et al.*, 2008). *Batrachochytrium dendrobatidis* pathogenesis can also be influenced by differences in lineages as evidence currently suggests that differences in mortality observed in wild populations can be attributed to the differences in lineages (Retallick and Miera, 2004). In a study conducted by Fisher *et al.* (2009) it was found that virulence of *B. dendrobatidis* was affected by genetic differentiation among different isolates. They also found that genetic differentiation between isolates had an influence on the size of the sporangia since a high-temperature/low-altitude/low host species diversity environment placed selection pressure on *B. dendrobatidis* resulting in smaller than average sporangia.

Infection with *B. dendrobatidis* can cause two types of lesions (1) structural damage or even loss of areas of the keratinized mouthparts as well as depigmentation of the keratodonts and rostrodonts, commonly found in tadpoles, (2) epidermal hyperplasia of cells containing keratin, keratosis (fusion of keratin layers) and strange behavioral patterns such as abnormal posture, lethargy and reduced response to tactile stimuli, commonly found in post-metamorphic amphibians (Daszak *et al.*, 1999; Forzán *et al.*, 2008; Rosenblum *et al.*, 2009). The lesions ultimately lead to the death of the infected amphibian due to the fact that the skin of amphibians is unique in the sense that it's actively involved in maintaining osmotic balance and is the primary defensive barrier against infection (Woodhams *et al.*, 2007). *Batrachochytrium dendrobatidis* subsequently interferes with the gas exchange, fluid exchange and ultimately the electrolyte balance of its hosts (Berger *et al.*, 1998; Voyles *et al.*, 2009). Infection in tadpoles decreases grazing efficiency and the amount of food they take in (due to the loss of areas of keratinized mouthparts), which may lead to reduced growth and developmental rates as well as a reduction in survival and ultimately leads to death (Parris *et al.*, 2004a; Parris and Cornelius, 2004; Smith *et al.*, 2007)

1.3.4 Distribution

Infection with this pathogen is listed as being a "Key Threatening Process", under the Environment Protection and Biodiversity Conservation Act 1999 (EPBC Act), for frogs worldwide (Skerratt *et al.*, 2008). The reason for this is that traded species are at risk of carrying diseases as well as parasites, allowing the crossing of geographical boundaries and thus the chance of infecting novel populations (Weldon and Fisher, 2011). The current wide-spread distribution of *B. dendrobatidis* can be explained by two hypotheses.

(1) The "novel pathogen hypotheses" (NPH) states that the disease originated and evolved in one geographical area where after it spread to new geographic areas as a result of the spread of amphibians (Weldon *et al.*, 2004; Fisher and Garner, 2007).

(2) The "endemic pathogen hypotheses" (EPH) states that *B. dendrobatidis* has always been present in the environment but that endemic amphibian hosts are becoming more susceptible to infection due to the recent increase in environmental changes, and as a result thereof *B. dendrobatidis* started to spread as the climate shifted to include new geographic areas. The range expansion of, for instance, the North American Bullfrog (*Lithobates catesbeina*) then facilitated the spread of *B. dendrobatidis* to other regions (Rachowicz *et al.*, 2005; Pounds *et al.*, 2006).

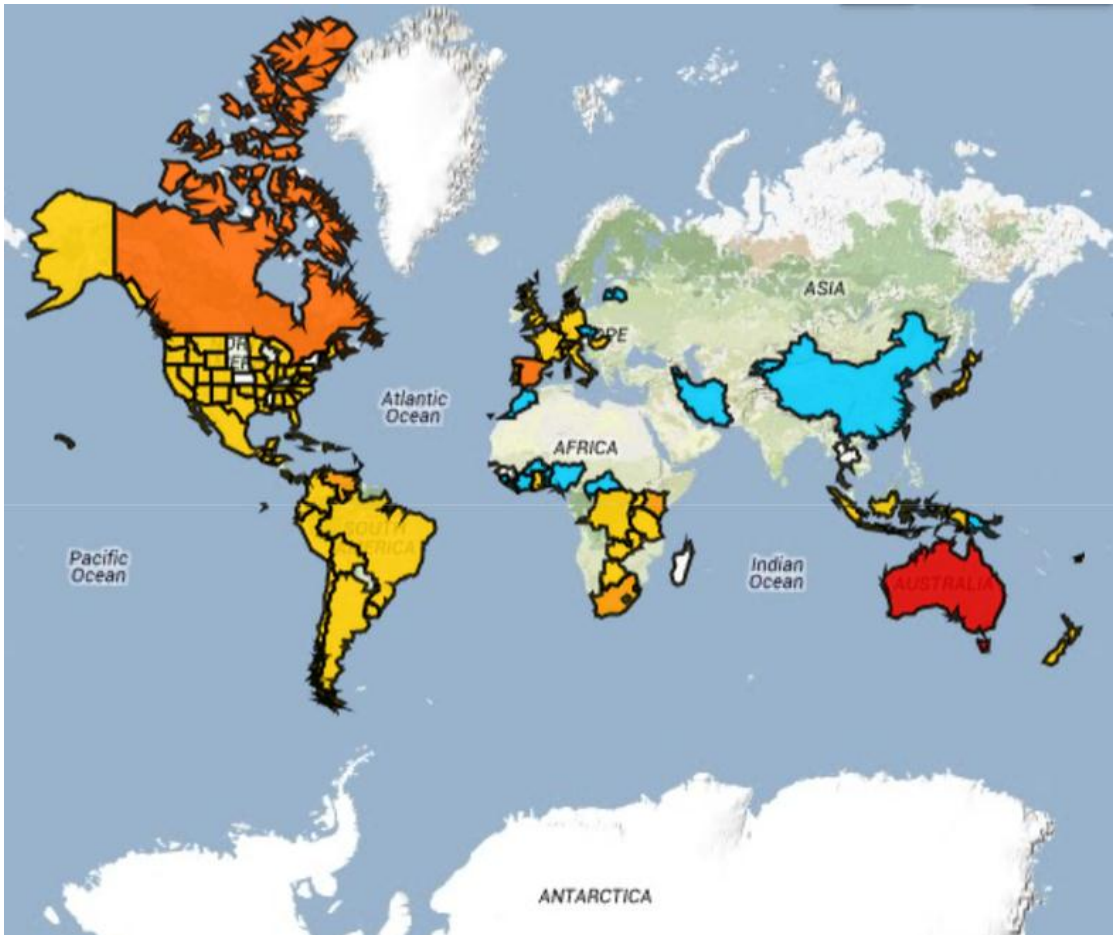
However, recent studies concluded that the NPH is more likely to be correct (Walker *et al.,* 2010) and in addition to the international pet trade, the transportation (local and international) of amphibians for bait trade, laboratories, zoo's and for bio-control purposes have indeed facilitated the spread of *B. dendrobatidis* to novel populations, causing detrimental effects and mass mortalities (Fisher and Garner, 2007; James *et al.*, 2009; Schloegel *et al.*, 2010). According to Weldon *et al.* (2004) one of the earliest reported cases of *B. dendrobatidis* was from a preserved *Xenopus laevis* from the 1930's, which incidentally was the same time that *Xenopus* first started being exported to Europe, Australia and the USA for pregnancy tests. From there the spread of *B. dendrobatidis* could have easily occurred through infected animals escaping or being released and coming into contact with wild populations.

The recent environmental changes could have also aided in the spread of *B. dendrobatidis* due to changes in ecological relationships that ultimately increases the rate at which pathogens spread and favours the ways that transmission and spread take place. The ecological alterations include the changes in geographical distribution of pathogens and hosts as well as changes in community structures (Harris *et al.*, 2009). According to Berger *et al.* (1998) major declines or even extinctions in population numbers can be expected when *B. dendrobatidis* is newly introduced into a novel population. These declines or extinctions can occur very suddenly and rapidly and can wipe out whole populations in only a few weeks (Lips *et al.*, 2006), which is a real cause for concern because *B. dendrobatidis* occurs on all continents except Antarctica and survives in a wide range of habitats and climates (Skerratt *et al.*, 2008).

The most recent statistics (table 1 and figure 1) according to the Global *B. dendrobatidis*-mapping project shows that a total of 56 out of 82 countries are currently *B. dendrobatidis*-positive and 520 out of 1252 species (41.53%) of amphibians have tested positive for *B. dendrobatidis*.

Table 1: Global summary (n >100 highlighted red for country or species) (www.bd-maps.net).

Country summary - countries with greater than 50 *B. dendrobatidis* + samples shown		**Species summary – species with greater than 50 *B. dendrobatidis* + samples shown**		
Country		**Species**	**IUCN status**	
United States	2259	*Alytes obstetricans*	Least Concern	886
Australia	2102	*Anaxyrus boreas*	Near Threatened	759
Puerto Rico	1004	*Lithobates catesbeianus*	Least Concern	668
Spain	787	*Eleutherodactylus coqui*	Least Concern	661
France	401	*Litoria wilcoxii*	Least Concern	457
Kenya	272	*Litoria pearsoniana*	Near Threatened	344
South Africa	266	*Litoria rheocola*	**Endangered**	249
Venezuela	246	*Eleutherodactylus portoricensis*	**Endangered**	213
Japan	243	*Xenopus laevis*	Least Concern	157
Canada	219	*Lithobates clamitans*	Least Concern	146
Peru	195	*Litoria genimaculata*	Least Concern	144
Switzerland	156	*Mixophyes fasciolatus*	Least Concern	117
Honduras	121	*Rana luteiventris*	Least Concern	115
Panama	115	*Hyperolius glandicolor*	Least Concern	108
Costa Rica	103	*Amietia fuscigula*	Least Concern	102
Mexico	58			
Germany	56			
Colombia	53			



>800 Positive
>600 Positive
>400 Positive
>200 Positive
<200 Positive
Negative
Negative (total sample size unknown)

Figure 1: A current snapshot of the global database of *Batrachochytrium dendrobatidis*. The map indicates countries where samples have been collected with red indicating the sites with the most positive samples for *Batrachochytrium dendrobatidis* (www.*bd*-maps.net) and white/blue the countries that are negative or unknown.

1.4. Amphibian disease mitigation

According to Kriger and Hero (2009) amphibian disease management can be defined in three simple steps namely:

1. Post-exposure measures which include the treatment of infected amphibians and environments as well as eradicating diseases from these environments and ensuring future resistance.
2. Pre-exposure measures which include adequate quarantine and testing methods for all animals that might be translocated.
3. Preventative measures such as banning any translocation of amphibians.

In 2005 several leaders in research and conservation convened for the Amphibian Conservation Summit and agreed upon the production of the Amphibian Conservation Action Plan (ACAP) (Gascon *et al.*, 2007) to ensure and assist in the implementation of conservation programs for amphibians and also to ensure that the necessary funds are available for these programs. According to the Amphibian Conservation Action Plan published in 2007 the conservation and research of amphibians can be divided into 11 priority actions (Gascon *et al.*, 2007) of which prevention and/or treatment of diseases such as *B. dendrobatidis* is one. These priority actions are a means to ensure that all the necessary steps are taken to successfully implement the conservation of amphibians. Furthermore ACAP is also responsible for identifying the necessary actions needed to understand and counteract the current global extinction of amphibians. As a result an Amphibian Survival Alliance was put into place to ensure the implementation of ACAP (Mendelson *et al.*, 2006) as well as the Amphibian Ark (AArk) in an attempt to unite all the different factions of the *ex situ* conservation community. The *ex situ* conservation community consists of individuals, organizations, researchers, scientists etc., whom all work with living organisms that are kept in captivity until such time as they can be released back into the wild. When it comes to amphibians, the *ex situ* conservation community primarily consists of zoos and aquariums, although other partners such as universities are also known to play significant roles in the conservation of amphibians (Zippel *et al.*, 2011).

Affected countries should try and keep conservation activities such as *ex situ* programs within that country where possible and employ local scientists and researchers to support all conservation activities. Besides national conservation activities in each country, regional centers for research regarding diseases as well as captive breeding

should also be initiated in zoos, aquariums, universities and government agencies (once again staffed by local researchers, conservationists and wildlife managers). Furthermore a global database should be created from all centers globally, including the research (e.g. *B. dendrobatidis* mitigation) and training available to promote amphibian conservation (Mendelson *et al.*, 2006). Until a valid, effective treatment protocol exists for *B. dendrobatidis*, countries should execute *in situ* actions such as monitoring, surveys and habitat protection programs along with *ex situ* programs involving husbandry and survival assurance/research colonies. Despite extensive efforts, some species of amphibian will always face threats in the wild that could lead to their extinction, in cases where immediate mitigation is not possible and certain species cannot be safeguarded *in situ* the only viable option left is ultimately *ex situ* conservation. For many species the only option left is *ex situ* programs since they are already teetering on the brink of extinction (Krajick, 2006). *Ex situ* programs have been instrumental in many successful reintroductions of threatened species back into the wild and in doing so prevented the extinction of those species. In order for these programs to be successful, there has to be unity between stakeholders from all different sectors including academic, conservation, zoo, policy, private sectors and international convention communities (Mendelson *et al.*, 2006; Zippel *et al.*, 2011).

1.4.1 Adequate quarantine and other pre-exposure measures

Pre-exposure methods are very important as they reduce the chance of infection by means of adequate bio-security measures including quarantine, batch-testing shipments, and diagnostic tests to confirm that animals being moved are indeed disease free as well as declaring farms, institutes and zoos as disease free locations. This is very important knowledge to obtain seeing as zoos and laboratories need to be able to move and translocate amphibians and can be a big contributor to spreading disease if their status is unknown (Pessier *et al.*, 1999; Parker *et al.*, 2002). In countries where amphibians form a large part of the food trade industry, proper quarantine and screening methods are of the upmost importance (Hanselmann *et al.*, 2004). In a study conducted by Johnson and Speare (2003) they found that water containing *B. dendrobatidis* can stay infective for up to seven weeks, which is an important factor to consider when formulating adequate quarantine measures for the translocation of amphibians as well as soil, water, equipment etc. that came into contact with amphibians. Countries relying solely on imports of amphibians should restrict these imports to dead amphibians or agree to strict quarantine periods and extensive disease testing and treatment of infected individuals as well as any and all equipment, water and soil (Kriger and Hero,

2009). Therefore any water, soil or objects that are being moved and came into contact with amphibians at some point should be regarded as infectious for *B. dendrobatidis* unless proven otherwise. The same should apply to new amphibians being added or released into the wild or *ex situ* programs. However, storage periods alone (quarantine) are not enough; possible infected water, soil; equipment etc. should be treated or disinfected before being discarded (Johnson and Speare, 2003).

1.4.2 Preventative measures

A loss of biodiversity along with outbreaks of disease in wildlife will continue to increase as long as there remains inadequate quarantine, surveillance, parasite and disease control programs (Skerratt *et al*., 2007). To control the spread of amphibian diseases it is vital to have an early detection system for these diseases (Pessier *et al*., 1999; Mutschmann *et al*., 2000; Parker *et al*., 2002; Mazzoni *et al*., 2003). Since *B. dendrobatidis* has been identified as one of the main drivers behind the recent declines in amphibian population numbers, it only stands to reason that any and all efforts should go towards preventing the spread of *B. dendrobatidis* to novel populations (Stuart *et al.*, 2004). This can only be done when the present and past global distribution of *B. dendrobatidis* is mapped and *B. dendrobatidis* subsequently identified at an early stage. In doing so novel populations can be identified more accurately and protected against infection (Boyle *et al.*, 2004). The ability to detect disease at an early stage would also assist in the protection of endangered species, the monitoring of novel environments along with promoting the reintroduction of *ex situ* bred populations back into the wild (Johnson and Speare, 2003). Furthermore, the necessary quarantine, screening and disinfection strategies could also be implemented in time if contaminated water, soil or animals are detected early enough. Therefore tests such as real-time Taqman assays can be used to accurately test and detect infection or contamination and thus aid in keeping diseases from spreading even more. According to Boyle *et al.* (2003): ”The ability of an assay to detect low levels of organisms at an early stage of infection is vital to prevent the spread of infection between enclosures in breeding facilities.” Not only will cross contamination be prevented in breeding facilities, but also for the movement of any animals between zoos, for the food trade, as pets and laboratory specimens (Boyle *et al.*, 2004).

1.4.3 Post exposure measures

The fact that there is still no effective control against *B. dendrobatidis* is a huge cause for concern and it is therefore important to develop a safe and effective treatment protocol that can be used by conservation programmes to cure threatened species of infection and maintain *B. dendrobatidis*-free populations (Garner *et al.*, 2009). Several treatments have already been tried, which include elevated temperatures (influences immune function of ectotherms and virulence of pathogens) (Woodhams *et al.*, 2003), formalin/malachite green (commonly used as an anti-parasitic for fish) (Parker *et al.*, 2002), salt (White, 2006), chloramphenicol (Bishop *et al.*, 2009) and numerous antifungals such as itraconazole, fluconazole and voriconazole (Nichols and Lamirande, 2000; Hadfield and Whitaker, 2005; Webb *et al.*, 2007; Martel *et al.*, 2011; Jones *et al.*, 2012). However, these treatments are only effective in some amphibians and are held back by physiological limitations since many of the species show adverse effects to increased salinity and temperature as well as toxic compounds. International and regional legislation also causes additional problems to the practical application of some of these treatments (Garner *et al.*, 2009; Jones *et al.*, 2012).

1.4.4 Possible control measures

1.4.4.1 Natural control

A. Temperature

Batrachochytrium dendrobatidis is known for its preference of cooler temperatures when grown in cultures, which indicates that specific environmental temperatures may be important for optimum survival of *B. dendrobatidis* in situ (Longcore *et al.*, 1999; Bradley *et al.*, 2002). *B. dendrobatidis* can survive and grow in culture when kept at temperatures between 6 and 28°C, but is unable to survive when kept at 32°C (Bradley *et al.*, 2002; Woodhams *et al.*, 2003). In a study conducted by Woodhams *et al.* (2003) they were able to prove that elevated temperatures affected the growth and progress of *B. dendrobatidis* in living amphibians and that fluctuating temperatures retarded the development of chytridiomycosis (Woodhams *et al.*, 2003). Elevated body temperatures in amphibians (even for short periods) were sufficient enough to eliminate the pathogen from the infected hosts. Chatfield and Richards-Zawacki (2011) also reported that they effectively rid infected amphibians of *B. dendrobatidis* by exposing them to 30°C for 10

days (Chatfield and Richards-Zawacki, 2011). However, this treatment also has a down side as several species of amphibians can't tolerate elevated temperatures; especially species acclimated to high altitudes and low temperatures.

B. Antimicrobial peptides

It is known that certain species of amphibians are able to coexist with *B. dendrobatidis* in nature and as a result don't suffer mass mortalities, but instead only have low infection intensities (Davidson *et al.*, 2003; Hanselmann *et al.*, 2004). According to Briggs *et al.* (2005) differences can occur within different populations of the same species to how they react to *B. dendrobatidis*. One population can coexist with *B. dendrobatidis*, whereas the other population can be driven to the brink of extinction. This can be the result of differences between their habitats and microclimates and the effect they have on the natural occurring skin peptides (Briggs *et al.*, 2005).

The skin of numerous amphibians is linked with defensive systems that differ in efficacy against both predators and pathogenic microorganisms (Rollins-Smith and Conlon, 2005). Amphibians also possess well-developed immune defenses, as well as adaptive and innate mechanisms such as antimicrobial peptides/skin secreted peptides and symbiotic skin bacteria and/or circulating granulocytes (Rollins-Smith *et al.*, 2006; Woodhams *et al.*, 2007; Richmond *et al.*, 2009; Lam *et al.*, 2010). These peptides are released through skin secretions in sometimes very high concentrations whenever the specimen is in stress or has an infection (Conlon *et al.*, 2007).

According to Ramsey *et al.* (2010) initial infection of *B. dendrobatidis* is prohibited by antimicrobial peptides and/or antibodies in the mucus of species that tend to be resistant to *B. dendrobatidis*. *In vitro* studies showed that purified antimicrobial peptides as well as enriched skin peptides from several different species are able to inhibit growth of *B. dendrobatidis* zoospores and sporangia (Rollins-Smith and Conlon, 2005; Rollins-Smith *et al.*, 2006; Woodhams *et al.*, 2006; Conlon *et al.*, 2007; Woodhams *et al.*, 2007; Rollins-Smith, 2009). In a study done by Rollins-Smith (2009) she found a 83% inhibitory response from 41 purified antimicrobial peptides tested against *B. dendrobatidis* (Rollins-Smith, 2009). A further defense mechanism in amphibians is their skin, which is protected by an adaptive immune system that is activated when antigens in the skin get transported to the spleen and T-cells start to proliferate (Carey *et al.*, 1999; Robert and Ohta, 2009).

However, not all species possess this innate ability to fend off chytridiomycosis and even species with antimicrobial peptides suffer some degree of population mortality. Recent studies conducted on the molecular makeup of *B. dendrobatidis* showed that *B. dendrobatidis* possesses a large serine-type peptidase gene family (Fisher, 2008; Rosenblum *et al.*, 2008). It is therefore possible that these peptidases are used by *B. dendrobatidis* to overcome some of the antimicrobial peptides of amphibians. The resistance of an amphibian species to *B. dendrobatidis* infection may therefore depend on its ability to degrade these *B. dendrobatidis* peptidases. It is further hypothesized that *B. dendrobatidis* has evolved certain genes that have the ability to mimic host proteins in order for the fungus to "hide" from the amphibians immune system within the skin cells (Fisher, 2008). Species lacking the necessary antimicrobial peptides to fight of *B. dendrobatidis* infection are more vulnerable to mass mortalities and thus possible extinction. Therefore urgent attention should be focused towards finding alternative ways of controlling *B. dendrobatidis* in these species.

C. Probiotics

Both plants and animals use microbes as a defence mechanism against pathogens. Hence the hypothesis that certain bacteria can be applied to amphibians in the hope that it will add the much needed defence against *B. dendrobatidis* infection (Bletz *et al.*, 2013). In a recent survey conducted by the Horizon Scan of Global Conservation, the use of probiotic therapy through bio-augmentation was herald as a new and important potential key in the fight against *B. dendrobatidis* (Harris *et al.*, 2009; Becker *et al.*, 2011; Bletz *et al.*, 2013). By applying these probiotics to amphibians' skin it could aid in fighting of chytridiomycosis and aid in the reintroduction of amphibians to areas where they have been extirpated (Bletz *et al.*, 2013). Although the use of probiotics shows a lot of promise the necessary laboratory and field experiments are still to be conducted in order to ensure that there are no unknown negative effects to the surrounding environment and organisms (Sutherland *et al.*, 2014).

D. Biological control agents

The interaction between hosts and their parasites can be affected by a number of other direct and indirect factors such as other members present in the community. One such member is predators which have the ability to impact infectious diseases and play a role in disease management and conservation (Johnson *et al.*, 2010). Usually within ecosystems parasites function as prey and therefore predation was suggested as a

means of biological control since *B. dendrobatidis* zoospores are within the ideal size range of prey for cladocerans such as *Daphnia* (Buck *et al.*, 2011). They are selective filter feeders feeding on fungi, bacteria, algae, protozoa etc. and are known to ingest the zoospores of any other species that happen to be small enough (Gleason *et al.*, 2014). *Daphnia magna* is also able to feed on zoospores of *B. dendrobatidis* and since *Daphnia* occurs globally in lentic habitats (all but the most extreme freshwater habitats) (Thorp and Covich, 2010) they possess the ability to decrease the number of infective zoospores in the environment and thus act as biological control agents (Buck *et al.*, 2011). In a study conducted by Searle *et al.* (2013) they proved that the presence of daphnia in water samples can severely reduce *B. dendrobatidis* levels depending on the species of daphnia as well as food concentration and grazing periods. Therefor daphnia is able to assist in the reduction of infection in amphibians globally.

1.4.4.2 Human induced treatments

A. Malachite green and formalin

Several drugs have been tested in the hope of finding an effective treatment against *B. dendrobatidis*, including a mixture of formalin and malachite green, which proved to be relatively successful in treating late stages of chytridiomycosis (Parker *et al.*, 2002). However, malachite green has been known to cause chromosomal fractures, respiratory toxicity, carcinogenesis as well as being cytotoxic in mammalian cells and a mutagen since it intercalates with double-stranded DNA and possesses the capacity to provoke cell transformation (Culp and Beland, 1996; Srivastava *et al.*, 2004). All these side effects aided in the decision to declare it a prohibited aquatic veterinary drug in North America and Europe (Andersen *et al.*, 2004) which does not make it a suitable solution for treating *B. dendrobatidis* infection.

B. Chloramphenicol

According to Bishop *et al.* (2009) certain species of amphibians can successfully be treated for chytridiomycosis by applying topical chloramphenicol. Archey's frogs from New Zealand that were treated with chloramphenicol showed no obvious adverse side effects and the infected specimens showed no visible signs of having chytridiomycosis. Furthermore the infected amphibians were able to eliminate the fungus, thus rendering the once infected amphibians' now *B. dendrobatidis* free. The chloramphenicol was applied both as a solution (in contact with the belly of the frog) and as an ointment (in

contact with the back of the frog). However, chloramphenicol is a banned substance in most developed countries for use in animals that serve as a food substance and therefore cannot be globally used as a treatment protocol especially not to treat frogs that are shipped or used for human consumption (Bishop *et al.*, 2009).

C. Antifungals

As the spread of *B. dendrobatidis* continues and amphibian population numbers keep plummeting, it is becoming abundantly clear that some sort of effective treatment protocol should be established. Especially since most of the current treatment protocols/methods for *B. dendrobatidis* have more disadvantages than advantages which include intolerance for elevated temperatures in some species or lethal toxicity for certain antifungal regimes in other species. Other chemicals recommended for use include the quaternary ammonium compound didecyl dimethyl ammonium chloride (DDAC), virkon, F10SC Veterinary Disinfectants, benzalkonium chloride, TriGenes, ethanol and sodium hypoclorite (bleach) (Young *et al.*, 2007).

Due to *B. dendrobatidis* being very sensitive to temperature in terms of growth and survival the rational conclusion would be to use this as a form of treatment against *B. dendrobatidis* (Woodhams *et al.*, 2003; Piotrowski *et al.*, 2004). Notwithstanding the potential that all the possible treatments (temperature, salt etc.) have for *B. dendrobatidis*, antifungal drugs remain the most commonly preferred and used method for treating captive amphibians (Chatfield and Richards-Zawacki, 2011). This is most likely due to the fact that even though elevated temperature is an effective way to treat *B. dendrobatidis*, the recommended temperatures (which ranges between 32°C and 37°C) (Woodhams *et al*., 2003) for effective treatment is above the thermal tolerance for many frog species, especially frogs from temperate regions (Duellman and Trueb, 1994).

Most antifungal drugs/treatments are tolerated by a huge number of different amphibian species and have been used successfully to treat infection with *B. dendrobatidis* (Nichols and Lamirande, 2001; Parker *et al.*, 2002; Garner *et al.*, 2009; Tobler and Schmidt, 2010) even at low concentrations (Pessier, 2008). However, the larvae of certain species are overly sensitive to exposure of typical veterinary drugs and it is not known if they will survive at concentrations needed to effectively kill *B. dendrobatidis* (Garner *et al.*, 2009) and there still remains some concerns about the possible side effects of these drugs to both amphibians and the surrounding environment (Woodhams *et al.*, 2003; Garner *et al.*, 2009).

However the treatment of amphibians while they are still in the larval stage is gaining more interest as most mortalities only occur after metamorphosis (Bosch and Martínez-Solano, 2006). Of the drugs that showed a lot of promise in the fight against *B. dendrobatidis* are chloramphenicol (Bishop *et al.*, 2009) and itraconazole which is a triazole antifungal agent with a wide-ranging spectrum of activity (De Beule and Van Gestel, 2001). However itraconazole sadly appears to be toxic to metamorphs and larvae for some species at the concentrations needed to effectively kill *B. dendrobatidis* (Nichols and Lamirande, 2000; Garner *et al.*, 2009) and was proven to cause depigmentation in certain specie's tadpoles after treatment and is therefore not generally recommended as a form of treatment (Garner *et al.*, 2009)

Even though the pathogen is susceptible to a variety of other chemical disinfectants, the concentration and exposure time still influences the efficacy of these disinfectants (Johnson *et al.*, 2003; Webb *et al.*, 2007). Although some are useful for the treatment of *B. dendrobatidis* most are better suited for the disinfection of equipment in the field and laboratory, but care has to be taken to prevent environmental contamination as many of these products are not environmentally friendly (Young *et al.*, 2007). However, regardless of the treatment/chemical selected, the thorough disinfection of enclosures and equipment should always remain a high priority to discourage disease transmission (Pessier, 2008). Especially since Young *et al.* (2007) found that no current antifungal treatment has the same consistent treatment outcome across different species and different life stages (Young *et al.*, 2007; Geiger *et al.*, 2011). Consequently a treatment that is effective across all life stages and different species with no side effects for both amphibians and the environment would be of great importance.

1.5. F10 Antiseptic solution

Due to the fact that the skin of amphibians is extremely permeable it makes the skin extremely sensitive and thus requires special disinfectants when treating amphibians (Phillott *et al.*, 2010). Normal disinfectants consist of or include by-products such as alcohol, phenol and iodine, which ultimately lead to the destruction of the mucus/wax layers of amphibians which prevents dehydration of amphibians as well as aiding in the prevention of microbial infections. Furthermore the extensive and continuous use of these disinfectants can lead to systemic absorption of the disinfectants by the amphibians (Wright, 2001).

According to Robertson *et al.* (2008) products or disinfectants such as F10, Virkon or ethanol aren't always the best option for treating amphibians but they remain useful and practical for the cleaning and disinfection of equipment, bench tops and tools used during field work or amphibian husbandry. However, some of these products can still be considered for the disinfection of infected amphibians if the correct treatment protocol can be established. There are several reasons why the disinfection of amphibians is so important, (1) for safety and biosecurity during captive husbandry and laboratory research, (2) prevention and reduction of possible cross-contamination, (3) to treat pathogen and fungal infections and (4) to disinfect tools, bench tops and equipment used on/for amphibians. The chosen disinfectant should thus be safe to use on amphibians without being harmful to the environment, or the animals being treated.

Health and Hygiene (Pty) Ltd in South Africa manufactures the F10 product range which consists of different products depending on the type of application. Some of these products include F10SC (Super Concentrate) Veterinary Antiseptic, F10 Germicidal Treatment Shampoo and F10 Germicidal Wound Spray. F10 is a broad spectrum veterinary disinfectant which is non-corrosive, non-irritant, biodegradable and non-toxic against non-target organisms. The F10 product range's active ingredients consist of ammonium and biguanidine compounds, which work together interactively to eradicate a wide range of viruses, bacteria, fungi and spores. F10 is a surfactant, which breaks down the surface membranes of the target organisms. These products show efficacy at low concentrations with minimal tissue irritation and short contact times. F10 has also been used in the treatment of a wide variety of vertebrates including mammals, birds, reptiles and even amphibians (Barrows, 2007; Webb *et al.*, 2007; Webb *et al.*, 2012).

Treatment application varies according to the specimen at hand and includes nebulisation - the aerosolisation of liquid F10; flushing – nasal flushes are used for the treatment of upper respiratory tract infections in avian and reptilian patients; fogging – as part of routine bio-security measures and can be done in the presence of animals and humans; and wound management – abscesses leaves cavities after draining or flushing it and these cavities can be filled with F10 ointment and left to heal by secondary intention. A wound spray is also available for application on short distances to animals that can't be easily restrained. F10 treatment shampoo has also been used for the successful treatment of dermatomycoses in some animals.

It has been determined that a 1:3000 dilution of F10 can be used as a prophylactic treatment against *B. dendrobatidis* for amphibians during their quarantine period (Webb *et al.*, 2007), although the study has only been done *in vitro*. Wild caught amphibians adjusting to a life in captivity are prone to secondary bacterial and fungal skin infections due to stress and bathing them in the prescribed dilutions will help prevent infection (Barrows, 2007).

According to a study conducted by Blooi *et al.* (2013), F10 Antiseptic Solution had an antifungal activity of >95 % after an exposure time of only 1 minute. However, the zoospores that remained motile after the observation eventually died as growth and development of these zoospores were affected by F10. This could be the result of a decrease in cell membrane integrity and damage that ultimately leads to the death of all the zoospores exposed to F10 (Blooi *et al.*, 2013). F10 subsequently shows the best promise for future use in the treatment of *B. dendrobatidis* as it is proven to be 100% effective in treating *in vitro B. dendrobatidis* at a 1:3000 dilution, and is more effective than many other disinfectants including TriGene and Betadine (Webb *et al.*, 2007; Webb *et al.*, 2012), while being safe for use on amphibians.

STUDY AIM AND OBJECTIVES

The aim of this study was to test the efficacy of F10 veterinary antiseptic as a treatment against the amphibian fungal pathogen *Batrachochytrium dendrobatidis*. Various sequential experiments were devised in order to address the aim. These experiments were grouped into four distinct objectives.

Objective 1: Attempt to infect the tadpoles and juveniles of *Amietophrynus gutturalis* with *B. dendrobatidis.*

Due to the fact that experimental animals are hatched in the lab from wild collected spawn, it is necessary to challenge them with *B. dendrobatidis* fungal cultures in order to induce infection.

Objective 2: Determine the level of toxicity of F10 on *B. dendrobatidis.*

B. dendrobatidis cultures have to be grown *in vitro* and then exposed to different concentrations of F10 in order to determine how long each concentration takes to kill all active zoospores present in each well.

Objective 3: Determine the level of toxicity of F10 on amphibians.

A number of different concentrations vs. different time intervals have to be tested in order to determine which combination has the lowest observed effect concentration (LOEC) to ensure that the treatment does not harm the host.

Objective 4: Establish if the proposed treatment protocol successfully treats *B. dendrobatidis* on live hosts. Hosts will first be infected with *B. dendrobatidis* and then treated with F10 according to the proposed protocol that was developed from the outcome of former objectives.

CHAPTER 2

Material and Methods

In order to fulfil the objectives of this study a phased approach was used to ensure all aspects of the research were covered systematically. By following these four phases (figure 2), we ensured that the minimum number of amphibians were exposed to lethal concentrations of F10, and that the chosen concentration ultimately killed *B. dendrobatidis* without having negative side effects on the experimental subjects.

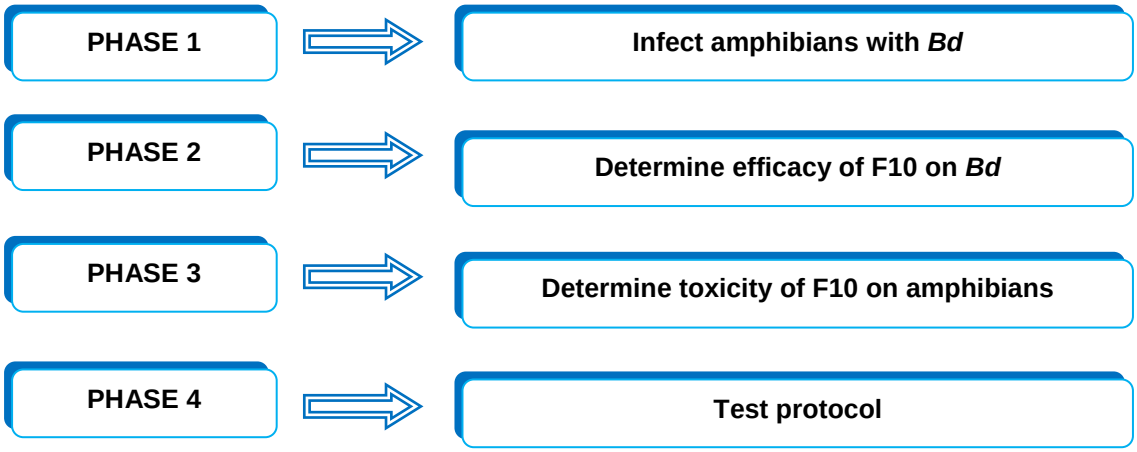


Figure 2: Outlines the four phases used in the methodology.

2.1 Culturing of *Batrachochytrium dendrobatidis*

Two isolates of *B. dendrobatidis* were used in this study, one from the African Amphibian Conservation Research Group (AACRG) collection (MG04, *Amietia fuscigula,* Silver Mine, South Africa) and a European isolate (IA II, Ibon Acherito, Spain) obtained from the Institute of Zoology, London, UK. The isolates are both from the global panzootic lineage (*Bd* GPL), and thus have a high infection potential and virulence. The cultures were kept at 4°C and passaged every 2-3 months since they are able to survive at temperatures between 4 to 6°C in nutrient broth for up to 5 months (Boyle *et al*., 2003, Longcore *et al*., 1999). For the duration of the experiment the cultures were maintained in 1% tryptone broth in 50 ml culture flasks and grown on 1% tryptone agar plates plates

(10g tryptone powder, 10g agar, and 1000ml distilled water) (figure 3) at 21°C and passaged weekly to enable an optimum zoospore yield.

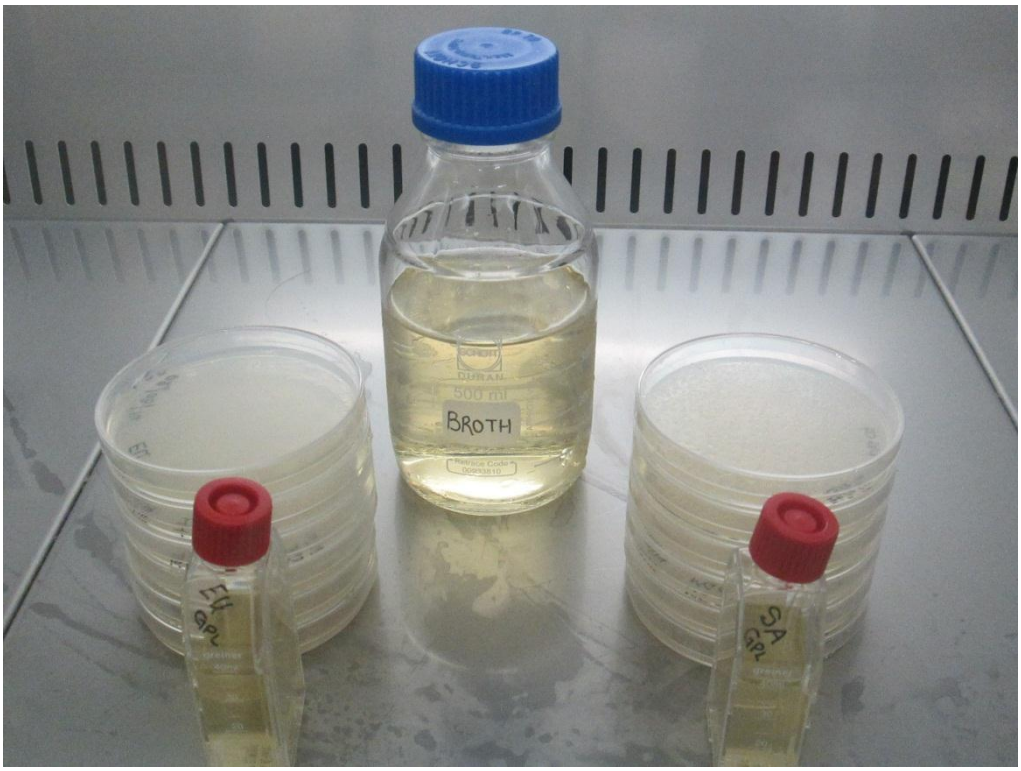


Figure 3: Example of *Batrachochytrium dendrobatidis* cultures kept in culture flasks and spread onto 1% tryptone agar plates.

2.1.1 Harvesting of *Batrachochytrium dendrobatidis* zoospores

Zoospores were cultivated for experiments following the method described in Boyle *et al*. (2004), but with a few modifications. Zoospore suspensions were prepared by adding 1ml of the chosen broth culture to 1% tryptone agar. Plates were left to dry in a laminar flow cabinet until most of the liquid media had evaporated or diffused into the agar, after which the plates were sealed with parafilm, inverted and incubated at 21°C for 4 days (figure 4). Zoospores were harvested by flooding the plates with 5ml of tryptone broth and leaving the plates for 30 min to allow the zoospores to become suspended in the broth. Zoospore density was determined with the aid of a haemocytometer (BOECO).

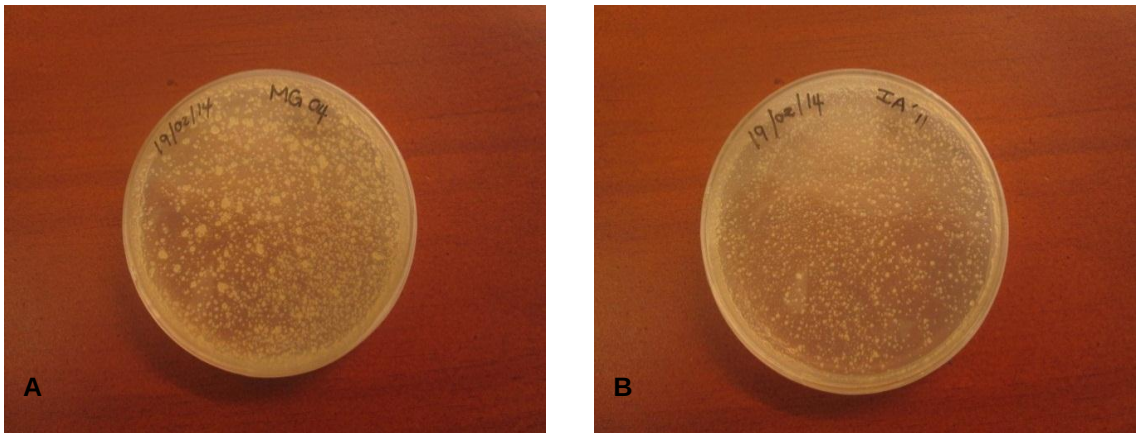


Figure 4: Tryptone agar plates with *Batrachochytrium dendrobatidis* growth used for harvesting zoospores. Plate A represents the South African GPL and plate B the European GPL strains.

2.2 Determining the toxicity levels of F10 on *Batrachochytrium dendrobatidis*

The toxicity protocol of Johnson *et al*. (2003) was followed to test the efficacy of F10 on *B. dendrobatidis*, except for minor modifications as given in the following description. Zoospores were harvested from three day old *B. dendrobatidis* cultures from a South-African strain and a European strain. A volume of 200 µl of broth containing approximately 10,000 zoospores was transferred to 96-well plates.The lids were placed back on the 96 well plates and then sealed with Parafilm and incubated at 21°C for 4 days until a biofilm developed at the bottom of the wells. The supernatant in each well was removed and replaced with a chosen concentration of F10.

The plates were subdivided into the various treatments as indicated in figure 5. *B. dendrobatidis* culture was exposed to different concentrations of F10 (1:5000, 1:7000, 1:10000, 1:15000, and 1:30000) with each concentration consisting of 12 replicates. A control group consisting of 16 wells, which did not receive F10, was also included on each plate. Experimental and control wells were separated by rows of empty cells (no *Bd* or F10) to prevent cross contamination. The control group received 1% tryptone broth instead of F10.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		**A**				**C**	**C**			**B**		
C						**O**	**O**					
D						**N**	**N**					
E						**T**	**T**					
F						**R**	**R**					
G		**C**				**O**	**O**			**D**		
H						**L**	**L**					

Figure 5: Diagrammatic representation of the 96 well plates with A-D indicating the blocks that received different concentrations of F10.

Microscopic observations were done on an inverted microscope (Nikon Eclipse TS100-F) to establish the time it took for all the zoospores within each well to die (no observable motile zoospores present in the well). The F10 was removed and replaced with 1% tryptone broth at different time intervals (see Table 2) with each treatment. After all the wells contained newly added 1% tryptone broth the plates were once again sealed with parafilm and incubated at 21°C for a further 24 hours. This was done to confirm that *B. dendrobatidis* was not able to survive the F10 treatment and reproduce after the F10 was replaced with culture media. Where no active swimming zoospores were observed, the respective treatment of F10 was deemed effective against *B. dendrobatidis*.

Table 2: Duration times for *B. dendrobatidis* exposures to different F10 concentrations. Each treatment/concentration consisted of 12 replicated wells for each of the time intervals tested.

Concentration	Block	Time Exposed
1:30000	A	150 min
	B	120 min
	C	90 min
	D	60 min
1:15000	A	10 min
	B	30 min
	C	60 min
	D	90 min
1:10000	A	5 min
	B	10 min
	C	15 min
	D	30 min
1:7000	A	5 min
	B	10 min
	C	15 min
	D	30 min
1:5000	A	5 min
	B	10 min
	C	15 min
	D	30 min
1:3000	A	5 min
	B	10 min
	C	15 min
	D	30 min

2.3 Collection and husbandry of amphibians

Egg strings from 5 clutches of the species *Amietophrynus gutturalis* (Guttural toad) were collected from a manmade pond in the Potchefstroom area (-26.679976, 27.095241) on 01/11/2013. The species *A. gutturalis* was chosen since this species has been used as a model species for doing *B. dendrobatidis* challenge experiments in our research group AACRG for the past five years. The collection methods, husbandry protocols and experimental procedures are known for this species and more importantly we know that it is susceptible to *B. dendrobatidis* infection. The eggs were housed in the AACRG bio secure laboratory in five 42cm x 28cm aerated plastic containers filled with approximately 4L of borehole water (until they hatched). Temperature in the lab was kept at a steady 20°C for the duration of the experiment. The tadpoles were moved to three 30cm x 40cm aerated aquaria (figure 6) with approximately 18L of aged borehole water one week after hatching from the different clutches (200 tadpoles per aquarium). Tadpoles were fed a steady diet of 4 grounded Tetra tabimin pellets (fish food pellets) per tank every day, and the water was changed every 2nd day. The tadpoles from one aquarium were used for the challenge experiment on tadpoles, while the tadpoles from the other two aquaria were allowed to complete metamorphosis undisturbed.

Figure 6: Tadpoles kept in aerated tanks until metamorphosis.

Tadpoles presenting with all four limbs were removed and placed in a separate slanted aquarium (figure 7). This was done to ensure the individuals had both aquatic and terrestrial habitats available. Once their tails were reabsorbed juveniles were individually kept in plastic tubs with wetted tissue paper (34cm circumference, figure 8). Juveniles were fed 3-5 pinhead crickets 3 times a week and the tissue paper changed twice weekly. Lighting for all experimental animals was set at 12 hours day/night cycles.

Figure 7: Slanted aquarium with aquatic and terrestrial habitats used to keep tadpoles presenting with four limbs.

.

Figure 8: Plastic tubs in which juvenile guttural toads were housed individually

2.4 F10 toxicity trials on *Amietophrynus gutturalis*

The lowest observed effect concentration (LOEC) of F10 was determined for *Amietophrynus gutturalis*. According to Crane and Newman (2000) the LOEC can be defined as: “The lowest test concentration having a mean response that differs significantly from that of the control”. The LOEC approach was thus used in this study to measure what the highest acceptable concentration of F10 is for both tadpoles and post-metamorphic amphibians of the focal species. By determining the LOEC we can be sure that only the minimum number of amphibian’s die from lethal concentrations of F10, and we can determine which concentrations are safe to use whilst remaining effective against *B. dendrobatidis*.

The final LOEC value in this study was obtained by doing 5 consecutive experimental trials aimed at minimizing the number of test animals that succumb from the experiment. During the first few tests only one amphibian was tested against different concentrations and different exposure times. Only at the final stage of the LOEC were the safe concentrations and exposure times tested on multiple individuals (see Crane and Newman, 2000). Each concentration vs. exposure time was terminated when one of the symptoms listed in table 3 occurred.

Table 3: LOEC endpoint symptoms used to determine toxicity of F10 on *Amietophrynus gutturalis*.

Tadpoles	Juveniles
Change in frequency/intensity of movement	Change in frequency/intensity of movement
Swimming upside down	Excessive shedding of skin
No swimming or other movement	Rapid breathing or eye blinking

2.4.1 Trial 1: Verification of the known 1:3000 effective concentration

According to Webb *et al*. (2007), F10 was 100% effective at killing *B. dendrobatidis* culture *in vitro* with a 1:3000 concentration after only 1 min of exposure time. A 1:250 stock solution of F10 was further diluted to 1:3000 concentration by adding 41.67 ml F10 to 458.33 ml dH_2O to obtain 500 ml of the dilution. The following equation was used to determine the amount of F10 required for 500 ml of the dilution:

$$\text{Amount Dilution Required} = \frac{\text{Strength required}}{\text{Stock strength}} \times 500 \text{ ml}$$

$$= \frac{1 \div 3000}{1 \div 250} \times 500 \text{ ml}$$

Tadpoles:

Five plastic beakers were filled with 100ml of the F10 dilution and five beakers were filled with borehole water. One tadpole (Gosner stage 25) per beaker was added to the beakers containing the F10 dilution and exposed for 0.5, 1, 2, 3 and 4 min respectively. The behaviour of each tadpole was observed to determine when adverse effects started to occur, which indicated the LOEC endpoint. Tadpoles were removed from the F10 dilution at the onset of any of these signs of stress and placed in the beakers containing the borehole water. Tadpoles (all experimental animals) were kept for 7 days to ascertain whether any latent adverse effects to F10 would occur, after which they were euthanized with Tricaine Methanesulfonate (MS-222). Specimens were stored in micro tubes containing 70% ethanol. This processing of individuals that survived exposure was repeated for all experimental trials on both tadpoles and juveniles.

Juveniles:

Since juveniles tend to climb onto the sides of beakers, petri dishes were used during exposure to ensure that they stayed in contact with the F10 dilution for the duration of the exposure. Five petri dishes were filled with 20ml of the F10 dilution and five were again filled with borehole water. Juveniles were placed in the petri dishes containing the F10 dilution and exposed for 3, 4, 5, 15 and 30 min respectively. The behaviour of each juvenile was observed under a stereo microscope to determine when adverse effects started to occur (LOEC endpoint). Juveniles were removed from the F10 dilution at the onset of any of these signs of stress and placed in the petri dishes containing the borehole water for 5 min in an effort to rinse them, after which they were returned to their individual enclosures.

2.4.2 Trial 2: Determination of the range of effective concentrations (range finding test)

By testing a wide range of concentrations with large steps between consecutive concentrations, it is possible to find those concentrations that are safe to use on the toads and their tadpoles. The following concentrations were used: 1:100, 1:500, 1:1000, 1:5000 and 1:10000 along with a control group exposed to borehole water. Dilutions were prepared and exposures conducted following the same method described in 2.4.1 except that the exposure time remained constant for a maximum of 5 min for each separate concentration.

2.4.3 Trial 3: Verification of the LOEC

F10 concentrations identified by the range finding test were used to determine the LOEC, which yields an even more precise safe concentration for the experimental animals. Tadpoles and juveniles were exposed to a series of three F10 dilutions with concentrations more diluted than the lowest lethal concentration, but more concentrated than the highest safe concentration. Again the same methods described in 2.4.1 were followed, except the experiment was conducted at 5 min and 15 min exposure times respectively.

2.4.4 Trial 4: Determine the effect of variable exposure times

The exposure times were increased using the LOEC for both tadpoles and juveniles respectively to determine if the animals can tolerate the safe concentration for a period of time that will be practical to apply. A treatment was deemed practical if it allowed the applicant enough time to treat at least 10

animals simultaneously, and still prevent over-exposure resulting from prolonged exposure to F10 that exceeded the maximum time allowed.

Ten different tadpoles were subsequently exposed to each of the respective safe treatments (combination of F10 concentration and exposure time) for different lengths of time to determine a concentration vs. exposure time combination that can still be deemed safe to use on the animals yet still be effective against *B. dendrobatidis*, and would thus present the lowest possible risk to both the tadpoles and juveniles. Once this combination was determined the experimental animals were exposed for 30 min to 1:10000 and 1:3000 concentrations respectively (the LOEC's for tadpoles and juveniles).

2.5 *Batrachochytrium dendrobatidis* challenge experiments

2.5.1 Exposure of tadpoles with *Batrachochytrium dendrobatidis*

The procedure for infecting tadpoles was consistent with the protocol of Garner *et al*. (2009), with infection taking place via zoospore inoculation, but with a few minor differences. The experiment consisted of two experimental units: a treatment group that was exposed to *B. dendrobatidis* culture (Both SA-*Bd* GPL as well as EU-*Bd* GPL) and a sham (control) group that received filtered *B. dendrobatidis* culture (culture containing active *B. dendrobatidis* zoospores was filtered with a sterile 0.2 µm single use syringe filter unit). It is more beneficial to the outcome of the experiment when a sham (inactive) treatment can be used instead of just a normal control group, this is due to the fact that it exposes all experimental groups to the most similar treatment regime (excluding only the factor that needs to be controlled which was live *B. dendrobatidis* culture in this case). The filtration procedure removed all live *B. dendrobatidis* thalli from the media, while still allowing all other *B. dendrobatidis* related metabolites to pass through the filter. In doing so the group that received the sham treatment was exposed to similar "discomfort" as the experimental treatment groups, apart from them receiving the infective pathogen.

Each group consisted of 100 randomly selected tadpoles between developmental stage 30 and 32 (Gosner, 1960), which were divided into 4 separate containers. All the containers received 12 hours of light per day and their water temperature was kept at 20°C. The developmental stage of all the tadpoles were identified with the normal table of Gosner (1960). The tadpoles were not weighed since we assumed a similar body mass for them as they were selected based on similar developmental stages.

The tadpoles in the treatment group were inoculated with active *B. dendrobatidis* zoospores for one hour each day for a total of 10 inoculations. Culture growth varied between days, which resulted in the slight variation in zoospore density of 35,000 to 50,000 zs/tadpole per inoculation. The same inoculation volume was used for the treatment group and the sham group in order to ensure that the only difference between the two treatments was the presence or absence of the transmissible zoospores.

Twenty tadpoles from each experimental group (5 tadpoles per container) were randomly selected to screen for *B. dendrobatidis.* Infection status was confirmed by microscopic screening of oral tissue prepared on wet mounts. The lower half of the oral disc (containing the lower keratodonts as well as rostrodont) was surgically removed with the aid of a stereo microscope. Temporary slides (wet-mounts/whole-mounts) were made by mounting the excised oral disc on a microscope slide in a drop of distilled water and covering it with a cover slip. A standard compound microscope was used to examine the wet mounts at 100x and 400x magnification. To positively identify the presence of *B. dendrobatidis* there had to be spheroid, septate, walled sporangia present in the host tissue (Smith *et al*., 2007).

2.5.2 Exposure of juveniles with *Batrachochytrium dendrobatidis*

The pathogen exposure of juveniles was consistent with the protocol used by Garner *et al*. (2009), with infection taking place via zoospore inoculation, but with a few minor differences. The experiment consisted of nine experimental units of 30 animals each, thus totalling 270 juvenile toads. All juveniles were weighed at the start and at the end of the experiment. Four groups were exposed to South African *B. dendrobatidis* GPL of which one received 5 x F10 treatments, another received 3 x F10 treatments, and the other one received only a single F10 treatment, with the fourth group serving as a positive control which did not receive any treatment with F10. The same F10 treatment groups were followed for the juveniles in the remaining groups (table 4), with the exception that they were exposed to European *B. dendrobatidis* GPL. The ninth (and

last) juvenile group served as a negative control, thus receiving only sterile culture media instead of live *B. dendrobatidis* culture followed by treatment with sterile water instead of F10.

Table 4: *Amietophrynus gutturalis* juvenile experimental units used to test the efficacy of F10 against *Batrachochytrium dendrobatidis*.

Group	Isolate	No. of F10 treatments
1 x F10 (SA-*Bd*)	MG 04	1
3 x F10 (SA-*Bd*)	MG 04	3
5 x F10 (SA-*Bd*)	MG 04	5
1 x F10 (EU-*Bd*)	MG 04	1
3 x F10 (EU-*Bd*)	IA 11	3
5 x F10 (EU-*Bd*)	IA 11	5
SA-*Bd*, No F10 (positive control)	IA 11	0
EU-*Bd*, No F10 (positive control)	IA 11	0
No *Bd*, No F10 (negative control)	BROTH	0

The body mass of each juvenile was determined by weighing them with a Scaltec SBC 32 (sensitivity 0,01g), which is in compliance with ISO 9001. The Tukey HSD test was calculated for each group to test for variance in body mass among the groups prior to the start of the experiment. Body mass was again taken at the end of the experiment to determine if any significant weight loss/gain took place during the experiment.

After being weighed the juveniles were individually exposed for five hours to either live *B. dendrobatidis* culture or sterile culture media in 90mm petri dishes containing 15ml borehole water as shown in figure 9 and figure 10. Juveniles were dosed with an average of 628000zs/ml every three days for a total of five doses. The juveniles were kept for seven days to allow an infection to establish before commencing with F10 treatments.

Figure 9: Juveniles were individually placed in petri dishes when dosed with *Batrachochytrium dendrobatidis* zoospores.

The F10 treatments were also performed on the juveniles in petri dishes containing 15ml borehole water (figure 9). Juveniles received an 8 min exposure to a 1:3000 concentration of F10 during each treatment. Groups that received more than one treatment received F10 on consecutive days. Juveniles that died during the experiment were placed in micro tubes with 70% ethanol. All the remaining survivors of all the various groups were kept for 5 days after their last F10 treatment, after which they were euthanized with Tricaine Methanesulfonate (MS-222) and stored in 1.5 ml micro tubes filled with 70% ethanol.

Figure 10: Juvenile being dosed with broth instead of active *Batrachochytrium dendrobatidis* zoospores.

2.5.3 Screening of juveniles for *Batrachochytrium dendrobatidis*

A non-invasive technique was used to screen for *B. dendrobatidis* before the first F10 treatment was performed, by means of swabbing the juvenile toads with sterile cotton swabs. The swab technique entails running the swab 5 times over the dorsal surface, the ventral surface, the thighs, and hind feet (adapted from Kriger *et al*., 2006). Gloves were changed after each juvenile was swabbed to ensure that no disease transmission took place between individuals and thus prevent false positives from occurring. Swabs were placed back in their original capsules and stored at 4°C until they were tested for *B. dendrobatidis* with quantitative/real-time polymerase chain reaction (qPCR). At the conclusion of the experiment all the juveniles were euthanized with Tricaine Methanesulfonate (MS-222) where after they were once again weighed and placed in microtubes containing 70% ethanol.

The swabs and whole frog tissue samples were subjected to qPCR for the diagnosis of *B. dendrobatidis* using a modified Boyle *et al*. (2004) protocol. Nucleic acids were extracted using 70 µl PrepMan Ultra (for the cotton tips) and 40 µl PrepMan Ultra (for the whole frog tissues). DNA was extracted by cutting the cotton from the swabs as well as cutting the hind limbs from the juveniles and adding them to microtubes containing the

PrepMan Ultra. The samples were then vortexed for 5 min, centrifuged for 1 min (16000 rcf), placed in a heating block for 10 min and once again centrifuged at 16000 rcf for 3 min. Once the DNA was extracted the Taqman PCR assay was followed, which consisted of mixing a master mix with the extracted DNA. The master mix is a solution containing forward primer, reverse primer, probe and double distilled water of which the quantities are provided in table 5. Once the master mix was completed 20µL of the master mix was added to 5µL of the DNA in a 96-well plate and loaded in the sequence detection system.

Table 5: Components used in the mix for real-time PCR of *Batrachochytrium dendrobatidis.*

	Working Dilution Concentration	Final Concentration	µL	24	24
Taqman	-	-	25	600	600
Forward primer	10 µM	10 pmol	2.5	2.5	60
Reverse primer	10 µM	10 pmol	2.5	2.5	60
Probe (light sensitive)	100 pmol	-	0.125	3	3
Double distilled water	-	-	9.875	237	237
Volume of master mix	-	Total	20	20	20
gDNA	-	-	5	5	5
Total volume of rxn	25 µL	Per tube	25	25	25

CHAPTER 3

Results

3.1 F10 killing effect on *Batrachochytrium dendrobatidis*

After incubating *Batrachochytrium dendrobatidis* for three days in 96 well-plates, a 100% growth rate was established in all of the experimental and control wells. A white biofilm was visible at the bottom of each well representing a layer of the fungal thalli. The wells exposed to the various F10 concentrations showed no survival after a certain time had elapsed (Table 6). The fungal colonies in each well were considered dead if no active zoospores were observed for a period of 1 min after the last swimming zoospore was observed, followed by 24 hours of incubation (F10 replaced with tryptone broth) where after the wells were declared 100% *B. dendrobatidis*-free if no signs of an active growing culture were observed. *B. dendrobatidis* survival time for both strains progressively decreased with increasing concentrations of F10. Differences existed in mean kill time interval for three of the concentrations, each time the European strain took longer than the South African strain. It took 1:5000 F10 just 5 min to kill both fungal strains (thus not necessary to test 1:3000 or less since 5 min would also kill 100% *B. dendrobatidis* for any stronger concentrations), while it took 1:30000 F10 30 min longer to kill the European strain at 150 min.

Table 6: Survival time of *Batrachochytrium dendrobatidis* isolates from both South Africa (MG04) and Europe (IA11) following exposure to different concentrations of F10. Each concentration had a total of 48 replicates per isolate.

F10 Concentration	Isolate Mean Time Interval Till Death	
	MG04	IA11
1:3000	5 min	5 min
1:5000	5 min	5 min
1:7000	5 min	10 min
1:10000	10 min	10 min
1:15000	10 min	30 min
1:30000	120 min	150 min

3.2 Safe dosage and treatment time of F10 for *Amietophrynus gutturalis*

The 1:3000 (theoretical standard) was first to be tested separately on tadpoles and juveniles where after the range finding test followed. Our range finding test indicated that juvenile toads survived 5 min of exposure to F10 concentrations of up to 1:500, but died 20 minutes after exposure to 1:100 (Figure 11). The juveniles didn't show any signs of side effects at the lower concentrations and survived seven days post exposure to F10. The juveniles were able to withstand concentrations up to 1:500 without any side-effects; however juveniles exposed to stronger concentrations than that, showed abnormal behaviour such as trying to escape from the exposure tubs, blinking rapidly and struggling to right themselves. The juveniles that survived the stronger concentrations had excessive skin shedding afterwards and showed signs of redness around the eyes, feet and hind legs. They also appeared uncoordinated as well as being sluggish and seemed to try and keep their legs away from their bodies. They also died soon after exposure and felt hard, rubbery and swollen when examined after death. All the individuals that survived 24 hours after exposures including all control animals remained alive until day 7 when they were euthanized. Although the highest concentration survived by juveniles was 1:500, it is suggested that a safer, yet effective working concentration would be from 1:1000 - 1:3000.

The outcome of the LOEC experiment for juvenile toads indicated that all concentrations were safe to use at 5 min exposures; although not 100% safe for 1:1000 (Table 7). However, longer exposure times (15 min) proved to be lethal at the high-end concentration of 1:1000. Concentrations lower than 1:3000 were not tested since a 100% survival rate was already obtained at concentrations as high as 1:2000.

Table 7: Lowest observed effect concentrations (LOEC) for toad juveniles when exposed to F10 for different dosage times.

Concentration	Survival (%)	
	5 min	15 min
1:1000	80	0
1:2000	100	100
1:3000	100	100

Tadpoles did not survive exposure concentrations greater than 1:5000, with survival times decreasing rapidly as concentrations increased (figure 11). Tadpoles that survived the stronger concentrations exhibited some of the LOEC signs such as being sluggish and swimming upside down with a slight reddening of the ventral part of their bodies. Once these symptoms were exhibited they weren't able to swim normally even after being rinsed and placed in clean borehole water. Some of the tadpoles were able to survive up until they were rinsed where after they died and became rigid and rubbery. Thus the safe effective concentration was estimated to be between 1:5000 - 1:10000 for the tadpoles to eliminate any toxic side effects that may occur.

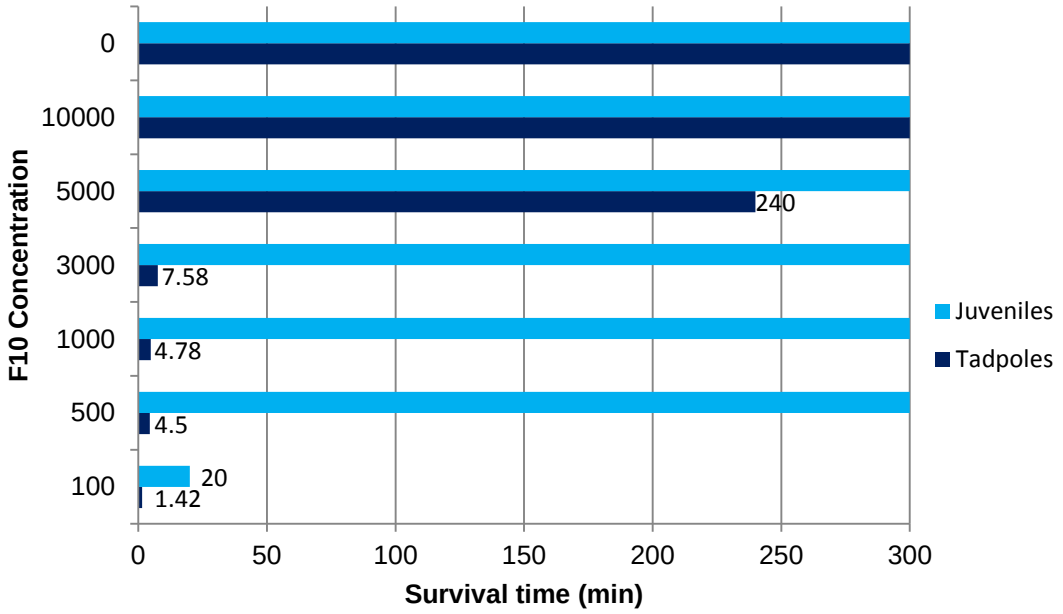


Figure 11: Survival time of tadpoles and juveniles when exposed to different concentrations of F10 (0=Control). Exposure time was kept constant for a maximum of 5 min. Individuals that survived up to 300 min post-exposure, survived for another seven days before being euthanized.

Tadpoles exposed to the stronger concentrations namely 1:100, 1:500, 1:1000 and 1:3000 had to be removed after only 3 sec, 21 sec, 23 sec and 40 sec exposures respectively as they were exhibiting toxicity symptoms. The LOEC experiment indicated that 1:10000 was the only safe F10 concentration for tadpoles across all exposure times, although still demonstrating slight toxicity at a prolonged exposure of 30 min (Table 8). The 1:7000 concentration was only safe to use on tadpoles for 5 min. Those

concentrations demonstrating less than 80% survival at any exposure time was deemed unsafe and exposures were no longer conducted for that concentration.

Table 8: Lowest observed effect concentrations (LOEC) for toad tadpoles when exposed to F10 for different dosage times.

Concentration	Survival (%)		
	5 min	15 min	30 min
1:5000	100	20	-
1:6000	100	30	-
1:7000	100	80	-
1:10000	100	100	80

3.3 Clearance of *Batrachochytrium dendrobatidis* using F10

3.3.1 *Amietophrynus gutturalis* susceptibility to *Batrachochytrium dendrobatidis*

All 20 tadpoles from both exposure groups (SA-*Bd* and EU-*Bd*) were infected in the region of the oral disc. Sporangia of various developmental stages (e.g. containing zoospores, discharge papilla visible, empty sporangia) were present between the tooth rows and on the jaw sheaths of the upper and lower jaws. None of the 20 randomly selected tadpoles from the sham group showed any signs of *B. dendrobatidis* infection and were therefore considered to be *B. dendrobatidis*-free. The average body mass of the tadpoles for the sham group was 0.1386g (SD = 0.04173) and 0.1501g (SD = 0.0509) for the combined experimental group, while the average Gosner stage was 30 for both groups.

The juveniles were measured prior to the *B. dendrobatidis* dose experiment to make sure the various groups had similar body length and body mass measurements. The body mass from the negative control group as well as both the SA-*Bd* and EU-*Bd* groups along with their respective positive control groups were tested for variability among groups. The p-values all showed that there was no significant difference in body length and body mass between any of the groups prior to the experiment since marked effects are only significant at $p < 0.05$ and there were none.

Both *B. dendrobatidis* groups suffered more deaths than initially anticipated during the experiment, especially the EU-*Bd* group. Increasingly more individuals from the EU-*Bd* group started dying seven days after the last *B. dendrobatidis* dose was administered (figure 12). On the day of swabbing prior to F10 treatment only 31.6% of the EU-*Bd* group was still alive of which almost half died immediately after swabbing because they were too weak to cope with the stress of swabbing. This left a remaining 19.1% of the original group to be euthanized rather than continue the experiment with them. The SA-*Bd* group also suffered mortalities, but had a 73.3% survival rate even after swabbing and could thus be used for treatment with F10. The control group however suffered no deaths during this time.

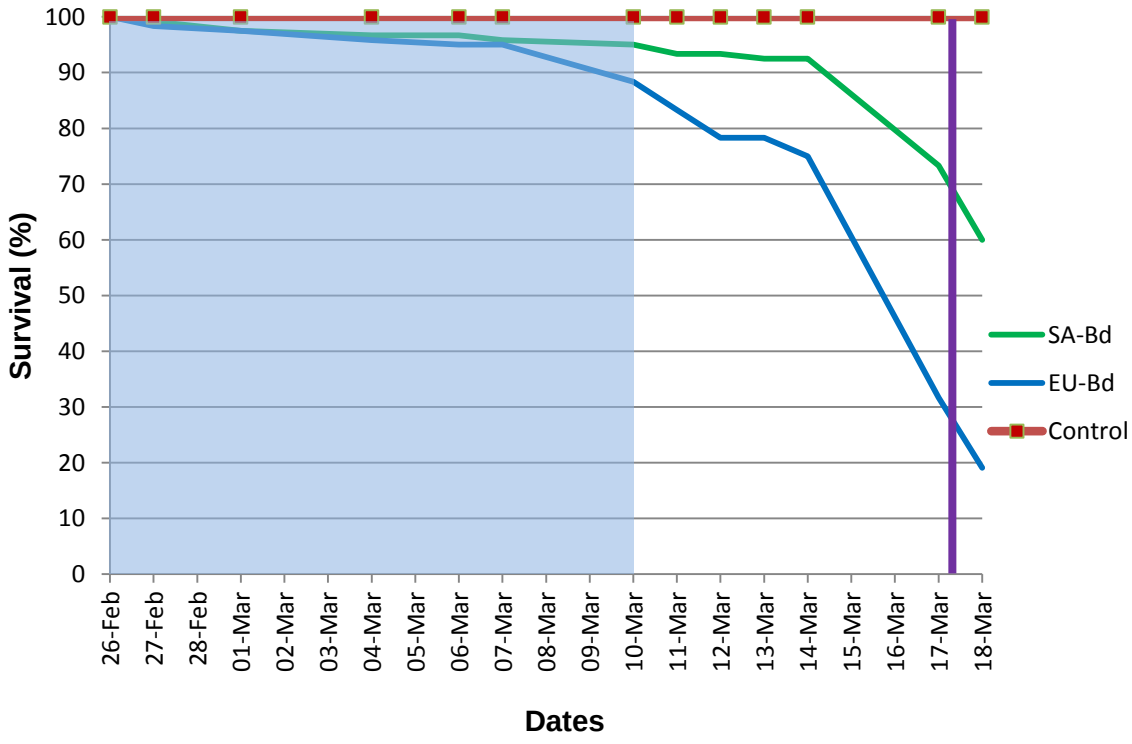


Figure 12: Percentage survival of *Amietophrynus gutturalis* during and following the experimental challenge with *Batrachochytrium dendrobatidis*. Dosing commenced on 26 February and ended 10 March (shaded area). A period of seven days was allowed for the infection to establish. Animals that were still alive by 17 March (purple line) were swabbed for *Batrachochytrium dendrobatidis* diagnosis.

According to the PCR results obtained the EU-*Bd* resulted in a 100% infection rate in all four of the exposed juvenile groups. Whereas the SA-*Bd* exposed groups never resulted in a 100% infection (Figure 13). Instead, infection prevalence ranged between 71 – 93% for the various SA-*Bd* exposed groups. The negative control group remained *B. dendrobatidis* free throughout the experiment.

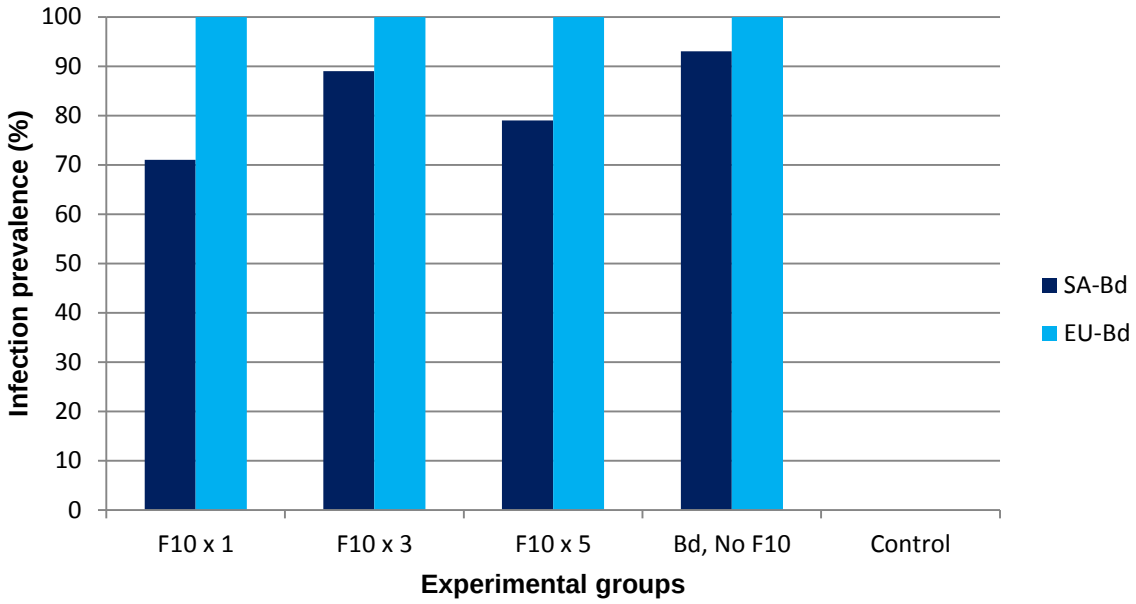


Figure 13: PCR results showing infection prevalence of *Amietophrynus gutturalis* juveniles following exposure to *Batrachochytrium dendrobatidis* and prior to F10 treatment. The control group was exposed to culture media only.

3.3.2 Clearance of infection with F10

As a general indication of body condition, the body mass of juveniles was measured before exposure to *B. dendrobatidis* and after treatment with F10. Mean body mass was lower in all of the treatment and control groups at the end of the experiment. The loss in body mass was evident for the time between the last exposure and the end of the treatment phase. Both toads that were exposed to live *B. dendrobatidis* as well as toads that were exposed to a sham solution experienced a loss in body mass (Table 9). This measured loss in body mass was only marginal, except in toads that were exposed to *B. dendrobatidis* and did not receive F10 treatment in which case it was almost significant ($p = 0,069$). The toads from this group were visibly emaciated and weak and could no longer successfully catch the prey items they were offered

Table 9: Mean body mass (Mb) of *Amietophrynus gutturalis* from the various treatment groups before *Batrachochytrium dendrobatidis* exposure, thus at the start of the experiment and their mean body mass (Mb) at the end of the treatment phase. Significant differences in body mass for each group were calculated by the Tukey HSD test ($p < 0.05$ indicates significant effects).

Treatment	Mean start Mb	SD	Mean end Mb	SD	P
1 x F10	0.099	0.0269	0.093	0.015	0.382
3 x F10	0.107	0.0272	0.103	0.028	0.675
5 x F10	0.104	0.0284	0.099	0.022	0.694
SA-*Bd*, No F10	0.119	0.0360	0.079	0.013	0.069
No *Bd*, No F10	0.107	0.0220	0.100	0.018	0.195

The remaining 73.33% juveniles of the SA-*Bd* groups received F10 treatment baths according to the schedule dictated by their treatment groups. All experimental groups were treated with a 1:3000 concentration of F10. The control group only received baths of borehole water instead of F10. The group that received only one dose of F10 had a *B. dendrobatidis*-clearance rate of only 60% (figure 14). The treatment groups that received multiple F10 doses had better success in clearing *B. dendrobatidis* with 88% and 86% clearance in the F10 x 3 and F10 x 5 groups respectively.

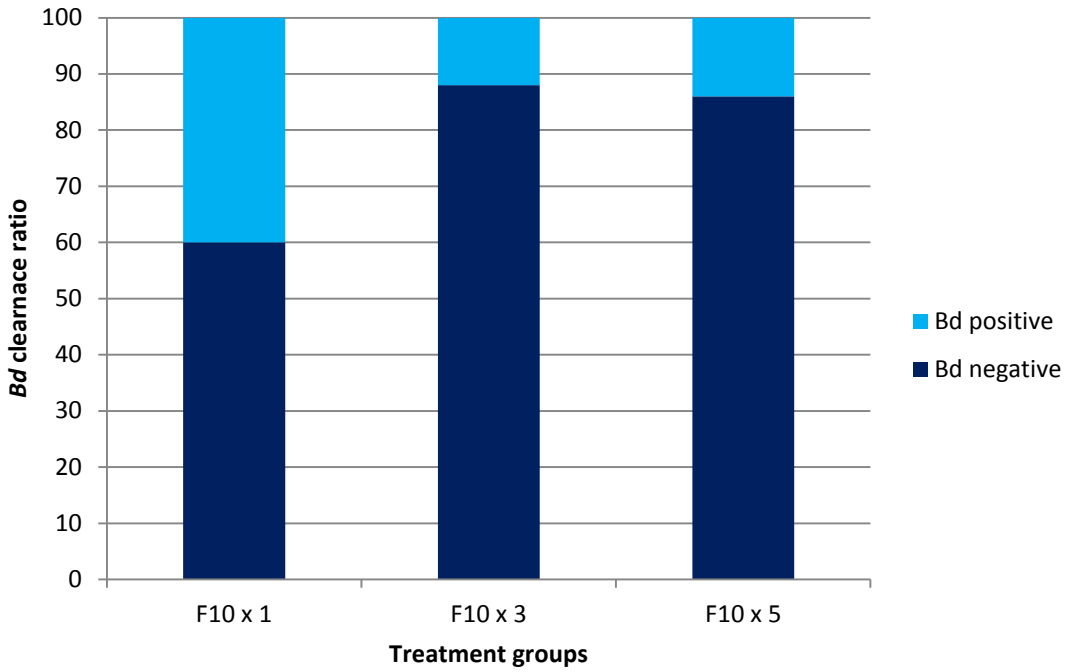


Figure 14: *Batrachochytrium dendrobatidis* clearance success in *Amietophrynus gutturalis* according to their F10 dosage.

All the groups that were formally exposed to *B. dendrobatidis* continued to experience some mortalities during the F10 treatment phase (figure 15), but the group that received no F10 treatment had the highest number of deaths, while no mortalities were recorded in the control group. More importantly the numbers of the surviving juveniles stabilized after treatment with F10, while the positive control group continued to suffer mortalities.

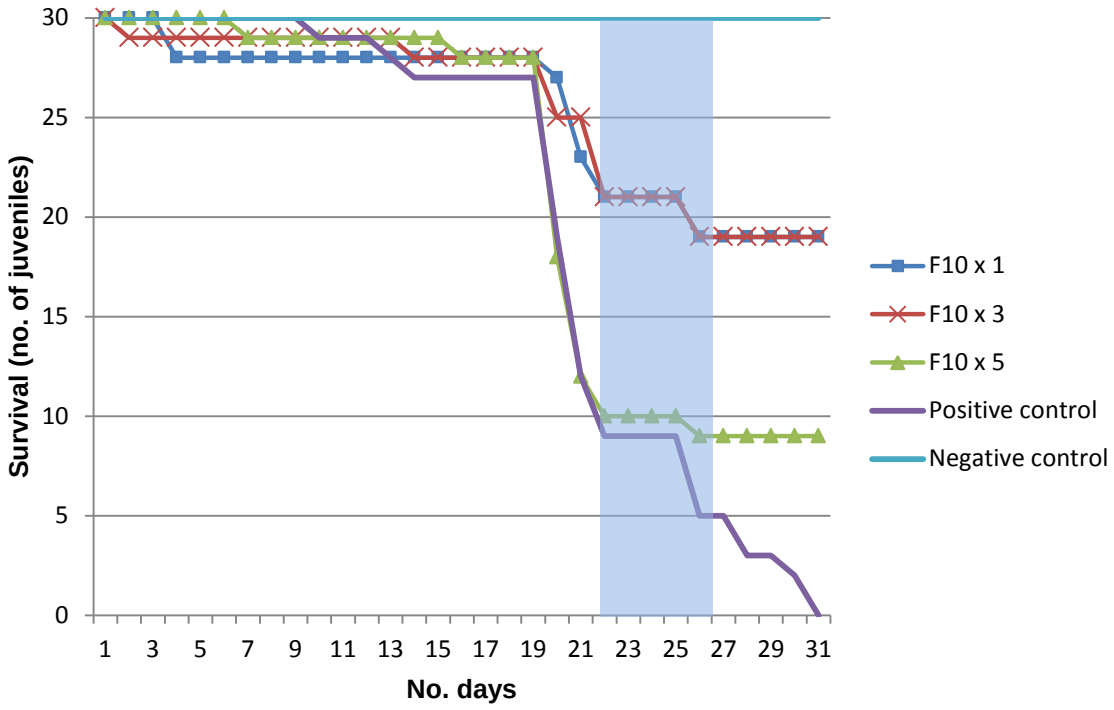


Figure 15: Death curve of *Amietophrynus gutturalis* juveniles following exposure to South African *Batrachochytrium dendrobatidis* and subsequent F10 treatment. The shaded area indicates the days in which F10 treatments were applied.

CHAPTER 4

Discussion

The sudden and catastrophic decline in amphibians is more than just the loss of an interesting and large group of species. They form a crucial part of many ecosystems and communities from which even mankind benefits, be it directly or indirectly. Their disappearance in ecosystems can cause whole ecological communities and food chains to collapse especially where they act as predators for flies, mosquitoes etc. or even as prey for certain mammals, birds and reptiles. They also comprise a near global distribution with highly permeable skin enables them to also act as ideal indicators of ecosystem health (Blaustein and Wake, 1995), which has become increasingly more important as environmental degradation and community instability increases with the increase in pollution (Warkentin *et al.,* 2009). These are only a few of the reasons why the protection and survival of amphibians are of the utmost importance. Since recent studies have shown that a large number of their declines can be attributed to factors such as pathogens (Alford and Richards, 1999; Stuart *et al.*, 2004; O'Rourke, 2007), it stands to reason that the study of these pathogens, especially *B. dendrobatidis*, should be just as important.

Several studies have been conducted in recent years on many aspects relating to *B. dendrobatidis* following its identification and description in 1999 (Longcore *et al.*, 1999), including its pathogenesis, biology and life cycle, transmission and infection as well as its distribution.

In an attempt to prevent the permanent loss of all amphibians the Amphibian Conservation Action Plan (ACAP) was established to ensure the conservation and disease management of amphibians. However in order to make the successful reintroduction of disease-free amphibians achievable as part of post-exposure, pre-exposure or preventative measures, *B. dendrobatidis* has to be removed or mitigated from the environment (Martel *et al.*, 2011). This will not only benefit wild populations but captive bred populations as well, especially amphibian species that are currently endangered. Methods to control this pathogen will help to increase population sizes through reintroduction programs and aid towards establishing disease-free populations.

Although there is currently published data on treating infected amphibians, none of it offers a permanent or adequate solution to the problem that is chytridiomycosis.

Nevertheless there are presently several disinfectants used worldwide when it comes to the *in vitro* treatment of *B. dendrobatidis*. Which includes bleach, Virkon, benzalkonium chloride (Johnson *et al.*, 2003), betadine, TriGene, Itraconazole (Garner *et al.*, 2009) and F10 (Webb *et al.*, 2007). In this chapter we discuss how many of these products were used in experimental studies in treating *B. dendrobatidis* infection (with variable success) as well as the outcome of experimentally infecting *Amietophrynus gutturalis* with *B. dendrobatidis* and subsequently treating them with F10. Ultimately an effective antifungal should not only be able to kill *B. dendrobatidis in vitro* but also be able to treat all amphibians suffering from chytridiomycosis regardless of species or life stage. In aquatic and semi-aquatic amphibian species for instance where tadpoles and adults share the same habitat, continued transmission between life stages can occur if both are not cleared of infection. However, practical application of the antifungal has to be considered when deciding on a concentration and exposure time. When using any type of chemical treatment or antifungal disinfectant, care must always be taken to avoid any and all possible environmental contamination (Johnson *et al.*, 2003). The idyllic chemical disinfectant or antifungal treatment should be affordable, readily available as well as practical and easy to apply without any harmful side-effects to the environment or treated animal. Short exposure times are also necessary when disinfection of equipment, tools and personnel is necessary and also to ensure that treatment doesn't become labour-intensive, especially when treating large groups of amphibians.

In order for us to achieve the aim of our study which was to test the efficacy of F10 veterinary antiseptic as a treatment against the amphibian fungal pathogen *B. dendrobatidis,* we had to address several study objectives. These objectives included the successful infection of tadpoles and juveniles *in vivo* with *B. dendrobatidis* zoospores, determining the toxicity of F10 on both *B. dendrobatidis* as well as amphibians and to test our proposed treatment protocol by experimentally infecting juvenile *A. gutturalis* juveniles with *B. dendrobatidis* and then treating them with F10.

4.1. F10 is toxic to *B. dendrobatidis*

Batrachochytrium dendrobatidis has become one of the most difficult pathogens to treat in recent years, however in culture *B. dendrobatidis* appears to be extremely sensitive to the majority of chemicals such as F10, TriGene, Betadine, Itraconazole, Bleach, Ethanol, Sodium Chloride, Potassium permanganate etc. However, differences exist among chemicals concerning the concentration that kills 100% of the zoospores and the concentration that allows growth and encystment (Berger *et al*., 2009). Several studies by numerous researchers have been done on possible treatment methods for *B. dendrobatidis*. Some of these treatments include the use of heat, antimicrobial peptides, probiotics, and chemical/antifungal treatments such as malachite green, chloramphenicol, itraconazole and F10 antiseptic solution. The most widely used disinfectant of all remains bleach at much higher concentrations than the other above mentioned products. However, bleach cannot be used on amphibians without causing negative side-effects and thus offers no permanent solution for chytridiomycosis.

A more suitable solution however could lie in the antiseptic F10, which in a study conducted by Webb (2007) was proven to be effective in killing *B. dendrobatidis* 100% with concentrations as little as 1:3000. Therefore we attempted to determine if concentrations other than 1:3000 are effective in killing *B. dendrobatidis in vitro* and at the same time be safe to use on amphibians as a possible treatment option for *B. dendrobatidis* infection. In order to determine the right combination of dose concentration and exposure time we had to test several concentrations of F10 on *B. dendrobatidis* grown *in vitro* in 96-well plates since the minimal inhibitory concentration (MIC) of F10 obtained may be lethal to live animals and in that case we had to have other suitable concentrations to use. Ultimately the EU-*Bd* proved more difficult to kill than the SA-*Bd* and subsequently longer exposure times were necessary to obtain a 100% death rate. Higher resilience of EU-*Bd* to F10 could be because of its propensity to grow more aggressively than the SA-*Bd*. F10 however proves to have the same effect on both these isolates when the concentration is 1:5000 or stronger and has a 100% death rate after only 5 min exposure. These results would suggest that a brief 1-5 day treatment with F10 at very low concentrations should be sufficient to eliminate *B. dendrobatidis* from infected amphibians at all life stages, although when treating infected amphibians the *B. dendrobatidis* strain should be taken into consideration if possible. This is just a precaution since our results would suggest that differences in the fungus ability to withstand treatment exist between isolates, even of the same strain. Since F10 is proven to be effective on more than one isolate of *B. dendrobatidis* it offers the ideal solution as

an effective antiseptic at ports, airports and all other border crossings that require quarantine of traded animals. Using F10 during routine quarantine and other mitigation processes can greatly decrease the chances of contamination/cross-contamination. In addition F10 can also be used against other fungal infections suffered by animals such as birds and reptiles as well as being effective against *B. dendrobatidis* even at low concentrations and could therefore be recommended for the cleaning of equipment, tools etc. during field studies. In addition small volumes of F10 are sufficient and F10 is environmentally friendly as well as biodegradable (Barrows, 2007), whereas bleach on the other hand is toxic to aquatic animals and the environment. Furthermore F10 proves to be 3.68 times more cost effective when comparing it to other treatments such as itraconazole (Anon, 2015a; Anon, 2015b). This makes it a more suitable choice for large scale quarantine and disinfecting procedures in the industry as well as nature.

The 96 well plates also remained clear of any *B. dendrobatidis* 24 hours post exposure and remained clear even seven days later which proves that F10 successfully kills this pathogen. Since our study proved that much lower concentrations of F10 are still effective in killing *B. dendrobatidis in vitro* we could use F10 to treat infected amphibians especially since lower concentrations poses less of a threat to the animals than higher concentrations and lowers any risk of environmental contamination. F10 also proved effective at different time intervals (table 6) as a factor of concentration, which allows for a treatment protocol to be adapted according to the requirements imposed by the situation, including time availability, no. of animals to be treated and host tolerance. Thus F10 in comparison to other disinfectants/antiseptics shows a lot of promise as being the ideal antiseptic to use in the battle against *B. dendrobatidis* infection worldwide.

4.2. Toxicity levels of F10 on amphibians

Various combinations of concentrations and time intervals have to be tested in order to determine which combination has the lowest observed effect concentration (LOEC) to ensure that the treatment does not harm the host or cause any side effects. Because it was found by Webb *et al.* (2007) that 1:3000 concentration of F10 is effective at killing 100% of *B. dendrobatidis in vitro* we decided to use these results as a base line concentration from which to explore the effects of higher and lower concentrations. Incidentally, the 1:3000 concentration proved to be lethal to tadpoles, however safe to use on juvenile toads. These findings allowed us to test lower concentrations for the

tadpoles and stronger concentrations for the juveniles. Thus given the right concentration *A. gutturalis* survives exposures to F10.

When *A. gutturalis* is exposed to toxic levels of F10, the effects are acute and clearly visible through a range of clinical symptoms such as cutaneous erythema, ulcerations, increased mucus production and changes in behaviour (Pessier, 2002), some of which were very evident in this experiment. Deaths usually set in within a few minutes after exposure, however individuals that were alive after six hours survived the seven day observation period for all respective concentrations. Yet none of these side-effects were observed at lower concentrations such as 1:500 and lower for the juveniles but were however observed with the tadpoles. Thus different sensitivities exist for tadpoles and for juveniles. Since 1:10000 proved to have none of the LOEC signs it was chosen as the most suitable concentration for tadpoles and the 1:3000 concentration for juveniles, as the risk of side-effects or death would be minimal to zero. The fact that the survival of the tadpoles as well as the juveniles were dependant on the length of exposure would suggest that exposure time would ultimately be the limiting factor when treating amphibians with F10. However this study serves only as a pilot for testing the efficacy of F10 on amphibians and it is therefore recommended that the effective treatments suggested in this study be repeated on other unrelated species to test for variation in results. However care should be taken when testing other species since the toxicity of F10 is ultimately not only a life-stage dependent concentration, but also a factor of exposure time. Prolonged or over exposure to normally safe concentrations can be just as toxic as shorter exposures to more concentrated F10 solutions. However as evident in the *in vitro* tests, a minimum exposure time of only 10 min at a concentration of 1:10000 and less than 5 min for 1:3000 was enough to effectively kill all *B. dendrobatidis* zoospores and the LOEC (table 8 and table 7) obtained for both life stages are strong enough to be effective against *B. dendrobatidis.*

4.3. Infectivity of *Amietophrynus gutturalis*

Specialised studies focusing on especially the transmission and effects of this pathogen on different species of amphibians as well as on all the life stages is necessary in order to comprehend the dynamics of chytridiomycosis (Rachowicz and Vredenburg, 2004). Studies such as these emphasize the necessity to successfully infect amphibians in the laboratory and shows that it is of the utmost importance since many of the current *B. dendrobatidis* research entails infected specimens for experiments and research towards understanding the origin of *B. dendrobatidis,* as well as understanding pathogenicity amongst strains. Because experimental animals are reared in the lab from collected wild spawn, it is necessary to challenge them with *B. dendrobatidis* fungal cultures in order to induce infection. In a study conducted by Rachowicz and Vredenburg (2004) on *Rana muscosa* it was demonstrated that infection with *B. dendrobatidis* can be obtained through exposing individuals to fungal zoospores or be transmitted from one infected life stage to another. We therefore set out to challenge tadpoles and juveniles of *A. gutturalis* with *B. dendrobatidis* zoospores in an attempt to infect them with *B. dendrobatidis* and found that they are indeed susceptible to infection.

The zoospore yield obtained for each dose tended to increase accumulatively which led to an average of 628 000 zs/ml. This is most likely as a direct result of the method we chose to follow when harvesting zoospores. By using broth culture that was at least 5 days old, more sporangia were able to encyct on the plates which allowed for a much higher zoospore yield in comparison to liquid broth cultures. Previous observations showed that *B. dendrobatidis* tends to grow much better and quicker on the 1% tryptone agar plates following flooding than in the 1% tryptone broth media. This high growth period can however only be maintained for short periods of time which is what makes it ideal to use during challenge experiments. The zoospore yield obtained during this study differs immensely from what could be expected in nature since the growth of *B. dendrobatidis in vitro* was done at optimal growth and reproduction conditions for *B. dendrobatidis*, regarding temperature, humidity and nutrients. Much lower numbers of zoospores can be expected in nature; however since we wanted to ensure positive *B. dendrobatidis* infection, larger zoospore yields were necessary.

No cross-contamination was evident in our study since the results show a 100% infection in our *Bd*-groups and a 0% infection in our sham group. Furthermore these results also prove that the tadpoles were indeed successfully inoculated with *B. dendrobatidis* zoospores and the *B. dendrobatidis* challenge experiment can be deemed

successful. The sham group further proves that adverse effects were as a result of zoospores infecting the host and not as a result of chemical cues released from *B. dendrobatidis* in the absence of infection.

The type of experimental setup we chose where we house the tubs containing the juveniles alongside each other has proved to be successful since no contamination was found in the control groups which were randomly placed between the infected tubs. Our method of infecting amphibians with *B. dendrobatidis* furthermore proved effective since we had a 100% infection in the tadpoles of both the SA-*Bd* and EU-*Bd* groups. Infection in the tadpoles could readily be identified by microscopic examination of excised mouthparts – see Smith *et al*. (2007). The juveniles however proved to be more difficult to infect since we had 100% infection in the EU-*Bd* groups but infection in the SA-*Bd* groups ranged between 71 - 93%. This is most likely due to the fact that the EU-*Bd* strain grows much faster and is able to infect amphibians quicker and more successfully than the SA-*Bd* strain and therefore exhibits differences in infectivity. This difference in growth and reproduction is also very evident when growing both these strains on 1% tryptone agar plates. Berger *et al. (*2009) experimentally infected juvenile *Litoria caerulea* frogs (isolate obtained from a wild *Nyctimystes dayi* from Tully) in order to treat them with antifungal compounds and only had an infection rate of 53% in post-exposure frogs before starting their treatment regime. Therefore induced infection in 83% of *A. gutturalis* juveniles in the SA-*Bd* groups was sufficient to start a treatment regime with F10 and also prove that *A. gutturalis* juveniles are highly susceptible to *B. dendrobatidis* infection when exposed to multiple doses of active *B. dendrobatidis* zoospores. Furthermore our study has also indicated that local resistance to SA-*Bd* does not exist. Our toads still became infected with *B. dendrobatidis*, just with lower infection intensity than the EU-*Bd* groups (which is explained above as a result of evolutionary relationships between co-evolved host/pathogen relationships).

The virulence of the different strains is also amplified by the rate at which mortalities occurred in the groups where a definitive drop in numbers occurred seven days after dosing stopped and the infection had time to establish (figure 12). Juvenile *A. gutturalis* is susceptible to chytridiomycosis and therefore becomes diseased and dies. The EU-*Bd* group suffered the most mortalities (the strains exhibited different virulence in *A. gutturalis,* with the EU-*Bd* strain being more virulent) while the control group suffered no mortalities suggesting that the mortalities were indeed due to chytridiomycosis.

Our control groups for both the tadpole and juvenile experiments however remained infection-free with no mortalities, which proves that the amphibians did not have *B. dendrobatidis* prior to dosing and that the infection was indeed as a result of challenging them with the fungus. Furthermore we showed that infection of *A. gutturalis* tadpoles and juveniles were indeed possible with exposure to fungal zoospores. We will however for future application propose to start treatment seven days after the first dose instead of seven days after the last dose. By doing this we lower the risk of exceeding the lethal infection intensity and thus ensure that the amphibians don't become moribund prior to treatment. Heavy infections also lead to high mortalities which can also be avoided if treatment commences sooner.

Effective dosing of amphibians with *B. dendrobatidis* will aid towards challenge experiments focused on differences in lineages and the effect that different lineages may have on each other as well as on different host species. Studying infected individuals will further aid in understanding the mechanisms behind *B. dendrobatidis* infection, as well as how to effectively control and manage *B. dendrobatidis* infections in highly susceptible species.

A. gutturalis juveniles also proved to be more susceptible to the EU-*Bd* than the SA-*Bd* as evident in the number of mortalities in the EU-*Bd* group in comparison to the SA-*Bd* group. This is likely due to an innate relationship between *A. gutturalis* and the SA-*Bd* strain since they both originate from the same general geographical location. Although both the SA-*Bd* and the EU-*Bd* isolates are from the same lineage, *A. gutturalis* reacted as a naive species would towards an invasive novel pathogen when exposed to EU-*Bd*. This poses an immense threat for any and all indigenous amphibians should any foreign *B. dendrobatidis* strains ever enter our ecosystem. This further illustrates just how important an effective treatment protocol is since chytridiomycosis could lead to an epidemic on the subcontinent if left untreated.

4.4. Treatment of infected juveniles according to the protocol

The disinfection of animals and equipment is done for reasons such as biosecurity and reduction of cross-contamination during research as well as for captive husbandry, and naturally as a quarantine procedure or part of a conservation management strategy for species threatened by disease. However disinfection of animals can also aid in the treatment of pathogens and fungal infections (Parker *et al.*, 2002; Webb *et al*. 2007). Therefore any disinfectant selected for use on equipment or animals should not have any harmful side-effects on the surrounding environment or the animals being treated.

A number of studies e.g. Garner *et al.* (2009), Nichols and Lamirande (2000) demonstrated that *B. dendrobatidis* infection could be treated with the correct antifungal regime. Garner *et al.* (2009) successfully treated *Alytes muletensis* tadpoles with Itraconazole, while Nichols and Lamirande (2000) cured infected *Dendrobates tinctorius* juveniles with an 11 day treatment of itraconazole baths. However, the application of Itraconazole has limitations in that it causes depigmentation in tadpoles (Garner *et al.,* 2009) and can therefore not be recommended as a viable treatment option for all life stages without further testing to determine the effects on melanin production in tadpoles. Further investigation of itraconazole was also recommended by Somchit *et al*. (2004) after they reported hepatotoxic side effects in mammals. In another study Berger *et al.* (2009) attempted to treat infected amphibians with benzalkonium and although the treatment did not rid the infected animals of their infection it did prolong their life-span and was able to reduce the infection intensity.

Some success was achieved by Bishop *et al.* (2009) who attempted to treat Archey's frogs (*Leiopelma archeyi*) suffering from *B. dendrobatidis* infection with chloramphenicol. After proving its efficacy *in vitro*, chloramphenicol was applied as a topical ointment and as a solution which was in constant contact with the ventral surface of the frog. According to Bishop *et al*. (2009) the study was successful based on the lack of adverse effects when comparing the treatment groups with the control groups. Daily observations included studying the behaviour and food consumption of the treated frogs. Controversially, chloramphenicol is banned in most developed countries (can cause anaemia in humans as well as bone marrow depression in animals along with other side effects which include vomiting, depression etc.) (Berendsen *et al*., 2010), which makes it unsuitable to use as a worldwide treatment regime for amphibians suffering from *B. dendrobatidis* infections.

Since very few chemicals/antifungals ever tested on amphibians were met with great success we decided to use an antiseptic not yet tested on amphibians in prior studies but proven to be effective against killing *B. dendrobatidis* 100% *in vitro* and which is readily available and affordable, namely F10 antiseptic solution. Johannesburg Zoo has been using F10 to successfully treat zoo animals with fungal infections as well as ring worms in reptiles, birds and mammals, but without formal testing of its efficacy (Michelle Burrows, pers. comm.). The zoo also uses F10 to disinfect equipment and enclosures. Thus F10 appears to be safe to use on a range of vertebrates as demonstrated by its current use in the animal health sector.

An overall infection rate of 71-93% was obtained in the SA-*Bd* group which was considerably lower than the 100% infection in the EU-*Bd* group. We propose that this difference in infection was as a result of the difference between the two strains and due to the fact that *A. gutturalis* was naive to the EU-*Bd* strain and was thus more susceptible to infection. The EU-*Bd* strain also appears to be much more virulent than the SA-*Bd* strain which could also lead to higher infections (especially in naive amphibian species) and higher mortalities. Therefore we could no longer use the EU-*Bd* group in the treatment phase as the mortalities suffered in this group were too high.

Juveniles from the SA-*Bd* group were separated into the three respective treatment groups prior to the start of the experiment and these groups were statistically comparable in body mass. It can therefore be deducted that any deaths suffered in a particular group was not because of differences in body mass but very likely due to chytridiomycosis. In a study conducted by Parris and Cornelius (2004) they found that although the pathogen does not directly affect survivorship it does however influence historical traits regarding growth and development which would explain why each of the three treatment groups in our experiment differed in population size (after the *B. dendrobatidis* challenge experiment) as well as infection (figure 13) at the start of the treatment phase. The body mass/size of the juveniles will however not influence susceptibility to *B. dendrobatidis* infection but rather infectivity and thus long term survival (Garner *et al*., 2011).

According to the results obtained at the end of the experiment the body mass of the three treatment groups were once again statistically comparable along with the negative control group (No *Bd*, No F10). We would however recommend that the body mass of the juveniles be determined three times instead of just two – thus weighing them again prior to the treatment phase but after the *B. dendrobatidis* challenge experiment. Nevertheless, the positive control group which received only *B. dendrobatidis* and no F10 treatment suffered a considerable loss in overall body mass. This group had considerable weight loss in comparison to the other groups (F10 treatment groups and the negative control group), indicating that the amphibians in the treatment groups as well as the negative control group were healthier and were able to sustain their body mass much better, indicating that the weight loss suffered was indeed as a result of chytridiomycosis and not due to husbandry.

After the amphibians of all the groups were weighed prior to the start of the experiment the bath treatments could commence. The groups were initially going to receive a bath of 1:3000 concentration of F10 for 10 min; but they started presenting with some of the LOEC signs at 8 min at which point they were removed from the F10 in order to ensure no harmful side-effects could occur as a result of F10 exposure. This result was contradictory to the previous F10 exposure experiments we had done on non-infected amphibians and we could only surmise that this was as a direct result from the *B. dendrobatidis* infection. Chytridiomycosis had already weakened the amphibians which could have resulted in an increased sensitivity to F10. We therefore adapted our treatment protocol and only exposed the juveniles for 8 min time periods. An examination of shed skin in the petri dishes after the 3rd and 5th F10 treatments showed no signs of any fungal sporangia (the control groups had no shed skin). This could perhaps be attributed to the effectiveness of F10 since the infected animals shed the infected skin at such an increased shedding rate that *B. dendrobatidis* is ultimately cleared from the animal. This excessive skin shedding did not however result in any abnormal behaviour in the juveniles after exposures, since they remained alive and healthy until the end of the experiment.

Our results have demonstrated that the clearance of *B. dendrobatidis* can be achieved through F10 treatment. We assume that the reason the F10 x 3 treatment group had a slightly higher percentage success rate may be as a result of the eventual difference in group size due to mortalities suffered prior to treatment. The F10 x 3 group had 25 juveniles at the start of the treatment experiment in comparison to the F10 x 5 which only had 18 juveniles. However, the F10 x 5 group still didn't achieve 100% success in

eliminating *B. dendrobatidis* and therefore we suggest that success is a factor of dose frequency and more than five doses are required to achieve clearance in 100% of individuals. Although the F10 x 1 treatment only had 60% success it did prolong the life expectancy even in the few individuals that remained infected. The mortalities suffered by the F10 x 1 group eventually stabilized along with the F10 x 3 group and the F10 x 5 group at day 26 (figure 15) where after no more mortalities occurred, but the positive control continued to suffer mortalities. The *B. dendrobatidis*-negative results we received could not have been due to an innate immunological response, since our positive control group that received only *B. dendrobatidis* and no F10, remained *B. dendrobatidis*-positive throughout the experiment and ultimately suffered high mortality rates as a result thereof. This would suggest that even if F10 should only be effective in clearing infected amphibians of chytridiomycosis at an estimate of 78%, it will however prolong their life span as well as the life span of the amphibians still infected along with reducing the infection to such a point that the infected amphibians could ultimately survive.

CHAPTER 5

Conclusion

The toad species *Ametiephrynus gutturalis* we endeavoured to treat with F10 could have been more effective if juveniles with lighter infections were treated, perhaps exposure to fewer zoospores or by commencing treatment with F10 at an earlier stage before they become too weak to be treated. To the best of our knowledge our study is presently the first report of the use of F10 for treating *Batrachochytrium dendrobatidis* infection in juveniles as well as the toxicity of F10 on both tadpoles and juveniles of *A. gutturalis*. F10 only appears to be an irritant to *A. gutturalis* at very high concentrations, especially for the tadpoles. The juveniles however appeared to be much more resilient and could withstand much higher concentrations of the antiseptic than the tadpoles. The concentrations needed to successfully kill 100% of the *B. dendrobatidis* infection proved to be safe for use on both the tadpoles and the juveniles as they exhibited no abnormal behavior or agitation and tadpoles even continued with normal foraging behavior during exposure. Furthermore, both tadpoles and juveniles were able to feed after being exposed to F10 with no signs of lethargy or excessive shedding.

Although we did not achieve a 100% success rate, F10 was still able to clear almost 90% of infected juveniles as well as prolonging the life-span of the few infected juveniles left. However, for future experiments we would recommend that all groups be equal prior to the treatment phase and not just prior to the *B. dendrobatidis* challenge experiment. This will ensure a more accurate result as to how many treatments with F10 is necessary to obtain a 100% success rate.

Provided below are summaries of the most important findings and associated protocols from this study that can serve as a quick reference guide to workers in the field of conservation medicine. The information is grouped into three boxes that explain: 1) *in vitro* toxicity trials on *B. dendrobatidis* (Box 1), 2) *B. dendrobatidis* challenge experiments on *A. gutturalis* (Box 2), and 3) treatment protocol for F10 (Box 3).

Box 1

Effect of F10 on *Batrachochytrium dendrobatidis (Bd)*

- Incubate *Bd* (10,000 zoospores) for three days in 96-well plates. Growth can be confirmed by a white biofilm visible at the bottom of the well.
- Expose the wells containing the *Bd* to various concentrations of F10 and observe zoospore activity under an inverted microscope.
- Note the time it takes for the various concentrations of F10 to achieve 100% mortality for different strains of *Bd*.

- F10 is able to kill *Bd* even at very low concentrations such as 1:30,000 and only takes 5 min or less at concentrations of 1:5,000 and stronger.
- Differences in the pathogen's ability to withstand the treatment exist between isolates (even if they are of the same strain). At very low F10 concentrations the European *Bd*GPL isolate takes twice as long as the South African *Bd*GPL to die. Thus the *Bd* strain and isolate should be taken into consideration when treating amphibians with F10.
- Efficacy at different time intervals as a factor of concentration allows for a treatment protocol to be adapted according to the requirements imposed by the situation, including the time availability, number of animals to be treated and host tolerance.

F10 is toxic to *Bd*.

Box 2

Infecting *Amietophrynus gutturalis* with *B. dendrobatidis (Bd)*

- In order to harvest enough zoospores to dose amphibians 1ml of a five day old broth culture (1% Tryptone broth media containing live *Bd*) has to be added onto a 1% tryptone agar plate and incubated at 21°C for 4 days.
- The incubated plates then have to be flooded with 5-10ml of sterile tryptone broth media and left for 30 min to allow the zoospores to become suspended.
- Zoospores are quantified and approximately 40,000 – 50,000 zoospores are used to dose individual tadpoles or juveniles.
- This procedure is repeated five times within 10-15 days.
- Infection in the tadpoles can easily be identified upon microscopic examination of excised mouthparts. Diagnosis in juveniles made from qPCR analysis of skin swabs.

- We obtained 100% infection in the tadpoles from both strains, and up to 93% infection in juveniles with the SA-*Bd*GPL strain vs. 100% infection with the EU-*Bd*GPL strain.
- Different isolates exhibit differences in infectivity of juveniles as well as differences in virulence.
- *A. gutturalis* reacted towards EU-*Bd* strain as a typical novel species would when encountering an invasive pathogen for the first time – resulting in high mortality.

Both tadpoles and juveniles from A. *gutturalis* are susceptible to infection with *Bd*.

Box 3

Treating infected juveniles with F10

- During the treatment protocol juveniles should be housed separately to avoid any contact between individuals.
- Juveniles must be placed in petri-dishes and bathed in a 10ml solution of 1:3000 F10 for 8 min.
- Juveniles are rinsed with water after receiving the bath treatments and returned to their enclosures.
- Repeat the treatment at least 5 times on 5 consecutive days.

- We achieved an almost 90% clearance of *Bd* using this protocol.
- We conclude that a 100% success rate was not achieved because of dose frequency limitation, and suggest that more than five doses are required to achieve 100% clearance.
- Even though not all the juveniles were *Bd* free after receiving treatment, their life-span was prolonged as a result of F10 reducing the infection to below the critical physiological threshold of the toads.
- Treatment with F10 also restricted the loss of body mass induced by chytridiomycosis.

Clearance of B*d* can be achieved through multiple F10 treatments of 1:3000 for 8 min.

Although clearance of *B dendrobatidis* can be achieved through F10 treatments, it is recommended that further studies should first be conducted on the long term effects of F10 on amphibians. Particularly an investigation on the health and development of amphibians treated with F10. Thereafter F10 can be utilized as a warranted veterinary treatment against *B dendrobatidis* infection, should the studies indicate that no long term side effects occurred as a result of being treated with F10.

REFERENCES

ALFORD, R. A. 1999. Ecology: Resource use, competition, and predation. *In:* MCDIARMID, R. W. & ALTIG, R. (eds.) *Tadpoles: The biology of anuran larvae.* Chicago: University of Chicago Press, 240-278.

ALFORD, R. A. & RICHARDS, S. J. 1999. Global amphibian declines: A problem in applied ecology. *Annual review of Ecology and Systematics*, 133-165.

ANDERSEN, W. C., ROYBAL, J. E. & TURNIPSEED, S. B. 2004. Determination of Malachite Green and Leucomalachite Green in Salmon with *In Situ* Oxidation and Liquid Chromatography with Visible Detection. US Food and Drug Administration Laboratory Information Bulletin, 4334, 1-13.

ANDRÉN, C., HENRIKSON, L., OLSSON, M. & NILSON, G. 1988. Effects of pH and Aluminium on Embryonic and Early Larval Stages of Swedish Brown Frogs *Rana arvalis*, *R. temporaria* and *R. dalmatina*. *Holarctic Ecology,* 11, 127-135.

ANON. 2015a. Meadow's Animal Healthcare. [Web:] http://www.mah-shop.co.uk/f10-antiseptic-solution---200ml-concentrated-166-p.asp. Date of access: 05/03/2015.

ANONb. 2015b. Pet Drugs Online. [Web:] http://www.petdrugsonline.co.uk/prescriptions/i/itrafungol-oral-solution. Date of access: 05/03/2015.

ASH, A. N. 1997. Disappearance and Return of Plethodontid Salamanders to Clearcut Plots in the Southern Blue Ridge Mountains. *Conservation Biology,* 11, 983-989.

BAILLIE, J. E., HILTON-TAYLOR, C. & STUART, S. N. 2004. IUCN Red List of Threatened Species: A Global Species Assessment, IUCN. Gland, Switzerland and Cambridge, UK.

BALMFORD, A., GREEN, R. E. & JENKINS, M. 2003. Measuring the changing state of nature. *Trends in Ecology & Evolution,* 18, 326-330.

BARR, D. 1990. Phylum chytridiomycota. *In:* MARGULIS, L., CORLISS, J. O., MELKONIAN, M. & CHAPMAN, D. J. (eds.) *Handbook of Protoctista.* New York: Lubrecht and Kramer, 137-248.

BARROWS, M. 2007. F10, A Novel Product Range Most Suited To Zoological Medicine. *The Facts*, 6, 1-4.

BAYLIS, A. D. 2000. Why glyphosate is a global herbicide: strengths, weaknesses and prospects. *Pest Management Science,* 56, 299-308.

BEATTIE, R. C. & TYLER-JONES, R. 1992. The Effects of Low pH and Aluminum on Breeding Success in the Frog *Rana temporaria*. *Journal of Herpetology,* 26, 353-360.

BECKER, C. G., FONSECA, C. R., HADDAD, C. F. B., BATISTA, R. F. & PRADO, P. I. 2007. Habitat Split and the Global Decline of Amphibians. *Science,* 318, 1775-1777.

BECKER, M., HARRIS, R., MINBIOLE, K. C., SCHWANTES, C., ROLLINS-SMITH, L., REINERT, L., BRUCKER, R., DOMANGUE, R. & GRATWICKE, B. 2011. Towards a Better Understanding of the Use of Probiotics for Preventing Chytridiomycosis in Panamanian Golden Frogs. *EcoHealth,* 8, 501-506.

BEEBEE, T. J. C. & GRIFFITHS, R. A. 2005. The amphibian decline crisis: A watershed for conservation biology? *Biological Conservation,* 125, 271-285.

BERENDSEN, B., STOLKER, L., DE JONG, J., NIELEN, M., TSERENDORJ, E., SODNOMDARJAA, R., CANNAVAN, A. & ELLIOT, C. 2010. Evidence of natural occurrence of the banned antibiotic chloramphenicol in herbs and grass. *Analytical and Bioanalytical Chemistry,* 397, 1955-1963.

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

BERGER, L., SPEARE, R., DASZAK, P., GREEN, E. D., CUNNINGHAM, A. A., GOGGIN, L. C., 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 rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences,* 95, 9031-9036.

BERGER, L., SPEARE, R., HINES, H., MARANTELLI, G., HYATT, A. D., MCDONALD, K., SKERRATT, L., OLSEN, V., CLARKE, J. & GILLESPIE, G. 2004. Effect of season and temperature on mortality in amphibians due to chytridiomycosis. *Australian Veterinary Journal,* 82, 434-439.

BERGER, L., SPEARE, R. & HYATT, A. D. 1999. Chytrid fungi and amphibian declines: overview, implications and future directions. *In:* CAMPBELL, A. (eds.) *Declines and disappearances of Australian frogs. Environment Australia, Canberra*, 23-33.

BERGER, L., SPEARE, R., MARANTELLI, G. & SKERRATT, L. F. 2009. A zoospore inhibition technique to evaluate the activity of antifungal compound against *Batrachochytrium dendrobatidis* and unsuccessful treatment of experimentally infected green tree frogs (*Litoria caerulea*) by fluconazole and bezalkonium chloride. *Research in Veterinary Science*, 87, 106-110.

BERNAL, M. H., SOLOMON, K. R. & CARRASQUILLA, G. 2009. Toxicity of Formulated Glyphosate (Glyphos) and Cosmo-Flux to Larval and Juvenile Colombian Frogs 2. Field and Laboratory Microcosm Acute Toxicity. *Journal of Toxicology and Environmental Health, Part A,* 72, 966-973.

BISHOP, P. J., SPEARE, R., POULTER, R., BUTLER, M., SPEARE, B. J., HYATT, A. D., OLSEN, V. & HAIGH, A. 2009. Elimination of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* by Archey's frog *Leiopelma archeyi*. *Diseases of Aquatic Organisms,* 84, 9.

BLAUSTEIN, A. R., HOFFMAN, P. D., HOKIT, D. G., KIESECKER, J. M., WALLS, S. C. & HAYS, J. B. 1994. UV repair and resistance to solar UV-B in amphibian eggs: a link to population declines? *Proceedings of the National Academy of Sciences,* 91, 1791-1795.

BLAUSTEIN, A. R., KIESECKER, J. M., CHIVERS, D. P., HOKIT, D. G., MARCO, A., BELDEN, L. K. & HATCH, A. 1998. Effects of Ultraviolet Radiation on Amphibians: Field Experiments. *American Zoologist,* 38, 799-812.

BLAUSTEIN, A. R. & WAKE, D. B. 1995. The puzzle of declining amphibian populations. *Scientific American,* 272.

BLETZ, M. C., LOUDON, A. H., BECKER, M. H., BELL, S. C., WOODHAMS, D. C., MINBIOLE, K. P. C. & HARRIS, R. N. 2013. Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecology Letters,* 16, 807-820.

BLOOI, M., MARTEL, A., VERCAMMEN, F. & PASMANS, F. 2013. Combining ethidium monoazide treatment with real-time PCR selectively quantifies viable *Batrachochytrium dendrobatidis* cells. *Fungal biology,* 117, 156-162.

BOLLINGER, T. K., MAO, J., SCHOCK, D., BRIGHAM, R. M. & CHINCHAR, V. G. 1999. Pathology, isolation, and preliminary molecular characterization of a novel iridovirus from tiger salamanders in Saskatchewan. *Journal of Wildlife Diseases,* 35, 413-429.

BOSCH, J. & MARTÍNEZ-SOLANO, I. 2006. Chytrid fungus infection related to unusual mortalities of *Salamandra salamandra* and *Bufo bufo* in the Penalara Natural Park, Spain. *Oryx,* 40, 84-89.

BOSCH, J., MARTíNEZ-SOLANO, I. & GARCíA-PARíS, 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.

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

BOYLE, D. G., BOYLE, D. B., OLSEN, V., MORGAN, J. A. T. & HYATT, A. D. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in

amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms,* 60, 141-148.

BRADFORD, D. F., SWANSON, C. & GORDON, M. S. 1992. Effects of Low pH and Aluminum on Two Declining Species of Amphibians in the Sierra Nevada, California. *Journal of Herpetology,* 26, 369-377.

BRADFORD, D. F., TABATABAI, F. & GRABER, D. M. 1993. Isolation of Remaining Populations of the Native Frog, *Rana muscosa*, by Introduced Fishes in Sequoia and Kings Canyon National Parks, California. *Conservation Biology,* 7, 882-888.

BRADLEY, G. A., ROSEN, P. C., SREDL, M. J., JONES, T. R. & LONGCORE, J. E. 2002. Chytridiomycosis in Native Arizona Frogs. *Journal of Wildlife Diseases,* 38, 206-212.

BRIGGS, C. J., KNAPP, R. A. & VREDENBURG, V. T. 2010. Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proceedings of the National Academy of Sciences,* 107, 9695-9700.

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, 3149-3159.

BRODIE, E. D., JR. & FORMANOWICZ, D. R., JR. 1987. Antipredator Mechanisms of Larval Anurans: Protection of Palatable Individuals. *Herpetologica,* 43, 369-373.

BRÜHL, C. A., SCHMIDT, T., PIEPER, S. & ALSCHER, A. 2013. Terrestrial pesticide exposure of amphibians: An underestimated cause of global decline? *Scientific Reports,* 3.

BRUNNER, J. L., RICHARDS, K. & COLLINS, J. P. 2005. Dose and host characteristics influence virulence of ranavirus infections. *Oecologia,* 144, 399-406.

BRUNNER, J. L., SCHOCK, D. M., DAVIDSON, E. W. & COLLINS, J. P. 2004. Intraspecific reservoirs: Complex life history and the persistence of a lethal ranavirus. *Ecology,* 85, 560-566.

BUCK, J. C., TRUONG, L. & BLAUSTEIN, A. R. 2011. Predation by zooplankton on *Batrachochytrium dendrobatidis*: biological control of the deadly amphibian chytrid fungus? *Biodiversity and Conservation,* 20, 3549-3553.

CAREY, C. & BRYANT, C. J. 1995. Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. *Environmental Health Perspectives Supplements,* 103, 13.

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

CARR, A. H., AMBORSKI, R. L., CULLEY, D. D. & AMBORSKI, G. F. 1976. Aerobic Bacteria in the Intestinal Tracts of Bullfrogs (*Rana catesbeiana*) Maintained at Low Temperatures. *Herpetologica,* 32, 239-244.

CHAPIN III, F. S., WALKER, B. H., HOBBS, R. J., HOOPER, D. U., LAWTON, J. H., SALA, O. E. & TILMAN, D. 1997. Biotic control over the functioning of ecosystems. *Science,* 277, 500-504.

CHATFIELD, M. & RICHARDS-ZAWACKI, C. 2011. Elevated temperature as a treatment for *Batrachochytrium dendrobatidis* infection in captive frogs. *Diseases of Aquatic Organisms*, 94, 235-238.

CHINCHAR, V. G., HYATT, A. D., MIYAZAKI, T. & WILLIAMS, T. 2009. Family *Iridoviridae*: Poor Viral Relations No Longer. *Current topics in microbiology and immunology,* 328, 123-170

COLLINS, J. P., BRUNNER, J. L., JANCOVICH, J. K. & SCHOCK, D. M. 2004. A model host-pathogen system for studying infectious disease dynamics in amphibians: tiger salamanders (*Ambystoma tigrinum*) and *Ambystoma tigrinum* virus. *Herpetological Journal,* 14, 195-200.

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,* 50, 498-506.

CULP, S. J. & BELAND, F. A. 1996. Malachite Green: A Toxicological Review. *International Journal of Toxicology,* 15, 219-238.

CRANE, M. & NEWMAN, M. C. 2000. What level of effect is a no observed effect? *Environmental Toxicology and Chemistry,* 19, 516-519.

CUNNINGHAM, A. A., LANGTON, T. E. S., BENNETT, P. M., LEWIN, J. F., DRURY, S. E. N., GOUGH, R. E. & MACGREGOR, S. K. 1996. Pathological and Microbiological Findings from Incidents of Unusual Mortality of the Common Frog (*Rana temporaria*). *Philosophical Transactions: Biological Sciences,* 351, 1539-1557.

CUSHMAN, S. A. 2006. Effects of habitat loss and fragmentation on amphibians: A review and prospectus. *Biological Conservation,* 128, 231-240.

DASZAK, P., BERGER, L., CUNNINGHAM, A. A., HYATT, A. D., GREEN, D. E. & SPEARE, R. 1999. Emerging infectious diseases and amphibian population declines. *Emerging infectious diseases,* 5, 735.

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

DAVIDSON, C. 2004. Declining downwind: Amphibian population declines in California and historical pesticide use. *Ecological Applications,* 14, 1892-1902.

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

DE BEULE, K. & VAN GESTEL, J. 2001. Pharmacology of Itraconazole. *Drugs,* 61, 27-37.

DELIS, P. R., MUSHINSKY, H. R. & MCCOY, E. D. 1996. Decline of some west-central Florida anuran populations in response to habitat degradation. *Biodiversity & Conservation,* 5, 1579-1595.

DENSMORE, C. L. & GREEN, D. E. 2007. Diseases of Amphibians. *Institute for Laboratory Animal Research Journal,* 48, 235-254.

DOCHERTY, D. E., METEYER, C. U., WANG, J., MAO, J., CASE, S. T. & CHINCHAR, V. G. 2003. Diagnostic and molecular evaluation of three iridovirus-associated salamander mortality events. *Journal of Wildlife Diseases,* 39, 556-566.

DONNELLY, M. & CRUMP, M. 1998. Potential Effects of Climate Change on Two Neotropical Amphibian Assemblages. *Climatic Change,* 39, 541-561.

DUELLMAN, W. E. & TRUEB, L. 1994. *Biology of Amphibians,* Baltimore, Johns Hopkins University Press.

DUFFUS, A. L. J., NICHOLS, R. A. & GARNER, T. W. J. 2013. Investigations into the Life History Stages of the Common Frog (*Rana temporaria*) Affected by an Amphibian Ranavirus in the United Kingdom. *Herpetological Review,* 44, 260-263.

DUSI, J. L. 1949. The Natural Occurrence of "Redleg", *Pseudomonas Hydrophila*, in a Population of American Toads, *Bufo Americanus*. *Ohio Journal of Science,* 49, 70-71.

ELMBERG, J. 1993. Threats to Boreal Frogs. *Ambio,* 22, 254-255.

EMERSON, H. & NORRIS, C. 1905. "Red-leg"—An infectious disease of frogs. *The Journal of Experimental Medicine,* 7, 32-58.

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

FISHER, M. C. 2008. Molecular toolkit unlocks life cycle of the panzootic amphibian pathogen *Batrachochytrium dendrobatidis*. *Proceedings of the National Academy of Sciences,* 105, 17209-17210.

FISHER, M. C., BOSCH, J., YIN, Z., STEAD, D. A., WALKER, J., SELWAY, L., BROWN, A. J., WALKER, L. A., GOW, N. A. & STAJICH, J. E. 2009. Proteomic and

phenotypic profiling of the amphibian pathogen *Batrachochytrium dendrobatidis* shows that genotype is linked to virulence. *Molecular Ecology,* 18, 415-429.

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.

FITE, K. V., BLAUSTEIN, A., BENGSTON, L. & HEWITT, H. E. 1998. Evidence of Retinal Light Damage in *Rana cascadae*: A Declining Amphibian Species. *Copeia,* 1998, 906-914.

FORMANOWICZ JR, D. R. & BRODIE JR, E. D. 1982. Relative Palatabilities of Members of a Larval Amphibian Community. *Copeia,* 1982, 91-97.

FORSON, D. D. & STORFER, A. 2006. Atrazine increases ranavirus susceptibility in the tiger salamander, A*mbystoma tigrinum*. *Ecological Applications,* 16, 2325-2332.

FORZÁN, M. J., GUNN, H. & SCOTT, P. 2008. Chytridiomycosis in an aquarium collection of frogs: diagnosis, treatment, and control. *Journal of Zoo and Wildlife Medicine,* 39, 406-411.

FREDA, J. & DUNSON, W. A. 1986. Effects of Low pH and Other Chemical Variables on the Local Distribution of Amphibians. *Copeia,* 1986, 454-466.

FREDA, J., SADINSKI, W. & DUNSON, W. 1991. Long term monitoring of amphibian populations with respect to the effects of acidic deposition. *Water, Air, and Soil Pollution,* 55, 445-462.

FRYDAY, S. & THOMPSON, H. 2012. Toxicity of pesticides to aquatic and terrestrial life stages of amphibians and occurrence, habitat use and exposure of amphibian species in agricultural environments. External Scientific Report. Food and Environment Research Agency, San Hutton. *EFSA Supporting Publications*, EN-343.

GAMRADT, S. C. & KATS, L. B. 1996. Effect of Introduced Crayfish and Mosquitofish on California Newts. *Conservation Biology,* 10, 1155-1162.

GARNER, T. W. J., GARCIA, G., CARROLL, B. & FISHER, M. C. 2009. Using itraconazole to clear *Batrachochytrium dendrobatidis* infection, and subsequent depigmentation of *Alytes muletensis* tadpoles. *Diseases of Aquatic Organisms,* 83, 257 - 60.

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,* 2, 455-459.

GARNER, T. W. J., ROWCLIFFE, J. M., FISHER, M. C. 2011. Climate change, chytridiomycosis or condition: an experimental test of amphibian survival. *Global Change Biology*, 17, 667–675

GASCON, C., COLLINS, J. P., MOORE, R. D., CHURCH, D. R., MCKAY, J. E. & MENDELSON III, J. R. 2007. *Amphibian conservation action plan: proceedings IUCN/SSC Amphibian Conservation Summit 2005,* Switzerland and Canbridge, UK, IUCN.

GEIGER, C., KUPFER, E., SCHAR, S., WOLF, S. & SCHMIDT, B. 2011. Elevated temperature clears chytrid fungus infections from tadpoles of the midwife toad, *Alytes obstetricans*. *Amphibia-Reptilia,* 32, 276 - 280.

GLEASON, F. H., LILJE, O., MARANO, A. V., SIME-NGANDO, T., SULLIVAN, B. K., KIRCHMAIR, M. & NEUHAUSER, S. 2014. Ecological functions of zoosporic hyperparasites. *Frontiers in Microbiology,* 5, 244.

GOSNER, K. L. 1960. A Simplified Table for Staging Anuran Embryos and Larvae with Notes on Identification. *Herpetologica,* 16, 183-190.

GRAY, M. J., MILLER, D. L. & HOVERMAN, J. T. 2009. Ecology and pathology of amphibian ranaviruses. *Diseases of Aquatic Organisms,* 87, 243-266.

HADFIELD, C. A. & WHITAKER, B. R. 2005. Amphibian emergency medicine and care. *Seminars in Avian and Exotic Pet Medicine*, 14, 79-89.

HAISLIP, N. A., GRAY, M. J., HOVERMAN, J. T. & MILLER, D. L. 2011. Development and Disease: How Susceptibility to an Emerging Pathogen Changes through Anuran Development. *PLoS ONE,* 6, 1-6.

HAN, B. A., SEARLE, C. L., BLAUSTEIN, A. R. 2011. Effects of an Infectious Fungus, *Batrachochytrium dendrobatidis*, on Amphibian Predator-Prey Interactions. *PLoS ONE,* 6, e16675.

HANSELMANN, R., RODRIGUEZ, A., LAMPO, M., FAJARDO-RAMOS, L., AGUIRRE, A. A., KILPATRICK, A. M., RODRíGUEZ, J. P. & DASZAK, P. 2004. Presence of an emerging pathogen of amphibians in introduced bullfrogs *Rana catesbeiana* in Venezuela. *Biological Conservation,* 120, 115-119.

HARP, E. M. & PETRANKA, J. W. 2006. Ranavirus in wood frogs (*Rana sylvatica*): Potential sources of transmission within and between ponds. *Journal of Wildlife Diseases,* 42, 307-318.

HARRIS, R. N., BRUCKER, R. M., WALKE, J. B., BECKER, M. H., SCHWANTES, C. R., FLAHERTY, D. C., LAM, B. A., WOODHAMS, D. C., BRIGGS, C. J. & VREDENBURG, V. T. 2009. Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *The ISME Journal,* 3, 818-824.

HARTE, J. & HOFFMAN, E. 1989. Possible Effects of Acidic Deposition on a Rocky Mountain Population of the Tiger Salamander *Ambystoma tigrinum*. *Conservation Biology,* 3, 149-158.

HECNAR, S. J. & M'CLOSKEY, R. T. 1996a. The effects of predatory fish on amphibian species richness and distribution. *Biological conservation,* 79, 123-131.

HECNAR, S. J. & M'CLOSKEY, R. T. 1996b. Regional Dynamics and the Status of Amphibians. *Ecology,* 77, 2091-2097.

HECNAR, S. J. & M'CLOSKEY, R. T. 1997. Patterns of Nestedness and Species Association in a Pond-Dwelling Amphibian Fauna. *Oikos,* 80, 371-381.

HELS, T. & BUCHWALD, E. 2001. The effect of road kills on amphibian populations. *Biological Conservation,* 99, 331-340.

HENRIKSON, B. I. 1990. Predation on Amphibian Eggs and Tadpoles by Common Predators in Acidified Lakes. *Holarctic Ecology,* 13, 201-206.

HILL, W. A., NEWMAN, S. J., CRAIG, L., CARTER, C., CZARRA, J. & BROWN, J. P. 2010. Diagnosis of *Aeromonas hydrophila, Mycobacterium* species, and *Batrachochytrium dendrobatidis* in an African Clawed Frog (*Xenopus laevis*). *Journal of the American Association for Laboratory Animal Science,* 49, 215.

HIRD, D. W., DIESCH, S. L., MCKINNELL, R. G., GORHAM, E., MARTIN, F. B., KURTZ, S. W. & DUBROVOLNY, C. 1981. *Aeromonas hydrophila* in wild-caught frogs and tadpoles (*Rana pipiens*) in Minnesota. *Laboratory animal science,* 31, 166-169.

HORNE, M. T. & DUNSON, W. A. 1994. Exclusion of the Jefferson salamander, *Ambystoma jeffersonianum*, from some potential breeding ponds in Pennsylvania: Effects of pH, temperature, and metals on embryonic development. *Archives of Environmental Contamination and Toxicology,* 27, 323-330.

HUBBARD, G. B. 1981. *Aeromonas hydrophila* infection in *Xenopus laevis*. *Laboratory Animal Science,* 31, 297-300.

HUSS, M., HUNTLEY, L., VREDENBURG, V., JOHNS, J. & GREEN, S. 2013. Prevalence of *Batrachochytrium dendrobatidis* in 120 Archived Specimens of *Lithobates catesbeianus* (American Bullfrog) Collected in California, 1924–2007. *EcoHealth,* 10, 339-343.

HYATT, A. D., BOYLE, D. G., OLSEN, V., BOYLE, D. B., BERGER, L., OBENDORF, D., DALTON, A., KRIGER, K., HERO, M., HINES, H., PHILLOTT, R., CAMPBELL, R., MARANTELLI, G., GLEASON, F. & COLLING, A. 2007. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms,* 73, 175-192.

JAMES, T. Y., LITVINTSEVA, A. P., VILGALYS, R., MORGAN, J. A., TAYLOR, J. W., FISHER, M. C., BERGER, L., WELDON, C., DU PREEZ, L. & LONGCORE, J. E. 2009. Rapid global expansion of the fungal disease chytridiomycosis into declining and healthy amphibian populations. *PLoS Pathogens,* 5, e1000458.

JANCOVICH, J. K., DAVIDSON, E. W., MORADO, J. F., JACOBS, B. L. & COLLINS, J. P. 1997. Isolation of a lethal virus from the endangered tiger salamander *Ambystoma tigrinum stebbinsi*. *Diseases of Aquatic Organisms,* 31, 161-167.

JANCOVICH, J. K., DAVIDSON, E. W., SEILER, A., JACOBS, B. L. & COLLINS, J. P. 2001. Transmission of the *Ambystoma tigrinum* virus to alternative hosts. *Diseases of Aquatic Organisms,* 46, 159-163.

JOHNSON, M., BERGER, L., PHILIPS, L. & SPEARE, R. 2003. Fungicidal effects 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. 2003. Survival of *Batrachochytrium dendrobatidis* in water: quarantine and disease control implications. *Emerging infectious diseases,* 9, 922.

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. & CHASE, J. M. 2004. Parasites in the food web: linking amphibian malformations and aquatic eutrophication. *Ecology Letters,* 7, 521-526.

JOHNSON, P. T. J., DOBSON, A., LAFFERTY, K. D., MARCOGLIESE, D. J., MEMMOTT, J., ORLOFSKE, S. A., POULIN, R. & THIELTGES, D. W. 2010. When parasites become prey: ecological and epidemiological significance of eating parasites. *Trends in Ecology & Evolution,* 25, 362-371.

JOHNSON, P. T. J., LUNDE, K. B., THURMAN, E. M., RITCHIE, E. G., WRAY, S. N., SUTHERLAND, D. R., KAPFER, J. M., FREST, T. J., BOWERMAN, J. & BLAUSTEIN, A. R. 2002. Parasite (*Ribeiroia Ondatrae*) infection linked to amphibian malformations in the western united states. *Ecological Monographs,* 72, 151-168.

JONES, D. K., HAMMOND, J. I. & RELYEA, R. A. 2011. Competitive stress can make the herbicide Roundup® more deadly to larval amphibians. *Environmental Toxicology and Chemistry,* 30, 446-454.

JONES, M., PADDOCK, D., BENDER, L., ALLEN, J., SCHRENZEL, M. & PESSIER, A. 2012. Treatment of chytridiomycosis with reduced-dose itraconazole. *Diseases of Aquatic Organisms,* 99, 243-249.

KATS, L. B., PETRANKA, J. W. & SIH, A. 1988. Antipredator Defenses and the Persistence of Amphibian Larvae With Fishes. *Ecology,* 69, 1865-1870.

KERR, J. B. & MCELROY, C. T. 1993. Evidence for large upward trends of ultraviolet-B radiation linked to ozone depletion. *Science,* 262, 1032-1032.

KIESECKER, J. M. & BLAUSTEIN, A. R. 1997. Population Differences in Responses of Red-Legged Frogs (*Rana Aurora*) to Introduced Bullfrogs. *Ecology,* 78, 1752-1760.

KILPATRICK, A. M., BRIGGS, C. J. & DASZAK, P. 2009. The ecology and impact of chytridiomycosis: an emerging disease of amphibians. *Trends in Ecology & Evolution,* 25, 109-118.

KOLOZSVARY, M. B. & SWIHART, R. K. 1999. Habitat fragmentation and the distribution of amphibians: patch and landscape correlates in farmland. *Canadian Journal of Zoology,* 77, 1288-1299.

KRAJICK, K. 2006. The Lost World of the Kihansi Toad. *Science,* 311, 1230-1232.

KRIGER, K., M & HERO, J.-M. 2009. Chytridiomycosis, Amphibian Extinctions, and Lessons for the Prevention of Future Panzootics. *EcoHealth,* 6, 6-10.

KRIGER, K. M., HINES, H. B., HYATT, A. D., BOYLE, D. G. & HERO, J. M. 2006. Techniques for detecting chytridiomycosis in wild frogs: comparing histology with real-time Taqman PCR. *Diseases of Aquatic Organisms,* 71, 141-8.

LAM, B., WALKE, J., VREDENBURG, V. & HARRIS, R. 2010. Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. *Biological Conservation,* 143, 529 - 531.

LESBARRÈRES, D., BALSEIRO, A., BRUNNER, J., CHINCHAR, V. G., DUFFUS, A., KERBY, J., MILLER, D. L., ROBERT, J., SCHOCK, D. M., WALTZEK, T. & GRAY, M. J. 2012. Ranavirus: past, present and future. *Biology Letters,* 8, 481-483.

LIPS, K. R. 1998. Decline of a Tropical Montane Amphibian Fauna. *Conservation Biology,* 12, 106-117.

LIPS, K. R. 1999. Mass Mortality and Population Declines of Anurans at an Upland Site in Western Panama. *Conservation Biology,* 13, 117-125.

LIPS, K. R., BREM, F., BRENES, R., REEVE, J. D., ALFORD, R. 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,* 103, 3165-3170.

LONGCORE, J. E., PESSIER, A. P. & NICHOLS, D. K. 1999. *Batrachochytrium dendrobatidis* gen. et sp. nov., a Chytrid Pathogenic to Amphibians. *Mycologia,* 91, 219-227.

MANN, R. M., HYNE, R. V., CHOUNG, C. B. & WILSON, S. P. 2009. Amphibians and agricultural chemicals: review of the risks in a complex environment. *Environmental Pollution,* 157, 2903-2927.

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.

MARTEL, A., SPITZEN-VAN DER SLUIJS, A., BLOOI, M., BERT, W., DUCATELLE, R., FISHER, M. C., WOELTJES, A., BOSMAN, W., CHIERS, K., BOSSUYT, F., PASMANS, F. 2013. *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *PNAS*, 110(38), 15325-15329.

MARTEL, A., VAN ROOIJ, P., VERCAUTEREN, G., BAERT, K., VAN WAEYENBERGHE, L., DEBACKER, P., GARNER, T. W. J., WOELTJES, T., DUCATELLE, R., HAESEBROUCK, F. & PASMANS, F. 2011. Developing a safe antifungal treatment protocol to eliminate *Batrachochytrium dendrobatidis* from amphibians. *Medical Mycology,* 49, 143-149.

MAZZONI, R., CUNNINGHAM, A. A., DASZAK, P., APOLO, A., PERDOMO, E. & SPERANZA, G. 2003. Emerging Pathogen in Wild Amphibians and Frogs (*Rana catesbeiana*) Farmed for International Trade. *Emerging infectious diseases,* 9, 995.

MENDELSON, J. R., LIPS, K. R., GAGLIARDO, R. W., RABB, G. B., COLLINS, J. P., DIFFENDORFER, J. E., DASZAK, P., IBÁÑEZ, R., ZIPPEL, K. C. & LAWSON, D. P. 2006. Biodiversity-Confronting amphibian declines and extinctions. *Science,* 313, 48-48.

MENIN, M., DE JESUS-RODRIGUES, D. & DE AZEVEDO, C. S. 2005. Predation on amphibians by spiders (Arachnida, Araneae) in the Neotropical region. *Phyllomedusa: Journal of Herpetology,* 4, 39-47.

MILLER, D., GRAY, M. & STORFER, A. 2011. Ecopathology of Ranaviruses Infecting Amphibians. *Viruses,* 3, 2351-2373.

MILLER, D. L., RAJEEV, S., BROOKINS, M., COOK, J., WHITTINGTON, L. & BALDWIN, C. A. 2008. Concurrent Infection with Ranavirus, *Batrachochytrium dendrobatidis*, and *Aeromonas* in a Captive Anuran Colony. *Journal of Zoo and Wildlife Medicine,* 39, 445-449.

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 increases the probability of host extinction. *Proceedings of the Royal Society B: Biological Sciences,* 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., 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. 2007. Population genetics of the frog-killing fungus *Batrachochytrium dendrobatidis*. *Proceedings of the National Academy of Sciences,* 104, 13845-13850.

MUTSCHMANN, F., BERGER, L., ZWART, P. & GAEDICKE, C. 2000. Chytridiomycosis in amphibians--first report in Europe. *Berliner und Munchener tierarztliche Wochenschrift,* 113, 380-383.

NICHOLS, D. & LAMIRANDE, E. Treatment of cutaneous chytridiomycosis in blue-and-yellow poison dart frogs (*Dendrobates tinctorius*). Proceedings: Getting the Jump on Amphibian Disease, Cairns, Australia, 2000. 51.

NICHOLS, D. & LAMIRANDE, E. 2001. Successful treatment of chytridiomycosis. *Froglog,* 46, 1.

OLSON, D. H., AANENSEN, D. M., RONNENBERG, K. L., POWELL, C. I., WALKER, S. F., BIELBY, J., GARNER, T. W., WEAVER, G., FISHER, M. C. 2013. Mapping the Global Emergence of *Batrachochytrium dendrobatidis*, the Amphibian Chytrid Fungus. *PLoS ONE,* 8(2), e56802.

O'ROURKE, D. P. 2007. Amphibians used in research and teaching. *ILAR Journal,* 48, 183-187.

PARKER, J. M., MIKAELIAN, I., HAHN, N. & DIGGS, H. E. 2002. Clinical diagnosis and treatment of epidermal chytridiomycosis in African clawed frogs (*Xenopus tropicalis*). *Comparative Medicine,* 52, 265-268.

PARMESAN, C. & YOHE, G. 2003. A globally coherent fingerprint of climate change impacts across natural systems. *Nature,* 421, 37-42.

PARRIS, M. J., BAUD, D. R. & QUATTRO, J. M. 2004a. Interactive Effects of a Heavy Metal and Chytridiomycosis on Gray Treefrog Larvae (*Hyla chrysoscelis*). *Copeia,* 2004, 344-350.

PARRIS, M. J. & CORNELIUS, T. O. 2004b. Fungal Pathogen Causes Competitive and Developmental Stress in Larval Amphibian Communities. *Ecology,* 85, 3385-3395.

PARRIS, M. J., DAVIS, A. & COLLINS, J. P. 2004c. Single-host pathogen effects on mortality and behavioral responses to predators in salamanders (Urodela: Ambystomatidae). *Canadian Journal of Zoology,* 82, 1477-1483.

PEARMAN, P. B., GARNER, T. W. J., STRAUB, M. & GREBER, U. F. 2004. Response of the italian agile frog (*rana latastei*) to a ranavirus, frog virus 3: A model for viral emergence in naïve populations. *Journal of Wildlife Diseases,* 40, 660-669.

PESSIER, A. P. 2002. An overview of amphibian skin disease. *Seminars in Avian an Exotic Pet Medicine*, 11, 162-174

PESSIER, A. P. 2008. Management of disease as a threat to amphibian conservation. *International Zoo Yearbook,* 42, 30-39.

PESSIER, A. P., NICHOLS, D. K., LONGCORE, J. E. & FULLER, M. S. 1999. Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp.) and White's tree frogs (*Litoria caerulea*). *Journal of Veterinary Diagnostic Investigation,* 11, 194-199.

PHILLOTT, A., SPEARE, R., HINES, H., SKERRATT, L., MEYER, E., MCDONALD, K., CASHINS, S., MENDEZ, D. & BERGER, L. 2010. Minimising exposure of amphibians to pathogens during field studies. *Diseases of Aquatic Organisms,* 92, 175-185.

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

POUNDS, A. J., 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 from epidemic disease driven by global warming. *Nature,* 439, 161-167.

POUNDS, J. A. & CRUMP, M. L. 1994. Amphibian Declines and Climate Disturbance: The Case of the Golden Toad and the Harlequin Frog. *Conservation Biology,* 8, 72-85.

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., FOGDEN, M. P. L., SAVAGE, J. M. & GORMAN, G. C. 1997. Tests of Null Models for Amphibian Declines on a Tropical Mountain. *Conservation Biology,* 11, 1307-1322.

POWELL, M. J. 1993. Looking at Mycology with a Janus Face: A Glimpse at Chytridiomycetes Active in the Environment. *Mycologia,* 85, 1-20.

RACHOWICZ, L. J., 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: Competing Explanations for the Origin of Emerging Infectious Diseases of Wildlife. *Conservation Biology,* 19, 1441-1448.

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

RAMSEY, J., REINERT, L., HARPER, L., WOODHAMS, D. & ROLLINS-SMITH, L. 2010. Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to

global amphibian declines, in the South African clawed frog, *Xenopus laevis*. *Infection and Immunity,* 78, 3981 - 3992.

RELYEA, R. & HOVERMAN, J. 2006. Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. *Ecology Letters,* 9, 1157-1171.

RELYEA, R. A. 2003. Predator cues and pesticides: a double dose of danger for amphibians. *Ecological Applications,* 13, 1515-1521.

RELYEA, R. A. 2004. Synergistic impacts of malathion and predatory stress on six species of North American tadpoles. *Environmental Toxicology and Chemistry,* 23, 1080-1084.

RELYEA, R. A. & JONES, D. K. 2009. The toxicity of Roundup Original Max® to 13 species of larval amphibians. *Environmental Toxicology and Chemistry,* 28, 2004-2008.

RELYEA, R. A. & MILLS, N. 2001. Predator-Induced Stress Makes the Pesticide Carbaryl More Deadly to Gray Treefrog Tadpoles (*Hyla versicolor*). *Proceedings of the National Academy of Sciences of the United States of America,* 98, 2491-2496.

RELYEA, R. A., SCHOEPPNER, N. M. & HOVERMAN, J. T. 2005. Pesticides and amphibians: the importance of community context. *Ecological Applications,* 15, 1125-1134.

RETALLICK, R. & MIERA, V. 2004. Strain differences in the amphibian chytrid *Batrachochytrium dendrobatidis* and non-permanent, sub-lethal effects of infection. *Diseases of Aquatic Organisms,* 75, 201 - 207.

RICHMOND, J. Q., SAVAGE, A. E., ZAMUDIO, K. R. & ROSENBLUM, E. B. 2009. Toward Immunogenetic Studies of Amphibian Chytridiomycosis: Linking Innate and Acquired Immunity. *BioScience,* 59, 311-320.

ROBERT, J. & OHTA, Y. 2009. Comparative and developmental study of the immune system in *Xenopus*. *Developmental Dynamics,* 238, 1249-1270.

ROBERTSON, H., EDEN, P., GAIKHORST, G., MATSON, P., SLATTERY, T. & VITALI, S. 2008. An automatic waste-water disinfection system for an amphibian captive-breeding and research facility. *International Zoo Yearbook,* 42, 53-57.

ROLLINS-SMITH, L. A. 2009. The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. *Biochimica et Biophysica Acta,* 1788, 1593-1599.

ROLLINS-SMITH, L. A. & CONLON, J. M. 2005. Antimicrobial peptide defenses against chytridiomycosis, an emerging infectious disease of amphibian populations. *Developmental & Comparative Immunology,* 29, 589-598.

ROLLINS-SMITH, L. A., DOERSAM, J. K., LONGCORE, J. E., TAYLOR, S. K., SHAMBLIN, J. C., CAREY, C. & ZASLOFF, M. A. 2002. Antimicrobial peptide

defenses against pathogens associated with global amphibian declines. *Developmental & Comparative Immunology,* 26, 63-72.

ROLLINS-SMITH, L. A., WOODHAMS, D. C., REINERT, L. K., VREDENBURG, V. T., BRIGGS, C. J., NIELSEN, P. F. & MICHAEL CONLON, J. 2006. Antimicrobial peptide defenses of the mountain yellow-legged frog (*Rana muscosa*). *Developmental & Comparative Immunology,* 30, 831-842.

ROSENBLUM, E. B., POORTEN, T. J., SETTLES, M., MURDOCH, G. K., ROBERT, J., MADDOX, N. & EISEN, M. B. 2009. Genome-wide transcriptional response of *Silurana* (*Xenopus*) *tropicalis* to infection with the deadly chytrid fungus. *PLoS One,* 4, e6494.

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*. *Proceedings of the National Academy of Sciences,* 105, 17034-17039.

SCHLOEGEL, L. M., DASZAK, P., CUNNINGHAM, A. A., SPEARE, R. & HILL, B. 2010. Two amphibian diseases, chytridiomycosis and ranaviral disease, are now globally notifiable to the World Organization for Animal Health (OIE): an assessment. *Diseases of Aquatic Organisms,* 92, 101-108.

SCHOCK, D. M., BOLLINGER, T. K., COLLINS, J. P. 2009. Mortality rates differ among amphibian populations exposed to three strains of a lethal ranavirus. *EcoHealth,* 6, 438-448.

SEARLE, C. L., MENDELSON, J. R., GREEN, L. E. & DUFFY, M. A. 2013. *Daphnia* predation on the amphibian chytrid fungus and its impacts on disease risk in tadpoles. *Ecology and Evolution,* 3, 4129-4138.

SEMLITSCH, R. D. & BODIE, J. R. 1998. Are Small, Isolated Wetlands Expendable? *Conservation Biology,* 12, 1129-1133.

SKERRATT, L., BERGER, L., SPEARE, R., CASHINS, S., MCDONALD, K., PHILLOTT, A., HINES, H. & KENYON, N. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *Ecohealth,* 4, 125 - 134.

SKERRATT, L. F., BERGER, L., HINES, H. B., MCDONALD, K. R., MENDEZ, D. & SPEARE, R. 2008. Survey protocol for detecting chytridiomycosis in all Australian frog populations. *Diseases of Aquatic Organisms,* 80, 85-94.

SMITH, K. G. & WELDON, C. 2007. A Conceptual Framework for Detecting Oral Chytridiomycosis in Tadpoles. *Copeia,* 2007, 1024-1028.

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

SOMCHIT, N., NORSHAHIDA, A. R., HASIAH, A. H., ZURAINI, A., SULAIMAN, M. R. & NOORDIN, M. M. 2004. Hepatotoxicity induced by antifungal drugs itraconazole and fluconazole in rats: a comparative *in vivo* study. *Human and Experimental Toxicology*, 23, 519-525.

SOTO-AZAT, C., CLARKE, B. T., POYNTON, J. C. & CUNNINGHAM, A. A. 2010. Widespread historical presence of *Batrachochytrium dendrobatidis* in African pipid frogs. *Diversity and Distributions,* 16, 126-131.

SOULÉ, M. E. 1986. Conservation biology. The science of scarcity and diversity. Sinauer Press, Sunderland, Massachusetts.

SRIVASTAVA, S., SINHA, R. & ROY, D. 2004. Toxicological effects of malachite green. *Aquatic Toxicology,* 66, 319-329.

STEPHAN, C. E. & ROGERS, J. W. Advantages of using regression analysis to calculate results of chronic toxicity tests. *In:* BAHNER, R. C. & HANSEN, D. J., (eds). Aquatic toxicology and hazard assessment: Eighth symposium, 1985 Philadelphia. American Society for Testing and Materials, 328-338.

STUART, S. N., CHANSON, J. S., COX, N. A., YOUNG, B. E., RODRIGUES, A. S., FISCHMAN, D. L. & WALLER, R. W. 2004. Status and trends of amphibian declines and extinctions worldwide. *Science,* 306, 1783-1786.

STUART, S. N., HOFFMANN, M., CHANSON, J. S., COX, N. A., BERRIDGE, R. J., RAMANI, P. & YOUNG, B. E. 2008. *Threatened Amphibians of the World,* Lynx Edicions, Barcelona, Spain; IUCN, Gland, Switzerland; Conservation International, Arlington, Virginia, USA.

SUTHERLAND, W. J., AVELING, R., BROOKS, T. M., CLOUT, M., DICKS, L. V., FELLMAN, L., FLEISHMAN, E., GIBBONS, D. W., KEIM, B., LICKORISH, F., MONK, K. A., MORTIMER, D., PECK, L. S., PRETTY, J., ROCKSTRÖM, J., RODRÍGUEZ, J. P., SMITH, R. K., SPALDING, M. D., TONNEIJCK, F. H. & WATKINSON, A. R. 2014. A horizon scan of global conservation issues for 2014. *Trends in Ecology & Evolution,* 29, 15-22.

TERHIVUO, J. 1988. Phenology of spawning of the common frog (*Rana temporaria* L.) in Finland from 1846 to 1986. *Annual Zoology Fennici,* 25, 165-175.

THOMPSON, D. G., WOJTASZEK, B. F., STAZNIK, B., CHARTRAND, D. T. & STEPHENSON, G. R. 2004. Chemical and biomonitoring to assess potential acute effects of Vision® herbicide on native amphibian larvae in forest wetlands. *Environmental Toxicology and Chemistry,* 23, 843-849.

THORP, J. H. & COVICH, A. P. 2010. Ecology and classification of North American freshwater invertebrates. *Aquatic ecology series.* San Diego: Academic Press.

TOBLER, U. & SCHMIDT, B. R. 2010. Within- and Among-Population Variation in Chytridiomycosis-Induced Mortality in the Toad *Alytes obstetricans*. *PLoS ONE,* 5, 1-8.

TODD, B. D., BERGERON, C. M., HEPNER, M. J. & HOPKINS, W. A. 2011. Aquatic and terrestrial stressors in amphibians: A test of the double jeopardy hypothesis based on maternally and trophically derived contaminants. *Environmental Toxicology and Chemistry,* 30, 2277-2284.

VERTUCCI, F. A. & CORN, P. S. 1996. Evaluation of Episodic Acidification and Amphibian Declines in the Rocky Mountains. *Ecological Applications,* 6, 449-457.

VITOUSEK, P. M., MOONEY, H. A., LUBCHENCO, J. & MELILLO, J. M. 1997. Human domination of Earth's ecosystems. *Science,* 277, 494-499.

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

VOYLES, J., YOUNG, S., BERGER, L., CAMPBELL, C., VOYLES, W. F., DINUDOM, A., COOK, D., WEBB, R., ALFORD, R. A. & SKERRATT, L. F. 2009. Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science,* 326, 582-585.

VREDENBURG, V. T., ROLAND, A. K., TUNSTALL, T. S., BRIGGS, C. J. & WAKE, D. B. 2010. Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proceedings of the National Academy of Sciences of the United States of America,* 107, 9689-9694.

WALKER, S. F., BOSCH, J., GOMEZ, V., GARNER, T. W. J., CUNNINGHAM, A. A., SCHMELLER, D. S., NINYEROLA, M., HENK, D. A., GINESTET, C., ARTHUR, C. P., FISHER, M. C. 2010. Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. *Ecology Letter,* 13, 372-382.

WARKENTIN, I. G., BICKFORD, D., SODHI, N. S. & BRADSHAW, C. J. A. 2009. Eating Frogs to Extinction. *Conservation Biology,* 23, 1056-1059.

WARNE, R. W., CRESPI, E. J. & BRUNNER, J. L. 2011. Escape from the pond: stress and developmental responses to ranavirus infection in wood frog tadpoles. *Functional Ecology,* 25, 139-146.

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.

WEBB, R., PHILIPS, A., SPEARE, R., CONNOLLY, J. & BERGER, L. 2012. Controlling wildlife fungal disease spread: *in vitro* efficacy of disinfectants against

Batrachochytrium dendrobatidis and *Mucor amphibiorum*. *Diseases of Aquatic Organisms,* 99, 119-125.

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

WELDON, C., DU PREEZ, L. H., HYATT, A. D., MULLER, R. & SPEARE, R. 2004. Origin of the amphibian chytrid fungus. *Emerging infectious diseases,* 10, 2100.

WELDON, C. & FISHER, M. C. 2011. The effect of trade-mediated spread of amphibian chytrid on amphibian conservation. *Fungal diseases: an emerging challenge to human, animal, and plant health*, 355-367.

WELSH, H. H., JR. 1990. Relictual Amphibians and Old-Growth Forests. *Conservation Biology,* 4, 309-319.

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

WILLIAMS, C. R., BRODIE, E. D., JR., TYLER, M. J. & WALKER, S. J. 2000. Antipredator Mechanisms of Australian Frogs. *Journal of Herpetology,* 34, 431-443.

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

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

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,* 89, 1627-1639.

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.

WOOLBRIGHT, L. L. 1996. Disturbance Influences Long-Term Population Patterns in the Puerto Rican Frog, *Eleutherodactylus coqui* (Anura: Leptodactylidae). *Biotropica,* 28, 493-501.

WRIGHT, K. 2001. Surgical techniques. *In:* WRIGHT, K. M. & WHITAKER, B. R. (eds.) *Amphibian Medicine and Captive Husbandry.* Krieger Publ. Co., Malabar, Florida.

WYMAN, R. L. & HAWKSLEY-LESCAULT, D. S. 1987. Soil Acidity Affects Distribution, Behavior, and Physiology of the Salamader *Plethodon Cinereus*. *Ecology,* 68, 1819-1827.

WYMAN, R. L. & JANCOLA, J. 1992. Degree and Scale of Terrestrial Acidification and Amphibian Community Structure. *Journal of Herpetology,* 26, 392-401.

YOUNG, S., BERGER, L. & SPEARE, R. 2007. Amphibian chytridiomycosis: strategies for captive management and conservation. *International Zoo Yearbook,* 41, 85-95.

ZIPPEL, K., JOHNSON, K., GAGLIARDO, R., GIBSON, R., MCFADDEN, M., BROWNE, R., MARTINEZ, C. & TOWNSEND, E. 2011. The Amphibian Ark: a global community for *ex situ* conservation of amphibians. *Herpetological Conservation and Biology,* 6, 340-352.

ZUG, G. R., VITT, L. J. & CALDWELL, J. P. 2001. *Herpetology: An Introductory Biology of Amphibians and Reptiles,* Academic Press., San Diego.