Disease dynamics in a metapopulation of *Amietia hymenopus*

A Pretorius

22792864

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Supervisor: Prof C Weldon

Co-supervisor: Dr R Antwis

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"But ask the animals, and they will teach you, or the birds in the sky, and they will tell you, or speak to the earth, and it will teach you, or let the fish in the sea inform you. Which of all these does not know that the hand of the LORD has done this? In his hand is the life of every creature and the breath of all mankind."

Job 12:7-10 (NIV)

"Reptiles and amphibians are sometimes

thought of as primitive, dull and dim-witted.

In fact, of course, they can be lethally fast,

spectacularly beautiful, surprisingly

affectionate and very sophisticated."

– David Attenborough

I would like to dedicate this dissertation to my parents and fiancé. You have been an inspiration to me. Nothing worth having comes easy and with your guidance and understanding the hard work has paid off.

Abstract

Batrachochytrium dendrobatidis (*Bd*), a fungal pathogen of amphibians capable of adversely affecting all levels of organisation up to community level. In South Africa *B. dendrobatidis* is widely distributed including in the Drakensberg Mountains where it infects Phofung river frogs, *Amietia hymenopus*. Our objective was to identify factors driving disease dynamics of *B. dendrobatidis* in *A. hymenopus*. We made use of a 10 year data set that resulted from monitoring this host-pathogen relationship in tadpoles from the Mont-aux-Sources region. Tadpoles were collected twice annually from four rivers: Vemvane, Tugela, Bilanjil and Ribbon Falls. Presence/absence of *B. dendrobatidis* was determined through qPCR analysis and cytological screening of tadpole mouthparts. We found no statistical significant difference between the sites, but infection was more consistent between years at sites situated along popular tourist hiking trails. Interestingly, infection prevalence, although higher in summer, did not differ significantly between seasons. High altitude coincides with moderate temperatures resulting in a repressed fluctuation on the pathogen's prevalence between warmer and colder months. Rainfall, however was negatively correlated with infection prevalence. Growth rate ratios of tadpoles indicated that tadpole size and not developmental stage is one of the main drivers of infection. Persistently low to moderate infection prevalence and low pathogen virulence implies that *B. dendrobatidis* acts as an endemic infection in *A. hymenopus*. Furthermore microsatellites were developed for this species during this study to aid in population genetics, unfortunately this was not possible, but it will be very useful for future conservation.

Key words: pathogen, prevalence, amphibian, disease dynamics, high altitude, microsatellites, conservation

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Declaration

I, Abigail Pretorius, declare that this dissertation is my own, unaided work, except where otherwise acknowledged. It is being submitted for the degree of M.Sc. to the North-West University, Potchefstroom. It has not been submitted for any degree or examination at any other university.

Abigail Pretorius

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Chapter outlay

Chapter 1 contains a general introduction to the study area, Mont-aux-Sources within the Drakensberg Mountain range in KwaZulu-Natal and Lesotho. It defines the target species for this study, *Amietia hymenopus* and the pathogen *Batrachochytrium dendrobatidis* that threatens amphibians around the world. It also contains the aims and objectives of this study.

Chapter 2 interprets the analysis of 10 years of data from a long term monitoring study. It describes the collection and processing of the tadpoles from this study area. It also discusses the results with a short discussion and conclusion to explain the outcome of the monitoring.

Chapter 3 describes the various steps to the methodology of developing microsatellites for *A. hymenopus*.

Chapter 4 contains the general conclusion of the entire study; including recommendations for possible improvements should the study continue.

Statement on the contribution of others

Chapter 2

This part of the study was started in 2006. From 2006 to 2007 Kevin Smith collected and processed samples. A paper was submitted and accepted by the journal: diseases of aquatic organisms in 2007. It was titled, Relationships among size, development, and *Batrachochytrium dendrobatidis* infection in African tadpoles. The research was funded by the North-West University, Potchefstroom campus. Authors for this paper include: Kevin G. Smith, Ché Weldon, Werner Conradie and Louis H. du Preez. From 2007 to 2009 Leon Meyer collected samples from the Drakensberg Mountains and processed them at the North-West University, Potchefstroom campus. From 2009 to 2015 samples were collected by Ché Weldon who did the processing at the same University. From 2015 to 2016 the samples were collected by me and different researchers that accompanied me during the different field work trips. Samples were processed at the North-West University, Potchefstroom campus and the Zoological Gardens of South Africa.

Chapter 3

This part of the study will be submitted as a paper to the journal Amphibia-Reptilia in November 2016. It is titled: Characterisation of 11 tri- and tetra-nucleotide polymorphic microsatellite loci for the Phofung river frog *Amietia hymenopus* using Illumina paired-end sequencing. The research was funded by the University of Manchester and the North-West University, Potchefstroom Campus. Authors for this paper include: Sarah Griffiths, Rachael Antwis, Abigail Pretorius, Graeme Fox, Ché Weldon and Richard Preziosi. Samples were collected in the Drakensberg Mountains around the Mont-aux-Sources area by the candidate and Ché Weldon. Fieldwork was funded by the National Research Foundation (NRF). Isolation of DNA and primer testing was done by the candidate and Rachael Antwis at the University of Manchester. Refinement of microsatelites were done by Sarah Griffiths also at the University of Manchester.

Chapter 1

Introduction and literature review

1.1. The Drakensberg Mountain range

1.1.1. Lesotho and the Drakensberg Mountains

Lesotho is the only country that is entirely located above 1,000 m above sea-level and it is landlocked within South Africa. It includes a vast amount of low hills and hi plateaus, including the Drakensberg Mountains and covers an area of just over 30,000 km^2. The highest known Mountain within Lesotho is known as Thabana Ntlenyana, which reaches a height of 3,482 m. The lowest point is found where the Orange and Makhaleng Rivers meet at 1,400 m. The watershed defines the border between South Africa and Lesotho and large rivers such as the Vaal and Orange Rivers flow from this mountain range in a Western direction. Some smaller rivers also flow from the mountain, in an Eastern direction, such as the Tugela (also spelt *Thukela* and *Uthukela*) River. The Tugela waterfall (Figure 1.1) is South Africa's highest waterfall with a total drop of 946 m from the summit plateau (Souchon, 2005). The cliffs, precipices and scattered peaks that collectively make up the Amphitheatre are the defining features of the Mont-aux-Sources area. With the Sentinel and Eastern Buttress towering up on either side, the Amphitheatre Wall rises majestically out of the enclosed Tugela Gorge, 800 metres high and four kilometres wide (Souchon, 2005). About 2.5 km inland, south of the falls, the land rises up to Mont-aux-Sources that reaches 3,282 m (Bristow, 2003). For many years this peak was regarded as the highest summit in South Africa although it actually falls well short of the republic's true high point, Mafadi at 3,451 m (Souchon, 2005).

Figure 1.1. The Tugela waterfall as seen from Beacon buttress.

The mountain formed approximately 182 million years ago as a result of the accumulation of lava that was more than 1 600 m thick. The Earth's crust ruptured and huge amounts of basaltic lava flowed out over the Clarens desert of that time (Linstrom, 1981). The lava field was extensive, probably covering most of Southern Africa and completely buried the former sand desert. Most of the lava had been removed later by erosion, but remnants still remain as the Drakensberg Mountains and the mountains of Lesotho (McCarthy & Rubige, 2005). The lava plates that still remain today are basalt rocks (a type of igneous rock) that have a maximum thickness of about 1,500 m. The basaltic rocks often form dark vertical cliffs such as the Amphitheatre's cliffs. In addition to its height, the area's significance is also geographical as it marks the pinnacle from which three of South Africa's major water basins begin. Basutoland missionaries Arbousset and Daumas named the peak in 1836 as they searched for the headwaters of the Orange and Caledon rivers. It is of non-technical grade and is often visited by trekkers and hikers. Unfortunately for these early missionaries, the true source of the Orange River lies further south, behind Mponjwana (Souchon, 2005).

As one of the Drakensberg's premier tourist and hiking destinations, the Mont-aux-Sources area has a good system of paths into Tugela Gorge and other lower-lying areas. A gravel road leads up to the base of Sentinel from the Free State side, offering a quick and easy 5 km route to the escarpment on good paths and a set of chain ladders (Figure 1.2). The original ladders were originally installed by Otto Zunckle in 1930 along with a hikers hut on the top of the plateau. The present study was conducted on top of the Drakensberg Mountains of Lesotho and KwaZulu-Natal.

Figure 1.2. A hiker summits the upper of the first section of the chain ladders.

1.1.2. Climate

The weather on the plateau fluctuates during the day and is usually quite unpredictable (Suchet, 2006). The Mountain range experiences four distinct seasons: summers are warm but there are frequent thunderstorms and possibly snow; autumns are cooler and are characterised by dense mist and low cloud cover with a chance of snow; winters are cold and dry, snow and gales can be expected and the rivers freeze (Figure 1.3); springs experience all of the above (Bristow, 2003). There is a chance of rain during the entire year. Temperatures may vary from -11 °C in winter to 30 °C in the summer, mostly in the valleys of the mountains. The annual temperature for this region is on average 5.8 °C. The Drakensberg plateau is the area in South Africa with the highest lightning frequency / km^2 and as a result veld fires are common (Nel & Sumner, 2008).

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Figure1.3. Headwater of the Tugela River covered with a sheet of ice.

An important factor contributing to the weather is the relative position of high- and low-pressure cells across the sub-continent and the winds they produce. During the summer there is usually a low-pressure system over the interior, with a high-pressure cell over the Indian Ocean. Wind tends to flow from high to low pressure bringing rain from the ocean. As the moist air reaches the Drakensberg it rises, cools and condenses to form storm clouds. During the winter this pattern is reversed; there is a high pressure system over the interior, while low pressure cells move across the country from the south-west (Bristow, 2003). Climate and weather mostly shape the environment. A lightning strike can crack into the basalt cliffs leaving a visible effect, while water erosion is an on-going process with results that can be seen over time. Often permafrost is the cause of the terrace-like erosion of steep hillsides at high altitudes. On cold nights moisture in the topsoil freezes and this literally lifts the top few centimetres of the ground. When it thaws, the top soil drops back and slides down creating a step. Overgrazing (Figure 1.3) greatly augments this process (Bristow, 2003).

Figure 1.4. Many domesticated animals are kept on the mountain plateau resulting in overgrazing.

1.1.3. Conservation status

Other than the animals that are herded in the grassland on top of the plateau, the area is relatively untouched. Tourism however is growing rapidly with a number of resorts and hotels being built at the foothills accompanied by various hiking trails. Figure 1.5 shows amazing views from the Drakensberg escarpment that are popular attractions. Today nearly the entire KwaZulu-Natal Drakensberg is contained within the uKhahlamba-Drakensberg Park and is controlled by the provincial parks board,

Ezemvelo KwaZulu-Natal Wildlife. Centralised conservation has also allowed for it to be declared as a World Heritage Site (Bristow, 2003; UNESCO, 2016) because of the natural beauty and cultural significance. The cultural component is due to the fact that there are about 40,000 pieces of San rock art in some 600 known locations in an area of 230,000 hectares (Bristow, 2003). The Drakensberg-Maloti Transfronteir Conservation Area was proclaimed in 2001 and includes the Sehlabathebe National Park in Lesotho. This is Lesotho's first World Heritage Site. The region is especially noteworthy for in situ conservation because of its biological diversity. Particularly plant species as it is recognised as a "Global Centre of Plant Diversity and Endemism". This environment is also important because it is known as an "endemic bird area". Species such as the Yellow-breasted Pipit is found in this area and it now a globally threatened species. The environment is truly unique and the number of valleys, rocky slopes and grassland protect a wide variety of threatened and endemic species (UNESCO, 2016).

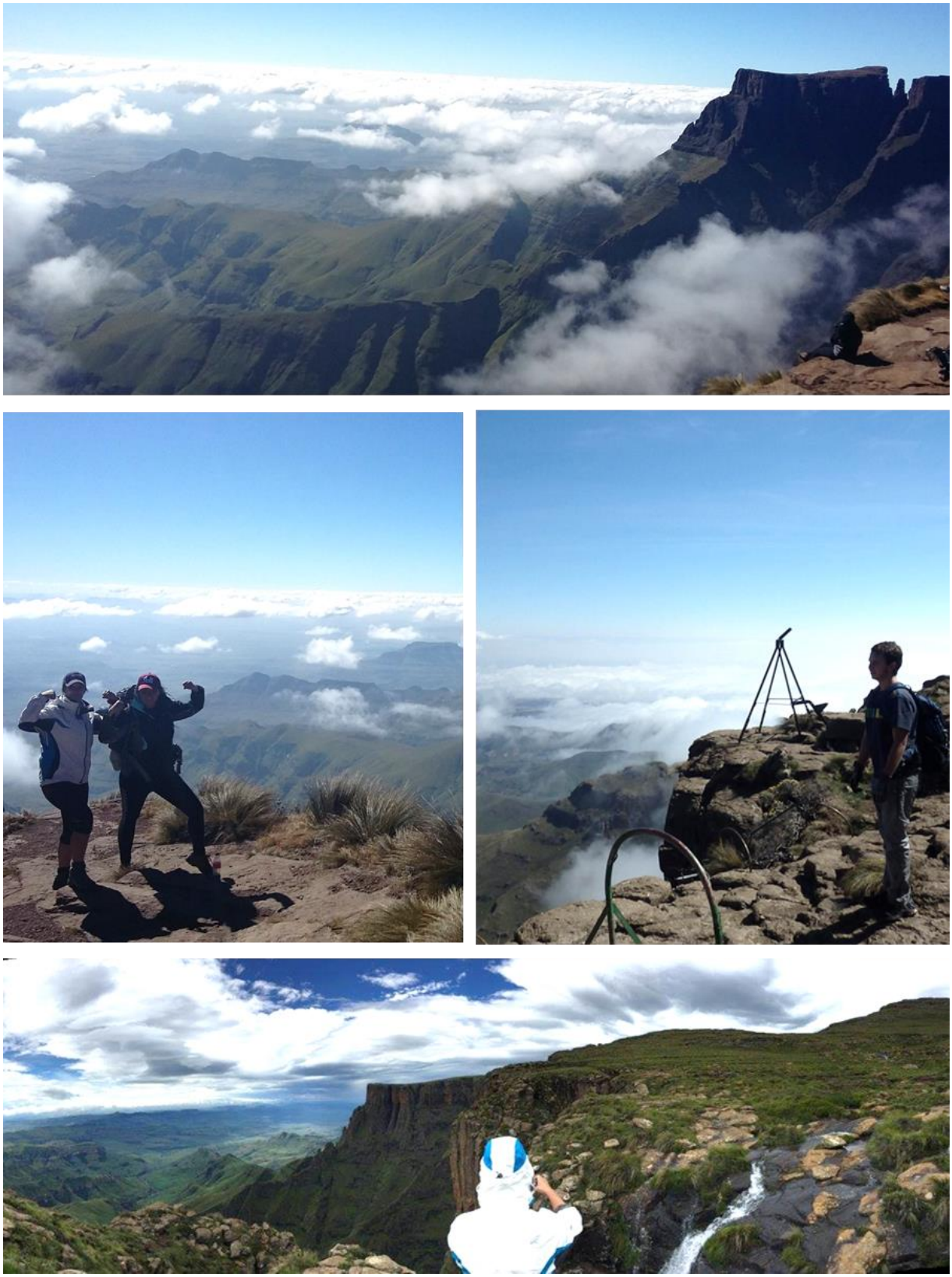


Figure 1.5. Vistas from the escarpment that are popular tourist attractions.

1.1.4. Fauna and flora

The vegetation of the Drakensberg is roughly described as being Afro-montane in the foothills below the plateau and Afro-alpine on the summit. These plants are mainly affected by altitude as it greatly influences temperature. With regard to attitude, north-facing slopes are hotter than south-facing slopes, therefore on the north-facing slopes it's more common to find open grasslands and scattered protea bushes and on the south-facing slopes there's usually forest and dense bush (Bristow, 2003). The montane belt includes both grasslands and temperate forests. The main trees that grow in the area are the highveld protea (*Protea caffra*) and the silver-leafed protea (*Protea roupelliae*). The main grasses include the red oat grass (*Themeda trindra*), which is a sweet grass and excellent for grazing and tussock grass (*Festuca costata*) which is a sour grass and therefore poor for grazing. Many bulbous plants, mainly watsonias and irises, bloom every spring, while delicate ground orchids rise in late summer (Bristow, 2003).

Animals that live in the mountains have to be specially adapted. Being extremely versatile and opportunistic feeders, jackals (*Canis mesomelas*) and baboons (*Papio ursinus*) survive in these harsh conditions. Retiles hibernate and the amphibians in this area are able to survive under sheets of ice in the winter months. Organisms such as the bearded vulture (*Gypaetus barbatus*) and grey rhebuck (*Pelea capreolus*) are specifically adapted physiologically and are highly specialised feeders. Some of the animals of the berg are found at all altitudes for example, the jackals, grey rhebuck and baboons. Others have a more specific habitat preference such as the bearded vulture that nests in the higher summits. Small antelope are also abundant at the foothills of the Drakensberg, such as bushbuck (*Tragelaphus scriptus*) that are found in the dense riverine bush and forest and the oribi (*Ourebia ourebi*) that are found in the grassland. Others include the klipspringer (*Oreotragus oreotragus*) and eland (*Taurotragus oryx*) (Bristow, 2003). Birds of the Drakensberg make up almost 40 % of all non-marine bird species of Southern Africa with some 300 species recorded (Alexander, 2016). The mountain pipit (*Anthus hoeschi*) is endemic to the Drakensberg Mountains while the buff-streaked chat *(Oenanthe bifasciata),* Drakensberg rock jumper *(Chaetops aurantius)*, Bush blackcap *(Lioptilus*

nigricapillus) and Drakensberg siskin *(Serinus symonsi)* are mostly found in the area (Chittenden, 2009). The berg adder (*Bitis arietas*), rhinkhals (*Hemachatus hemachatus*) and Drakensberg crag lizard (*Pseudocordylus melanotus*) are some of the reptiles that are found in this habitat (Marais, 2004; Marais & Alexander, 2008). These are only few of the wide variety of animals found above and below these mountains.

1.1.5. Frog diversity

Not many frog species are found on the plateau around the Mont-aux-Sources area. The Clicking Stream frog (*Stronylopus grayii*) has a wide distribution and can be found almost anywhere in Southern Africa, including habitats at high altitudes. Their calls are short and soft, like a monotonousness hollow click. Within a large chorus it is more of a continuous rattle. Males mainly call at night but calls are often heard throughout the day as well, from a concealed location, especially during cloudy weather (Du Preez & Carruthers, 2009). This species breeds in any aquatic habitat from ditches with water and shallow seeps to ponds and small dams. They tolerate water of any quality, even brackish pools along the coats' spray zone. This is one of the unique species that breed both in both summer with summer rainfall and winter with winter-rainfall. Some 300 eggs are then laid about 30 cm from the water's edge in the vegetation. This frog species is of least concern and needs no conservation action as they are abundant although difficult to find (Minter *et al*., 2004). During this study a few of these frogs were heard calling but never in choruses.

The Common River Frog (*Amietia delalandii*) is also found throughout most of Southern Africa. Their call consists of a series of short clicks followed by a short croak. Males call during the day and night, all year round, either from a concealed position within vegetation or along the water's edge. These frogs are found in the grassland, forest and savannah biome, on banks of rivers or pools. Often found in a wide variation of wetland habitats or even garden ponds (Du Preez & Carruthers, 2009).

Figure 1.6. An adult *Amietia delalandii* caught in the Vemvane River on top of the Drakensberg Mountain plateau.

The Maluti River frog (*Amietia vertebralis*) is found in habitats known as “Afro-montane grassland” at altitudes of above 1,700 m in the Drakensberg Mountains and Lesotho. They can tolerate extremely cold water and live under sheets of ice in the winter, but they cannot withstand high temperatures. This species is predominately aquatic and call while nearly submerged in water. Their calls sound like hollow knocks followed by a soft stuttering groan (Du Preez & Carruthers, 2009). During this study one individual (Figure 1.6) was caught and examined and then released again.

Figure 1.7. A juvenile *Amietia vertebralis* caught in a slow flowing stream within Lesotho.

The Phofung River frog (*Amietia hymenopus*) is the target species of this study (Figure 1.8) and is also found at high altitudes in the Drakensberg Mountains and Lesotho. The word Phofung has a Sotho origin and means mist. The grassland above the plateau is also known as the Phofung plains which is quite fitting for both as the area is frequently covered with a blanket of mist. The frogs' maximum snout vent length (SVL) is 65 mm and has a relatively broad head in comparison to its body (Du Preez & Carruthers, 2009). Adults are mostly found under rocks submerged in the river and can stay submerged for a number of days, while juveniles may be seen at the edge of the river (Lambris, 1988). Since this species is found at high altitudes, it has an umbraculum in its eyes to protect them since the UV index is much higher in that area. Their calls consist of intermitted, softly produced

clicks from the edges of the river or gently flowing side streams (Du Preez & Carruthers, 2009). Similar to *A. vertebralis*, *A. hymenopus* does not tolerate high temperatures but they do survive under sheets of ice in the winter (Lambris, 1989).

This species is an opportunistic breeder, as tadpoles are found throughout the year, although they mostly breed from September to February. They then attach eggs to submerged vegetation in the rivers with stony and sandy substrates (Lambris, 1989). According to Van Dijk, (1996) tadpole development is a slow process with a duration of a few months, sometimes exceeding a year. In this time, the tadpoles survive in the rivers and are well adapted to life in fast-flowing water. Its large sucker-like mouth allows it to have a firm grip on the smooth rock and its flat body and strong yet narrowly-webbed tail lets it swim against the stream.

Originally *A. hymenopus* was categorised as *restricted* (Branch, 1988), later however more localities of this species were found, especially within Lesotho (Bates, 2002). Now, according to the latest ICUN assessment (IUCN 2017), this species is listed as *Near Threatened*. There are known threats to the species but it is unlikely that any will cause significant declines within the populations (Minter *et al*., 2004). The frogs are especially protected by the habitat they are found. If more people and their livestock move up the rivers into areas of high altitude, it may threaten this species as increased water pollution and erosion will occur and *A. hymenopus* only occurs in pristine habitat and do not move over land (Mouton 1996).

According to O'Grady (1998) there was a mass mortality of *A. hymenopus* in 1998 reported by hikers in the Drakensberg escarpment. That year the river had been reduced to a series of isolated pools due to an exceptionally dry year but the cause of death was undetermined. After investigation, the fungal pathogen *Batrachochytrium dendrobatidis* was thought to be the cause as it was found in museum specimens of that species from that area (Weldon *et al*., 2004). This does not however prove that the pathogen was responsible for the mortalities. It may be seen as a plausible explanation as Berger *et al*. (2000) states that chytridiomycosis

epidemics often do follow after periods of severe drought and affect high-altitude species such *A. hymenopus*.

Figure 1.8. *Amietia hymenopus* adults and tadpoles found on the Drakensberg Mountain's escarpment which is the focal species of this study.

1.2. Amphibian chytrid

In Australia and Central America, montane amphibian populations were observed declining in the year 1993. No environmental causes were evident and there was no other evidence at the time (Richards *et al*., 1993). The presence of an unknown fungus was then detected among sick and dead amphibians in 1998 and was isolated from a blue poison dart frog (*Dendrobates auratus*) in 1999. It was identified and described and placed in the phylum Chytridiomycota which is the only phylum

that reproduce with zoospores (motile spores). These fungi are referred to as chytrid with about 1,000 known species distributed around the world. They are propelled by a single flagellum at the posterior end. Further the fungi was placed in the class Chytridiomycetes and the Order Chytridiales (Longcore *et al.*, 1999; James *et al*., 2009; Hyatt *et al.*, 2007). According to Powell (1993) these fungi can be found anywhere from the tropics to the arctic tundra, including even deserts. They are usually found in aquatic systems for example, rivers or dams. Also found in estuarine and marine environments but mostly in terrestrial ecosystems such as agricultural or desert soils and forests (Barr, 1990).

In the environment these fungi play an important role as bio-degraders as they reduce chitin found in insects, pollen, cellulose which is found in plant material and keratin found in skin and hair of organisms (Barr, 1990). According to Longcore *et al*. (1999) the only known chytrid parasite that affects vertebrates is known as *B. dendrobatidis* and is the leading contributor to the population declines and extinctions described in both wild and captive amphibian species infected by this cutaneous disease (Berger *et al* 1998; Berger *et al.*, 1999; Pessier *et al.*, 1999; Bosch *et al.*, 2001; Skerrat *et al*., 2007; Crawford *et al*., 2010). Subsequent surveys have demonstrated that *B. dendrobatidis* is now found on every continent except Antarctica (Berger *et al.*, 1999; Longcore *et al.*, 1999; Bosch *et al.*, 2001; Weldon *et al.*, 2004). In 2013 Huss *et al*. (2013) collected 120 archived American bullfrog (*Lithobates catesbeianus)* specimens that were collected between 1924 and 2007 in California (USA) and Baja California (Mexico). He used a qPCR assay to test for *B. dendrobatidis* and found 19.2 % to be infected. The earliest positive specimen from that study was collected in Sacramento Country, California in 1928. From another study, historical specimens were found positive that were collected in 1911 from Wonsan in North Korea (Fong *et al*., 2015). A study conducted by Talley *et al*. (2015) tested (using qPCR) 1028 specimens collected in Illinois (USA) between 1888 and 1989 and found the earliest specimen to be infected by *B. dendrobatidis* was collected in 1888 and is to date the earliest positive archived specimen. In South Africa Weldon *et al*. (2004) tested for *B. dendrobatidis* in 697 archived *Xenopus* specimens collected from 1879 to 1999. He found 2.7 % to be infected; the earliest positive specimen was collected in 1938.

1.2.1. Origin and diversity

Although it is possible to determine when *B. dendrobatidis* was first discovered, it remains difficult to determine the origin of *B. dendrobatidis* in spite of both population genetic and historical survey approaches that have been used to do this (Weldon *et al.,* 2004; Walker *et al*., 2008; James *et al*., 2009). From amphibians collected in Africa that were infected with *B. dendrobatidis* it was found that the pathogen had a wide distribution within Africa in the preceding century (Fisher *et al*., 2009b). Soto-Azat*et al*. (2010) suggested that Southern Africa may be the source of *B. dendrobatidis* and it has recently spread to all the corners of the earth with the trade of *Xenopus* spp. There are currently two hypotheses that attempt to clarify the global spread of *B. dendrobatidis*. The first states that the pathogen was only found in one geographical area and dispersed with the spread of amphibians. It is known as the "novel pathogen hypothesis" (Weldon *et al*., 2004; Fisher & Garner, 2007). The second hypothesis states that the pathogen is not new to the environment in others words, it has always been there, amphibians are only now more susceptible because of recent changes to the environment. Therefore as climate change alters the environment it facilitates the dispersal of *B. dendrobatidis* to new geographic areas. This is known as the "endemic pathogen hypothesis" (Rachowicz *et al*., 2005; Pounds *et al*., 2006). The novel pathogen hypothesis is the most widely accepted hypothesis as novel amphibian populations have been found to be infected due to human-assisted influences (Fisher & Garner, 2007; James *et al*., 2009; Schloegel *et al*., 2010).

Batrachochytrium dendrobatidis has a wide diversity which is made up of at least five different phylogenetic and phenotypic lineages (Farrer *et al*., 2011; Bataille *et al*., 2013). Three of these lineages, known as; *Bd*Cape, *Bd*Brazil and *Bd*CH, are known to have a lower virulence and differ morphologically to *Bd*GPL. *Bd*Cape is from Southern Africa and the island of Mallorca, *Bd*Brazil is from the Atlantic Forest in Brazil and *Bd*CH is from Switzerland, each of which is restricted in their distributions (Farrer *et al*., 2011; Bataille *et al*., 2013). *Bd*GPL however has a high virulence and

has recently expanded to a global distribution. It has also driven most chytrid-related mass mortalities to this day (Ghosh & Fisher 2016), and is associated with the arrival of *B. dendrobatidis* in North and Central America, Europe, Australia and the Caribbean (Farrer *et al*., 2011). The fifth lineage is known as *Bd*Korea and has an unknown virulence. So far it seems to be endemic to Asia (Bataille *et al*., 2013). Recombination in *B. dendrobatidis* is also possible and was first describes by Morgan *et al*. (2007). It appears the highly virulent *Bd*GPL came from a recombination of once isolated lineages and has spread through anthropogenic means across the world (Farrer *et al*., 2011). It has spread by means of food trade (Mazzoni *et al*., 2003), pet trade with infected amphibians (Aplin & Kirkpatrick, 1999), contaminated water (Rowley & Alford, 2007) or scientific trade (Parker *et al*, 2002; Weldon & Fisher, 2011). Therefore amphibians infected with *B. dendrobatidis* are placed under the "Environment Protection and Biodiversity Conservation Act 1999 (EPBC Act)" for being a "Key Threatening Process" (Skerratt *et al*., 2008).

1.2.2. Life cycle and transmission

Batrachochytrium dendrobatidis starts out with a sessile and reproductive zoosporangium which releases a motile and uniflagellated zoospore. The zoospores can only move over distances of about two centimetres and aren't active for very long (Berger *et al*., 2005; Kilpatrick *et al*., 2009). The zoosporangia possess cells containing discharge papillae which are responsible for the release of zoospores. They are then able to disperse through infected water and infect other individuals by host-to-host contact (Forzán *et al*., 2008; Pessier, 2008). A colony of *B. dendrobatidis* usually arises by asexual amplification as a sporangium is able to develop from a single zoospore (Berger *et al*., 2005). Sporangia then develop in the outer epidermal layers of amphibian skin, known as the *stratum granulosum* and the *stratum corneum.* Mature and empty zoosporangia are mostly found on the outside layers while immature sporangia occur deeper inside the more viable cells (Piotrowski *et al*., 2004). The zoospore forms a cyst underneath the skin's surface when it comes into contact with its host and then starts its reproductive life cycle, followed by what is known as clustering of zoospores (Piotrowski *et al*., 2004). The

aggregation of clusters in the cells is thought to be a colonisation strategy of the organism as they expand concentrically from the originally infected area. This results in a core devoid of sporangia within the hyperkeratosis tissue (Weldon & Du Preez, 2006).

In infected tadpoles the sporangia are spread on the mouthparts which are keratinised and as the tadpole develops it spreads to other keratinised parts, such as the epidermis (Marantelli *et al.,* 2004). Therefore it is necessary for a multi-layered keratinised skin for an adult frog to be infected by this pathogen (Berger *et al*., 1998; Marantelli *et al*., 2004). Adult frogs then often end up being infected from their tadpole stages (Rachowicz & Vredenburg, 2004). Amphibians become infected with *B. dendrobatidis* when their skin comes into direct contact with another host species or infected water and substrate that contain zoospores.

1.2.3. Pathogenesis

Many abiotic factors influence the pathogenesis of *B. dendrobatidis*, for example, water temperature. If temperatures are below the optimal preference for *B. dendrobatidis* it will inhibit the pathogen's development whereas if temperatures are too high, the pathogen may perish (Woodhams *et al*., 2008). According to Retallick & Miera (2004) different *B. dendrobatidis* lineages also influence its pathogenesis and *Bd*GPL has a higher pathogenesis compared to the other lineages. It is often seen that tadpoles infected with *B. dendrobatidis* have structural damage to their mouthparts as the pathogen causes epidermal hyperplasia to the cells containing keratin. Signs such as strange behaviour, keratosis, reduced response to stimuli and lethargy are often seen in infected post-metamorphic amphibians (Daszak *et al*., 1999; Forzán *et al*., 2008; Rosenblum *et al*., 2009). In a study conducted by Vieira *et al*. (2013) it was reported that loss of the keratinized mouthparts as well as depigmentation of the keratodonts and rostrodonts in tadpoles is a sure sign of *B. dendrobatidis* infection. The loss of keratinized mouthparts inhibits proper grazing and reduces the amount of food the tadpoles are able to ingest, resulting in delayed growth and development of the tadpoles which may eventually lead to their death

(Parris *et al*., 2004; Parris and Cornelius, 2004; Smith *et al*., 2007). In adult frogs *B. dendrobatidis* inhibits cutaneous osmoregulation because of lesions that damage the epidermis. This involves electrolyte imbalance, impaired gas exchange and fluid exchange and can ultimately lead to the death of the amphibian (Berger *et al*., 1998; Voyles *et al*., 2009).

The rate of mortality is influenced by many different factors, for example, age and species of the host, the temperature as well as the fungal dose (Berger *et al*., 1999; Berger *et al*., 2005). Some species though, tolerate *B. dendrobatidis* infection, for example the Mountain yellow-legged frog (*Rana muscosa*) has a high mortality rate due to the pathogen after metamorphosis but show no signs of infection while still a tadpole. Many species become sub-clinically infected, in other words, they are infected by *B. dendrobatidis* but do not develop chyridiomycosis and therefore they have a relative tolerance to the pathogen. A few of these species include the African clawed frog (*Xenopus laevis*), the American bullfrog (*Lithobates catesbeianus*) and the Tiger salamander (*Ambystoma tigrinum*) (Daszak *et al*., 2003; Davidson *et al*., 2003; Weldon *et al*., 2004; Garner *et al*., 2006; Fisher and Garner, 2007). This supports the theory that *B. dendrobatidis* has been distributed around the world by anthropogenic means. Exported and imported amphibians may have been sub-clinically infected and could therefore be reservoirs of *B. dendrobatidis* for susceptible and often novel species (Pessier, 2008).

1.2.4. Epidemiology

Zoospores can survive for months, even without a host, in the right conditions, such as when temperature is optimal and the environment is moist (Johnson & Speare 2005). New outbreaks of *B. dendrobatidis* are being observed recently as global warming shifts ambient temperatures to the optimum growth temperature of the pathogen, especially in highland areas (Bosch *et al*., 2007; Pounds *et al*., 2006). It strives in temperatures ranging from 4° C to 29° C (Longcore *et al*.,1999), and is often found at high elevations, for example the Rocky Mountains, the Sierra Nevadas and the Andes (Seimon *et al*., 2007; Pilliod *et al*., 2010; Vredenburg *et al*., 2010) as

well as at high latitudes (Schock *et al*., 2010). According to Knapp *et al*. (2011) cold weather environments do not always inhibit *B. dendrobatidis*, although other research argue that it does have an effect on its pathogenesis (Muths *et al*., 2008; Pilliod *et al*., 2010). The fact remains that there have been substantial amphibian declines at high altitudes were the environment is usually cooler but the relationship is not always that straight-forward and will become even more complicated as the climate changes (Fisher *et al*., 2009).

1.3. Aims and objectives

The aim of the study is to determine factors that influence pathogen dynamics in frogs at high altitudes from the Drakensberg Mountains. Through the use of *B. dentrobatidis* infection intensity, environmental variables and host genetics this study attempts to understand threats to the persistence of the frog populations found in the Drakensberg Mountains.

Objective 1: Determine the infection profile of *B. dentrobatidis* in *A. hymenopus* from the Mont aux Sources region of the Drakensberg in its environmental context by analysing 10 years of long term monitoring data.

Objective 2: Develop host species microsatellites to use for population genetics that can identify the evolutionary significance of this species and contribute to understanding the relationship between host genetics and disease incidence.

Chapter 2

Long term monitoring

2.1. Introduction

The major loss of biodiversity is becoming a great concern globally. The exact numbers of species lost is unknown but it is estimated that because of this rapid rate of loss we are currently experiencing the greatest mass extinction in the last 100,000 years (Eldridge, 1998). Anthropogenically caused habitat destruction plays a major role in the loss of species, but in many cases concerning amphibians, it is not always the leading cause (Alford & Richards 1999; Houlahan *et al.* 2000). Some of the other causes include chemical pollution, global warming, introduced alien species and infectious diseases (Blaustine *et al.*, 2004). Infectious diseases may include various pathogens for example; bacteria, parasites such as worms, protozoans, oomycetes and fungi (e.g., Blaustein *et al.* 1994; Drury *et al.,* 1995; Jancovich *et al*., 1997; Kiesecker & Blaustein, 1995; Berger *et al*., 1998; Longcore *et al*., 1999; Pessier *et al*., 1999; Blaustein & Johnson, 2003; Daszak *et al*., 2003). The fungus *Batrachochytrium dendrobatidis* is considered the leading pathogen in amphibian declines (Skerratt *et al.,* 2007).

Results from several laboratory experiments showed that *B. dendrobatidis* can infect and kill post metamorphic amphibians and can infect larval stages (Berger *et al.*, 1998; Berger *et al.*, 1999; Nichols & Lamirade, 2001). Tadpoles are seen as reservoir hosts for the pathogen as they are not killed by it granting *B. dendrobatidis* the ability to persist in an area even when the density of the amphibian population is low or absent and only tadpoles remain in the system (Daszak *et al.,* 1999, 2003).

Amphibian populations tend to decline at high altitudes (Young *et al.*, 2001; Stuart *et al.*, 2004; La Marca *et al.*, 2005), often in connection with the presence of *B. dendrobatidis* in montane areas (Berger *et al.,* 1998; Bosch *et al.,* 2001). High altitudes and cooler temperatures are now frequently associated with *B. dendrobatidis* outbreaks leading to fatal chytidiomycosis time and again (Fisher *et al.*, 2009).

Amietia hymenopus (the focal species of this study) is endemic to the Drakensberg of KwaZulu-Natal. Population declines have not been noted for this species and it is categorised as Least Concern, although intermittent mortality have been observed. Monitoring this high altitude species then becomes important because external stressors, for example; pollution or climate change, may aggravate the effects of *B. dendrobatidis* which in turn may cause population losses (Tarrant, 2013). Climate change is especially dangerous to species found in montane habitats (Thuiler, 2000).

Long term monitoring is often the process of comparing species presence and abundances of sampling sites that were sampled 50 to 100 years earlier. The data from these programmes are then compared to external factors such as global warming or habitat destruction (Thuiler, 2000). These programmes can also be complemented by experiments, for example research in microcosms. In these cases factors such as rainfall or temperature are manipulated to establish certain outcomes and expose unexpected responses (Thuiler, 2000). The most used approach to long term monitoring is to observe and re-survey sites over a certain time period. In some regions seasonal fluctuations of flowers and migratory animals have been recorded over time periods and special ranges. The data collected from the long term monitoring programme is then measured against known temperatures of rainfall collected in the same area over the same time span. From these approaches the problem remains that it is still challenging to establish the cause for the observed correlation (Thuiler, 2000).

Extensive efforts have been directed at identifying the pathogenicity and understanding the spread of *B. dendrobatidis* since chytridiomycosis is most likely

the reason for some of the worst amphibian population declines. The dynamics of *B. dendrobatidis* are still not completely understood in some regions of the world. It is also important to monitor reservoir hosts and dispersal agents in an attempt to help mitigate *B. dendrobatidis* spreading to more vulnerable species (Piotrowski *et al.,* 2004; Rodríguez-Brenes *et al.,* 2016).

This study focuses on the dynamics of *B. dendrobatidis* in the Drakensberg Mountains over a time span of 10 years. It aims to use the principles of long term monitoring to identify the fluctuations of the pathogen under different environmental and biological conditions in a very unique habitat and frog species. Long term monitoring in this way is useful as it allows trends between host-pathogen-environment to emerge and allows the possibility to predict future events. Over time it also becomes possible to observe which environmental factors influence each other.

2.2. Materials and methods

2.2.1. Site allocations, sampling intervals

All together there were nine possible sites investigated, four of which were selected for long term monitoring (Figure 2.1).

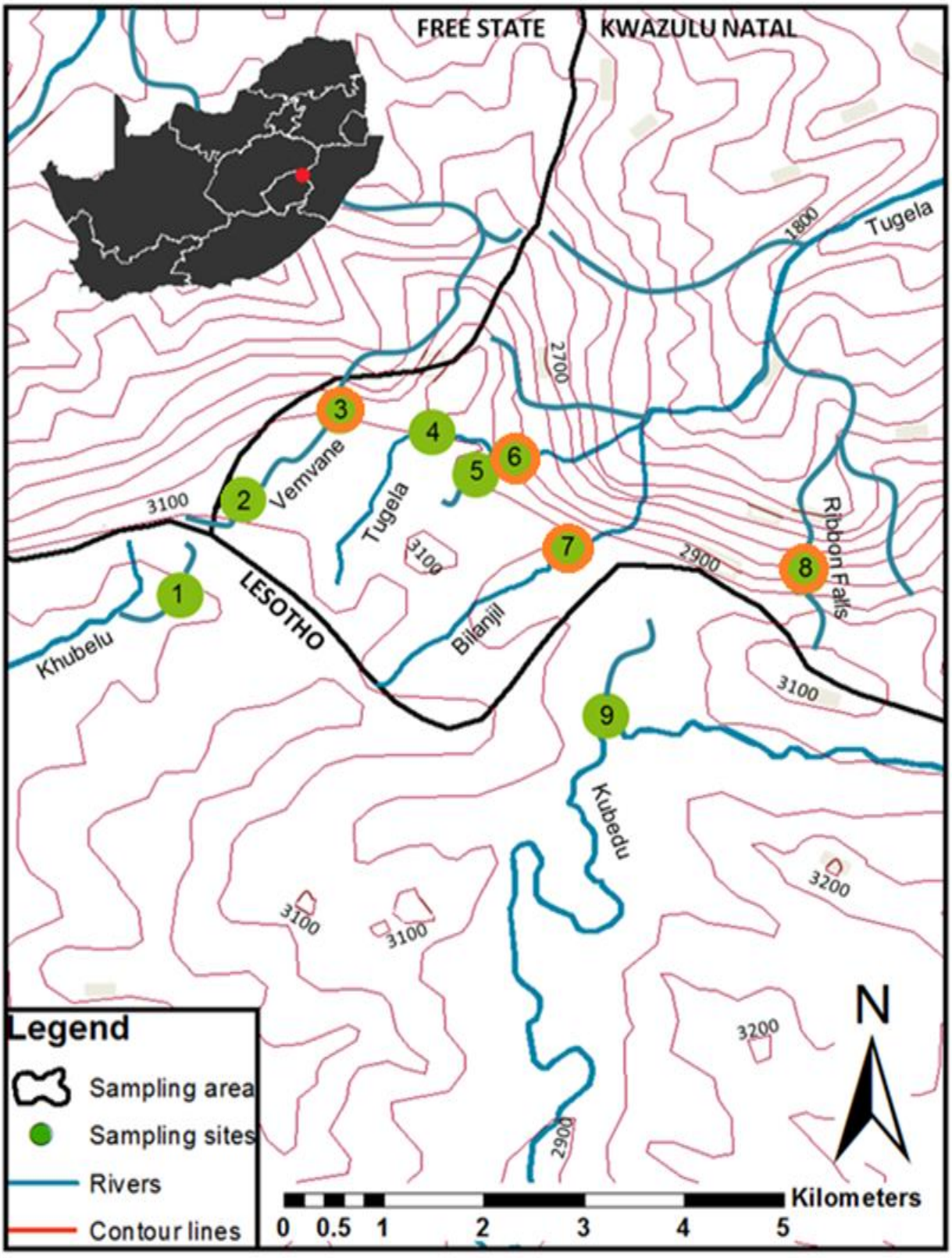


Figure 2.1. A GIS map indicating topography, the sample area and sites selected for sampling. The sites with an orange border indicate the sites that were used for long term monitoring.

The sampling sites were all selected in the origin of rivers along the Amphitheatre escarpment, ranging from 2,600 to 3,000 m above sea-level. The sites that were chosen are spread over a distance of about 15 km with valleys and hills in between within the province of KwaZulu-Natal. To reach the sites it was necessary to hike from the car park to the chain ladders, and ultimately hiking to each site individually, often in harsh weather conditions such as dense fog or very strong consis10t winds and rain. Temperatures were also known to drop immensely and without warning and often rivers in which were sampled would have a layer of ice on the surface. Lightning storms were also cause for alarm and sampling was carried out cautiously in these conditions.

Sampling was conducted for 10 years in summer and winter intervals. Summer sampling times ranged from September to February, which is when it is most likely to rain and winter sampling times ranged from March to July, in the low rainfall months. Rainfall data was retrieved from the historical maps of the South African Weather Service website (SAWS, 2016).

2.2.2. Collection of tadpoles

Samples from 2006 to 2011 were already in the long term monitoring database and therefor the collection for new samples took place from 2012 to 2016 at the different sites in the Drakensberg Mountains. At each site between 10 and 30 *A. hymenopus* tadpoles were sampled using 200 x 150 mm nets with a mesh size of 1 mm^2. Tadpoles were identified based on morphological characteristics described by Kruger *et al*. (2011). Tadpoles were collected using the dip-net method described by Shaffer *et al*. (2001) by overturning rocks in the river and sweeping underneath with the net as well as sweeping under overhanging vegetation on the banks of the river as shown in Figure 2.2. Once the tadpoles were collected, they were euthanized using MS222 (tricaine methanesulfonate) and preserved either in 70 % ethanol in a 20 ml polypropylene tube for PCR analysis or 10 % NBF (formalin) for cytological screening.

Figure 2.2. Collecting tadpoles within the rivers, sweeping under rocks and overhanging vegetation.

2.2.3. Measurements and screening for *B. dendrobatidis*

Once the tadpoles were brought to the lab, their body length was measured. This is the length from their mouth to the base were the tail begins as illustrated in Figure 2.3.

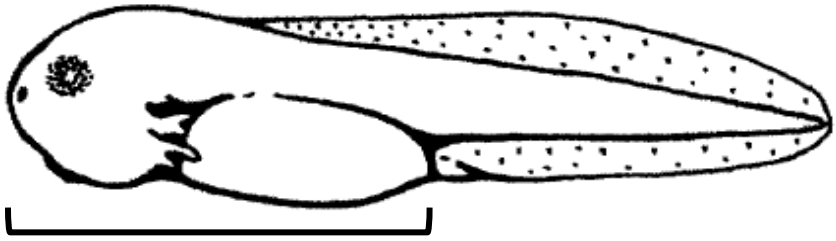
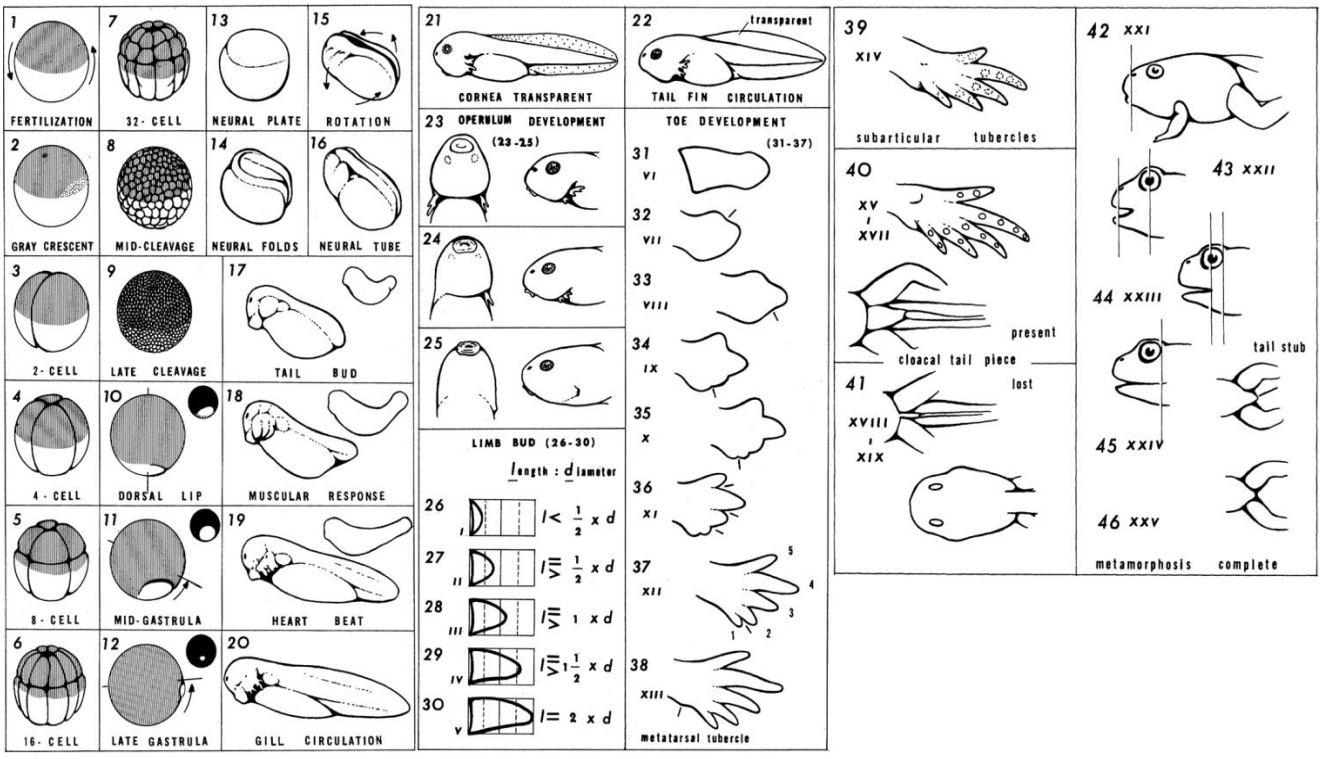


Figure 2.3. Body length of tadpoles. Image adapted from Gosner (1960)

Tadpoles were then staged using a dissecting microscope with 0.8 - 5 x 10 lenses according to Gosner (1960). This was done in order to identify at which stage they are in their development as they undergo morphological changes. Tadpoles undergo 46 stages to complete metamorphosis from embryo to adult frog, illustrated in Table 2.1.

Table 2.1. The different stages of tadpole development (Gosner, 1960).



Cytological screening and qPCRs (quantitative Polymerase Chain Reaction) were then used to diagnose *A. hymenopus* tadpoles. Although qPCRs are more accurate it was only necessary to determine if the individual was infected or not for this study

and therefore this method was used to a lesser extent. The qPCR method was therefore only used for samples that were preserved in ethanol.

2.2.4. Cytological screening

The Cytological screening technique was modified from the California Centre for Amphibian Disease Control's protocol (2007); testing for *Batrachochytrium dendrobatidis*. After tadpoles were measured and staged, their mouthparts were removed with a pair of sterilised scissors. Between individual tadpoles the scissors were sterilize over a flame to prevent cross contamination. Mouthparts were then transferred onto a glass slide to expose the keratodont rows and rostrodonts (see Figure 2.4).

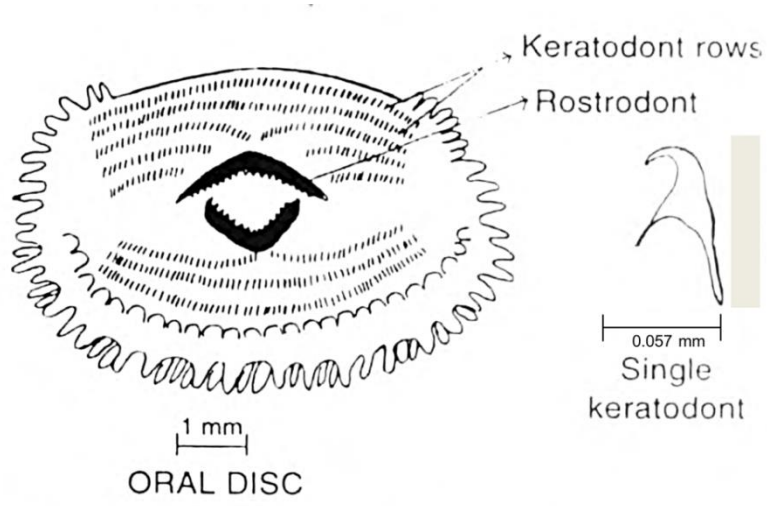


Figure 2.4. Illustration of mouthparts from a tadpole (Bordoloi *et al.,* 2001).

A drop of water in then placed on the wet mount followed by a cover slip. Using a standard compound microscope the slides were then screened under 100x and 400x magnification. A tadpole was diagnosed as infected when spherical sporangia could be seen (Longcore *et al.,* 1999). Most tadpoles were diagnosed in this manner.

2.2.5. Screening by means of qPCR

Batrachchytrium dendrobatidis diagnosis by means of qPCR was done with a modified protocol from Boyle *et al.* (2004), using a Taqman Assay. Tadpole mouthparts were surgically removed from the tadpoles using a sterilised scalpel blade. Between every tadpole a new sterilized blade was used to avoid cross contamination. The working area was also cleaned continuously with 96 % ethanol.

DNA was extracted by placing the sample tissue in a sterile 1.5 ml reaction tube containing 40 µl PrepMan Ultra. The samples were all labelled correctly and then vortexed for five minutes, centrifuged for one minute at 16,000 rcf, and placed in a heating block at 100 °C for 10 minutes. After the samples have cooled down at room temperature for two minutes they were again centrifuged at 16,000 rcf for three minutes. As much as possible supernatant was then pipetted into a new set of sterile 1.5 ml reaction tubes and they were labelled again. 36 µl of ultrapure water (ddH_2O) was then pipetted into a new set of reaction tubes, labelled again and four µl of the previously collected supernatant was added and mixed well.

The Master Mix was then prepared in a sterile 1.8 ml CryoTube according to the concentrations in Table 2.2. To ensure reliability the samples were run in duplicate, therefore the concentrations were doubled. After the final primer was added to the master mix it was mixed well by pipetting up and down a few times and then briefly vortexed.

Table 2.2. Master Mix composition used for q PCRs

Components	Single run (µl)	Duplicate run (µl)
Taqman	12.5	25
Forward primer	1.25	2.5
Probe (light sensitive)	0.0625	0.125
Reverse primer	1.25	2.5
dH2O	4.937	9.875
Total	**20**	**40**

A 96-well plate was then set up according to Table 2.3. The negatives are illustrated in red and the standards in blue. 20 µl of Master Mix was pipetted into each well, including the negatives and standards followed by 5 µl of DNA. Instead of DNA, sterile water was added to the negative wells. The plate was then placed in the PCR thermocycler.

Table 2.3. A typical set up of a 96-well PCR plate for qPCR purposes run in duplicate.

1a	2a	3a	4a	5a	6a	7a	8a	9a	10a	11a	12a
1b	2b	3b	4b	5b	6b	7b	8b	9b	10b	11b	12b
13a	14a	15a	neg	S100	S10	S1	S0.1	16a	17a	18a	19a
13b	14b	15b	neg	S100	S10	S1	S0.1	16b	17b	18b	19b
20a	21a	22a	23a	24a	25a	26a	27a	28a	29a	30a	31a
20b	21b	22b	23b	24b	25b	26b	27b	28b	29b	30b	31b
32a	33a	34a	35a	36a	37a	38a	39a	40a	41a	42a	43a
32b	33b	34b	35b	36b	37b	38b	39b	40b	41b	42b	43b

Only tadpoles that were collected in the summer survey of 2015 were diagnosed in this manner as they were preserved in ethanol.

2.2.6. Statistical analyses

Statistical analyses were performed on the data set using GraphPad Prism 5. Significance of the analyses mentioned below was tested through having a p-value smaller than 0.05 ($P < 0.05$). *Batrachochytrium dendrobatidis* prevalence was calculated as the percentage of the total number of frogs per sample set that were infected. The specific analyses performed were chosen based on composition normality of the data. Normality was tested using Shapiro-Wilk & D'agostino Pearson tests. The Mann-Whitney test was performed on the *B. dendrobatidis* prevalence for each year's data set, as it is a nonparametric test and does not require a normal distribution in the data. The test was used to identify whether one of two random variables were stochastically larger than the other (Mann & Whitney, 1947). No error bars are reported for this data set as the prevalence of *B. dendrobatidis* is calculated only from presence or absence of the fungus in individual frogs. When *B. dendrobatidis* prevalence was measured against each site the Kruskal-Wallis test was used as well as the Dunn's Mulitple comparison post-hoc test. The Kruskal-Wallis test is also a nonparametric test and significance would indicate that one sample stochastically dominates the other (Kruskal & Wallis, 1953), while the Dunn's test analyses the specific samples for stochastic dominance (Dunn, 1964). A two-way anova was performed on the data set where years were clumped together against *B. dendrobatidis* prevalence as it determines the influence of two different categorically independent variables on one continuous dependent variable. It was also used to assess the effect of each independent variable as well as the interaction between them. When sites were clumped together against *B. dendrobatidis* prevalence, another two-way anova was performed with a Bonferroni post-hoc test to counteract errors from multiple comparisons. When *B. dendrobatidis* was measured against season a paired t-test was used to determine if the data sets were significantly different from each other. In doing so the test statistic follows a t-distribution under a null hypothesis of any statistical hypothesis test. When the

tadpoles' growth ranges were measured against each other and % *B. dendrobatidis* prevalence, Pearson's correlation (Pearson product-moment correlation coefficient) was used to measure the degree of linear dependence between two variables. Three different tests were performed using this statistical analysis.

2.3. Results

Infected tissue was often accompanied by depigmentation of the keratinised mouthparts (Smith & Weldon, 2007). Figures 2.5 A & B were taken during the screening process to illustrate infected tadpoles.

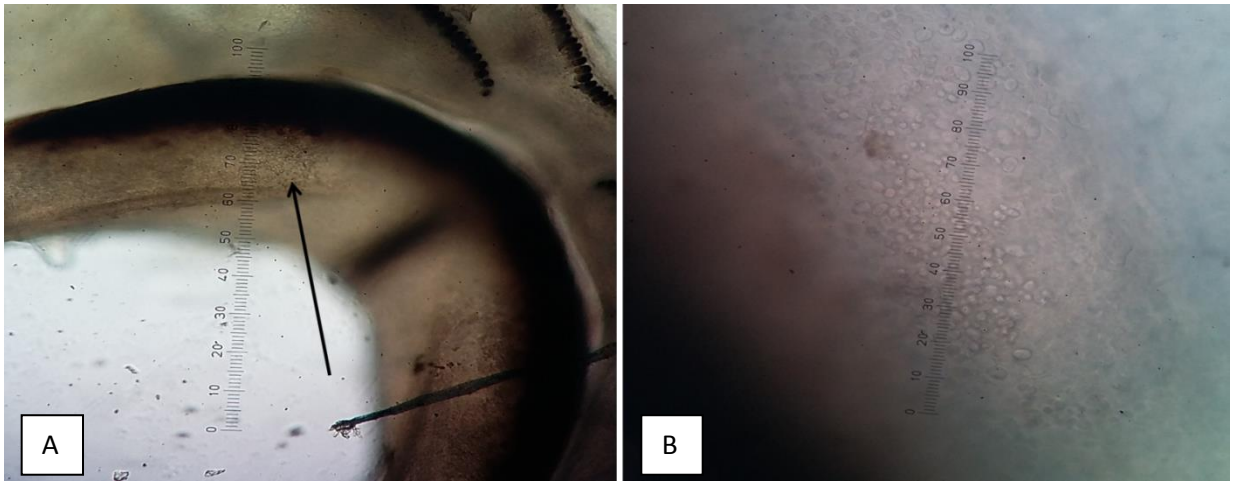


Figure 2.5. A. Infected rostrodont of *A. hymenopus* at 100x magnification. The arrow indicates the infected area. B. Spherical sporangia cluster of *B. dendrobatidis* clearly seen on the tadpole's rosrodont at 400x magnification.

The *B. dendrobatidis* prevalence in a certain area is affected by many environmental aspects. Some of these variables include; temperature, annual precipitation, species richness and biome (Fisher *et al.,* 2009). In this study the effects of seasonal changes, different sites within the study area, average monthly rainfall, growth and development of the host were examined to identify the disease dynamics in the Drakensberg Mountains.

Altogether 1,473 tadpoles were sampled and screened for *B. dendrobatidis* over a duration of 10 years, excluding 2013 when there were no sampling surveys. The data in Table 2.4 indicates the number of tadpoles collected each year in summer and winter surveys.

Table 2.4. The number of *A. hymenopus* tadpoles sampled over a duration of 10 years with summer and winter intervals.

Year	Summer survey (n)	Winter survey (n)
2006	84	12
2007	249	220
2008	142	218
2009	30	110
2010	40	No survey
2011	41	40
2012	20	43
2013	No survey	
2014	40	40
2015	23	41
2016	40	40

2.3.1. Influence of site on *B. dendrobatidis*

No significant difference was found in *B. dendrobatidis* prevalence over the duration of the study compared to each site individually (Figure 2.6), the P value of the medians are bigger than 0.05 ($P < 0.05$). From the graph it can be seen that Bilanjil and Ribbon Falls have the most variation in *B. dendrobatidis* prevalence. These two

sites also have the highest and lowest incidences. Tugela had the highest mean value although it was very close to the mean value of Vemvane. Tugela had the most consistent occurrence of *B. dendrobatidis* prevalences. From the graph it can also be seen that Tugela and Vemvane have never been *B. dendrobatidis* free during the last 10 years.

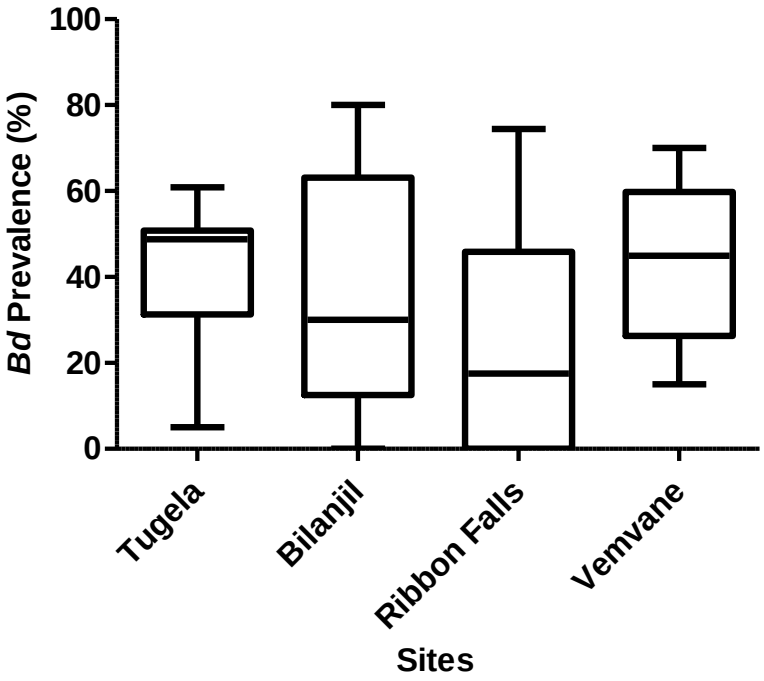


Figure 2.6. The % *B. dendrobatidis* provenance for each site over the duration of ten years.

Figure 2.7 gives a breakdown of site prevalence for each year. It shows that the years do differ significantly from one another versus *B. dendrobatidis* prevalence with a P value of 0.002. Statistical analysis, by means of a two-way anova, also shows that 52.8 % of the total variation in the data is attributed to the year. However there is no significant difference between B. *dendrobatidis* prevalence for each site (P value of 0.15) over the entire duration of the study and within each year. In 2010 and in 2016 Bilanjil and Ribbon Falls had 0 % *B. dendrobatidis* incidence. In 2015 Ribbon Falls had 0 % *B. dendrobatidis* incidence.

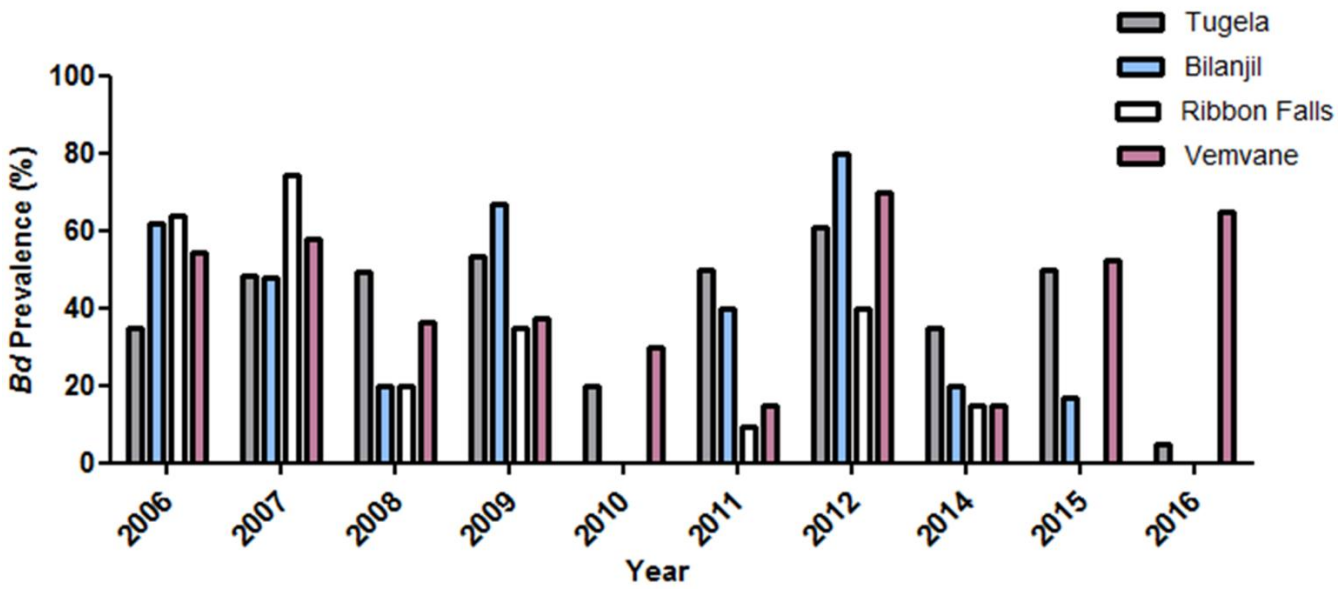


Figure 2.7. The *B. dendrobatidis* prevalence for each year with a comparison for each site within that year.

2.3.2. Seasonal influences on *B. dendrobatidis*

The following graph (Figure 2.8) shows the *B. dendrobatidis* prevalence for the sites combined over each year and for season separately. Although there is no statistical significant difference (P value of 0.24) between summer and winter surveys, it still appears from the graph that summer surveys tend to have higher *B. dendrobatidis* incidences than winter surveys. Winter only had a higher prevalence in 2006 (58 % in winter vs. 53 % in summer), and 2014 (32 % compared to 10%). *Batrachochytrium dendrobatidis* prevalence from 2006 to 2016 also shows no trend, 2-3 year peaks however do occur. The highest peak was in 2015 (100%) followed by 2012 at 95 % and then 2009 with 76 %, all within summer surveys. The lowest *B. dendrobatidis* prevalence were found in 2010 at 12 % followed by 2011 with 12.5 %, 2014 at 10 %, and 2015 that had 0 % in the winter survey. In 2016 both summer and winter surveys had the same *B. dendrobatidis* prevalence of 17.5 %.

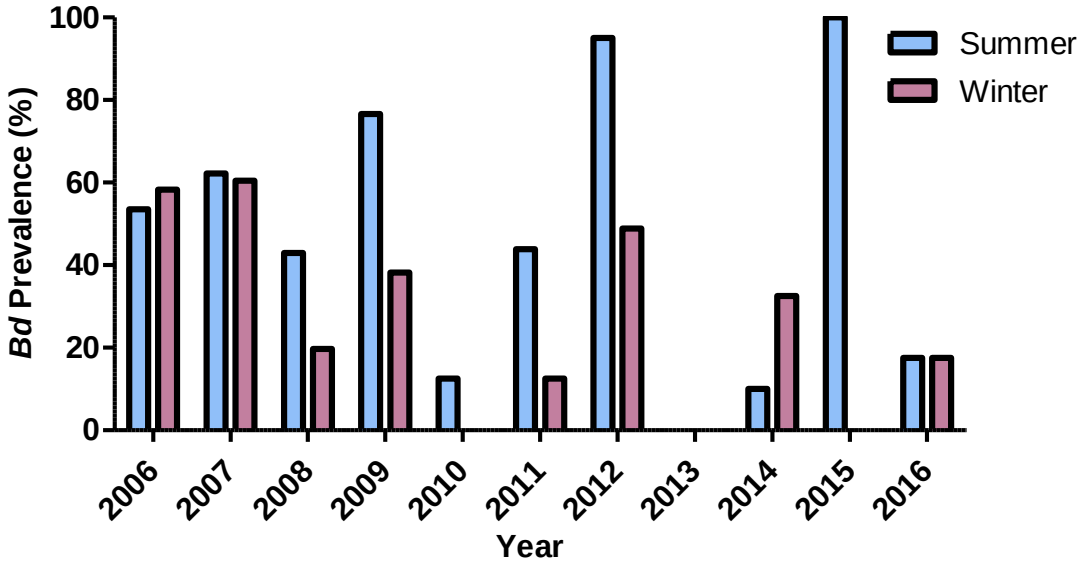


Figure 2.8. The *B. dendrobatidis* prevalence for each year within summer and winter sampling intervals.

Figure 2.9 also illustrates data pertaining to season similar to that of Figure 2.8, only in a chronological order according to the month when sampling was conducted. There is no statistical difference between comparable surveys, with specific regard to summer and winter surveys. This graph only aids in understanding the trends because sampling was not conducted at the same time each year. In some years the summer survey was carried out before winter and during other years, after winter.

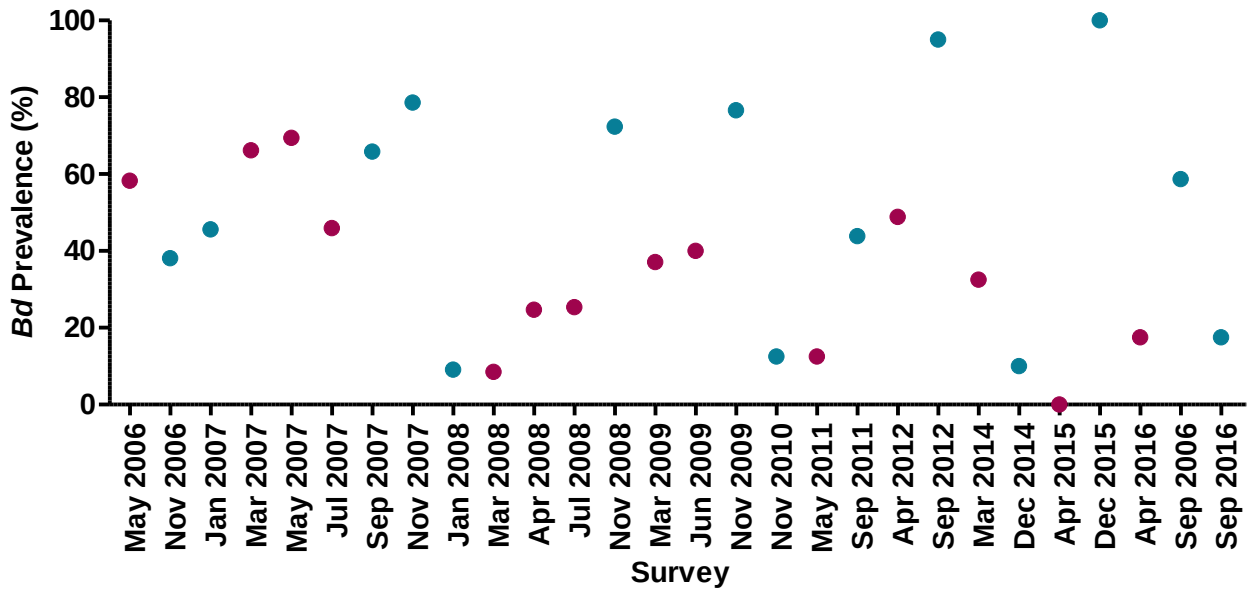


Figure 2.9. The chronological order of surveys per month for *B. dendrobatidis* prevalence for each year. The blue dots indicate summer surveys and purple dots indicate winter surveys.

For the 10 years, summer and winter surveys were then grouped together in order to analyse for a comprehensive seasonal difference (Figure 2.10). There was still no significant difference found using a paired t test between the seasonal data sets (P value of 0.08). Although summer does have slightly higher mean *B. dendrobatidis* prevalence than winter, but with larger variation. Summer also has a higher variation in *B. dendrobatidis* prevalence over the length of the study than winter has.

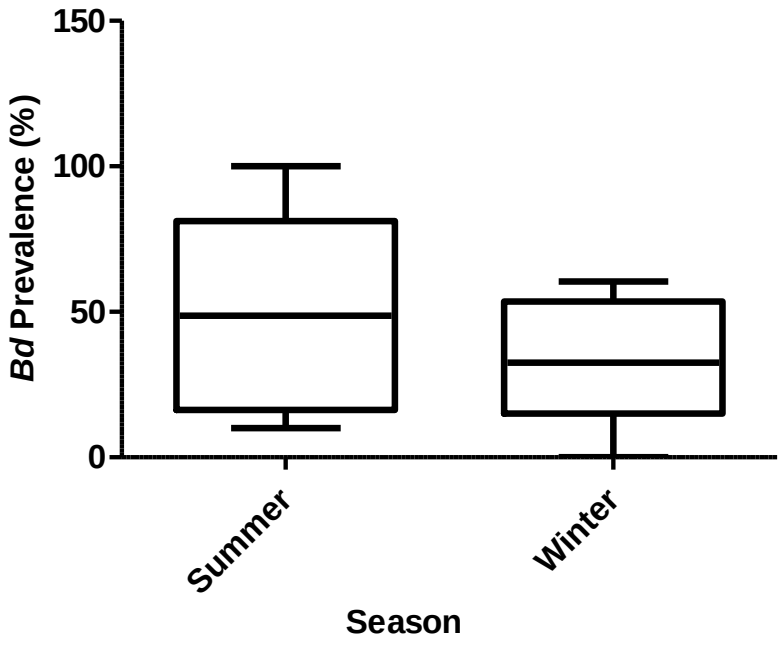


Figure 2.10. Box-whisker plot of the cumulative *B. dendrobatidis* prevalence for summer and winter surveys over 10 years.

2.3.3. Influence of rainfall on *B. dendrobatidis*

In order to interpret the trends of the *B. dendrobatidis* prevalence over the course often years, it was compared to rainfall of the corresponding sampling period (Figure 2.11). Rainfall data for May 2006 and November 2008 was unavailable unfortunately. Although no statistical significance was found when comparing prevalence and rainfall per survey, from the graph it appears that every time *B. dendrobatidis* prevalence peaks, there is little rainfall and every time rainfall peaks, *B. dendrobatidis* prevalence is lower. The third highest *B. dendrobatidis* prevalence was 78 % in November 2007 and only 60 mm average rainfall was recorded for that month. September 2012 had the second highest *B. dendrobatidis* incidence over the 10 year duration but only had 100 mm average rainfall for that month. No sampling was done in 2013. In December 2014 there was the highest rainfall of 500 mm average for the duration of the study and the third lowest *B. dendrobatidis* incidence with only 10 % infected tadpoles. In December 2015 there was 100 % *B. dendrobatidis* prevalence with only 50 mm average rainfall for that month. In 2016

rainfall and *B. dendrobatidis* incidence stayed constant with a *B. dendrobatidis* prevalence of 17.5 % and 50 mm average rainfall.

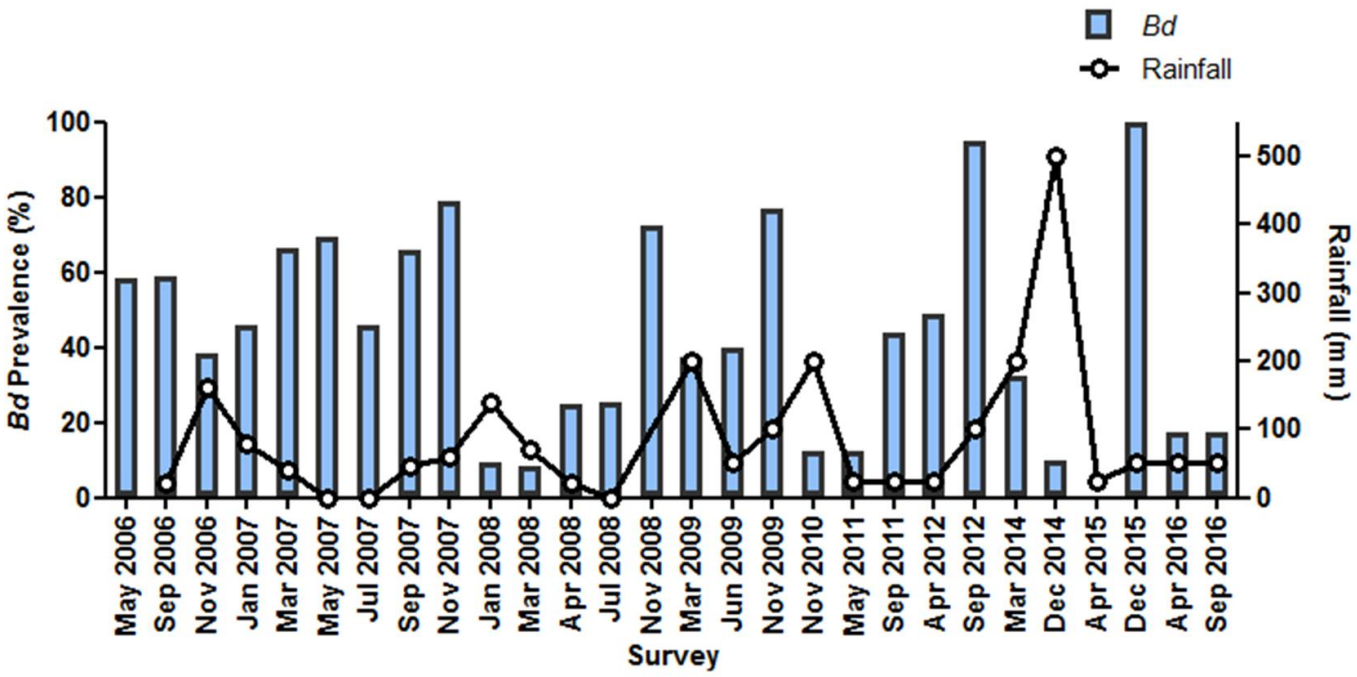


Figure 2.11. The *B. dendrobatidis* prevalence in comparison to the average rainfall per month (mm) for each survey.

In Figure 2.12 the *B. dendrobatidis* prevalence was compared to the amount of rainfall in a scatterplot. Although no distinct correlations could be found, some visual tendencies can be seen. Data points below 100 mm rainfall range between 0 and 100% *B. dendrobatidis* prevalence, but where the data points are above 100 mm rainfall the *B. dendrobatidis* prevalence is 40 % or lower indicating a clear threshold rainfall level.

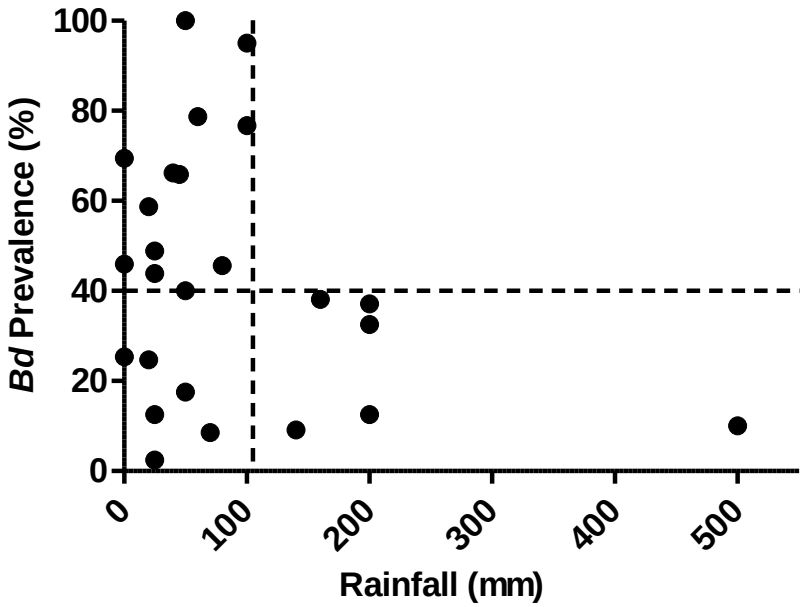


Figure 2.12. A scatterplot comparing *B. dendrobatidis* prevalence to rainfall (mm). The vertical dotted line (100mm) indicates the threshold rainfall level where a significant limit in *B. dendrobatidis* prevalence (horizontal dotted line; 40%) was observed.

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2.3.4. Influences of tadpoles growth and development on *B. dendrobatidis*

In Figure 2.13 *B. dendrobatidis* prevalence was compared to the Gosner stage of the tadpoles collected over the duration of the study. A strong linear positive correlation was found with a Pearson R of 0.96 ($p = <0.0001$). From statistical analysis it can also be said that 93 % of the data points fit within the 95 % confidence intervals with $R^2 = 0.93$. As the tadpole develops and stages increase, so does the *B. dendrobatidis* prevalence.

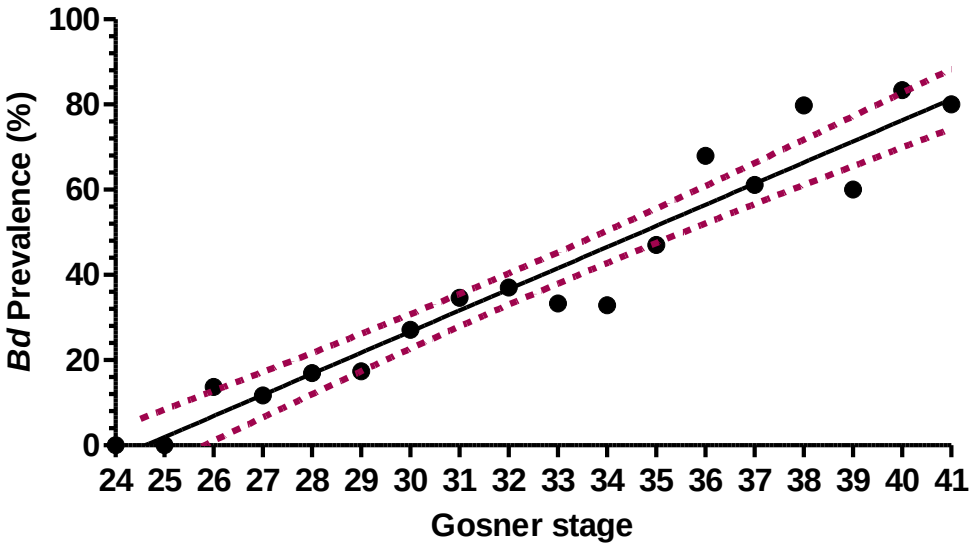

Figure 2.13. Pearson's correlation of *B. dendrobatidis* prevalence and development (Gosner stage) of the tadpoles. The linear regression line with 95 % confidence interval bands (red dotted lines) are also indicated.

The *B. dendrobatidis* prevalence was also compared to the body length of the tadpoles collected over the duration of the study, shown in Figure 2.14, to measure the influence of growth. A statistically significant P value of <0.0001 was given for Pearson's correlation on these data. It has a stronger linear correlation than the previous correlation with a Pearson R of 0.98. The R^2 is also higher at 0.96. Thus 96 % of the data point fit within the 95 % confidence intervals. As the tadpole grows and increases in size, the *B. dendrobatidis* prevalence increases.

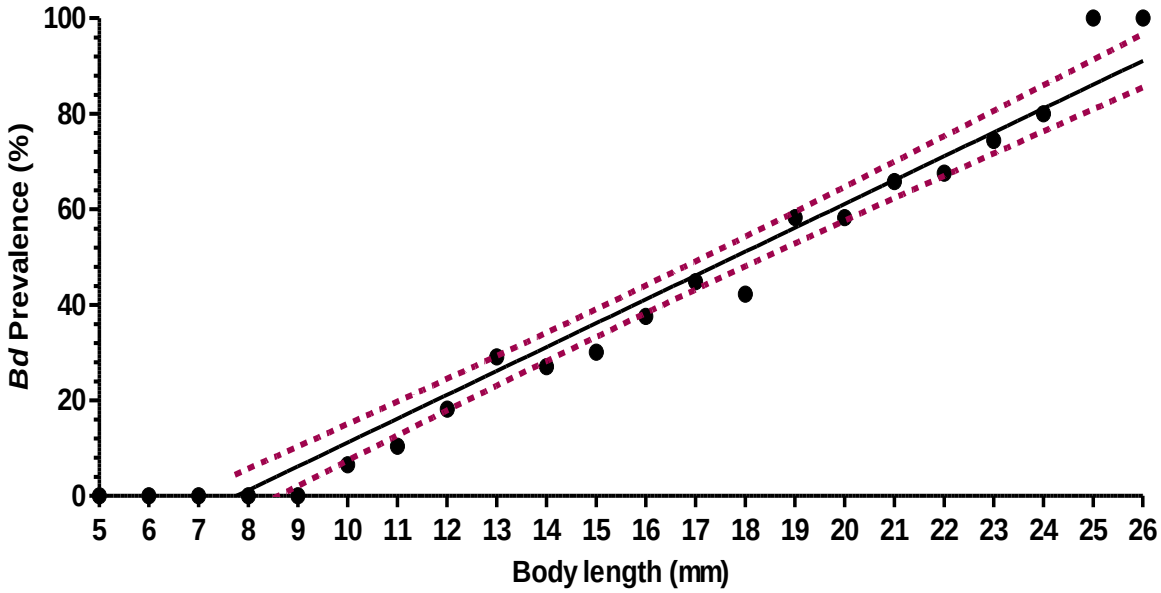

Figure 2.14. A Pearson's correlation of the *B. dendrobatidis* prevalence in comparison to the body length of the tadpoles. The linear regression line with 95 % confidence interval bands (red dotted lines) are also indicated.

Figure 2.15 represents the tadpoles' growth rate by comparing the Gosner stage with body length. As the tadpoles develop and their Gosner stage increases so do the sizes and therefore the body length of the tadpoles. Pearson's correlation indicated a significant correlation ($P < 0.0001$), but due to the high variation of sizes per single Gosner stage, the Pearson R is lower at 0.85 and the $R^2 = 0.72$.

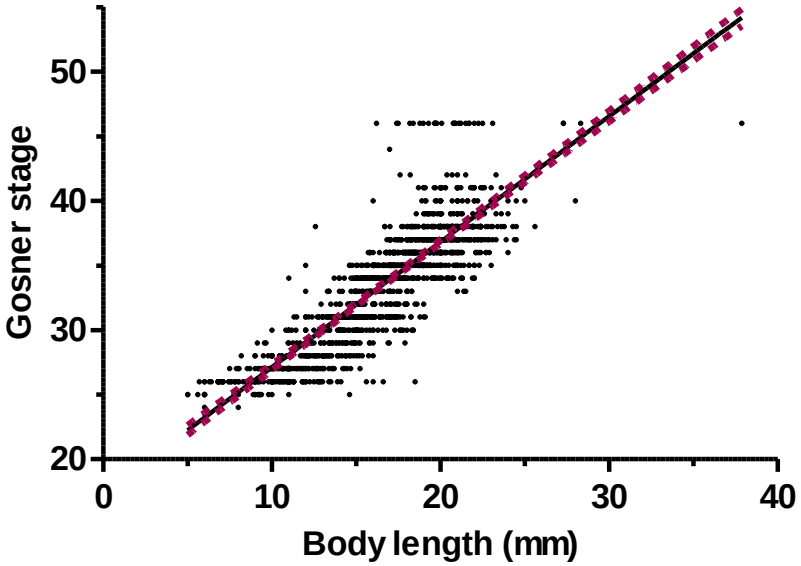

Figure 2.15. Pearson's correlation of the Gosner stage in comparison to the body length of the tadpoles showing the variance in size per developmental stage. The linear regression line (black) and 95% confidence intervals (red dotted lines) are also indicated.

To take the variation from Figure 2.15 into account and to determine which of these two interdependent aspects is the driving factor for *B. dendrobatidis*, a Pearson correlation between the body length/Gosner stage ratio and *B. dendrobatidis* prevalence was calculated and is illustrated in fig 2.16. The Pearson's correlation was significant with $p < 0.0001$. There is again a strong correlation as the Pearson R is at 0.96 and the R^2 at 0.93. As the tadpoles grow per development stage, the *B. dendrobatidis* prevalence increases.

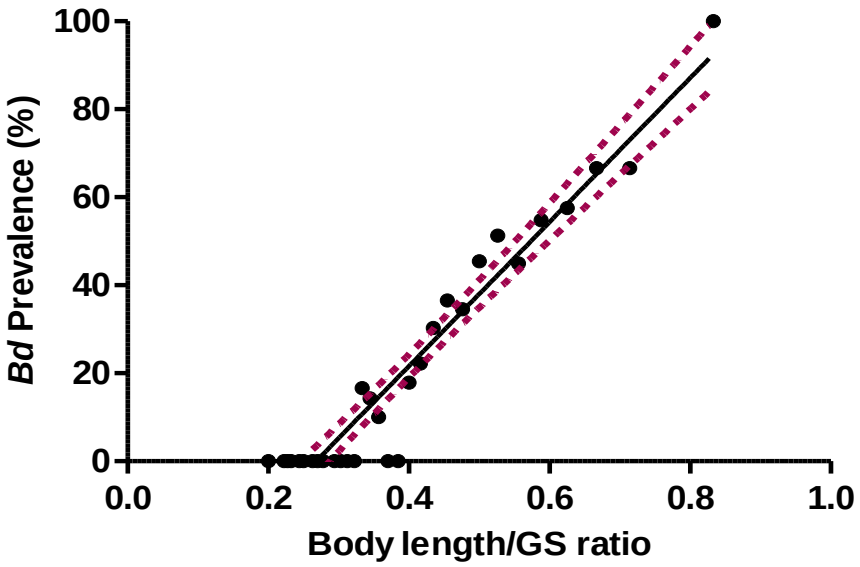

Figure 2.16. A Pearson's correlation between the *B. dendrobatidis* prevalence and body length/GS ratio (growth ratio). Linear regression line and 95 % confidence intervals (red dotted lines) are also indicated.

2.4. Discussion and conclusion

2.4.1. *Batrachochytrium dendrobatidis* occurrence among sites

This study focused on the tadpoles of the species *A. hymenopus*, in an area that was predicted to have high *B. dendrobatidis* prevalence (Tarrant *et al.,* 2013). From the map in Figure 2.1 it can be seen that all four highlighted sites are found within the high probability areas from Figure 2.17 below. The predictive modeling done by Tarrant *et al.,* (2013) shows that *B. dendrobatidis* is found highly concentrated along the KwaZulu-Natal escarpment and the highlands surrounding Lesotho.

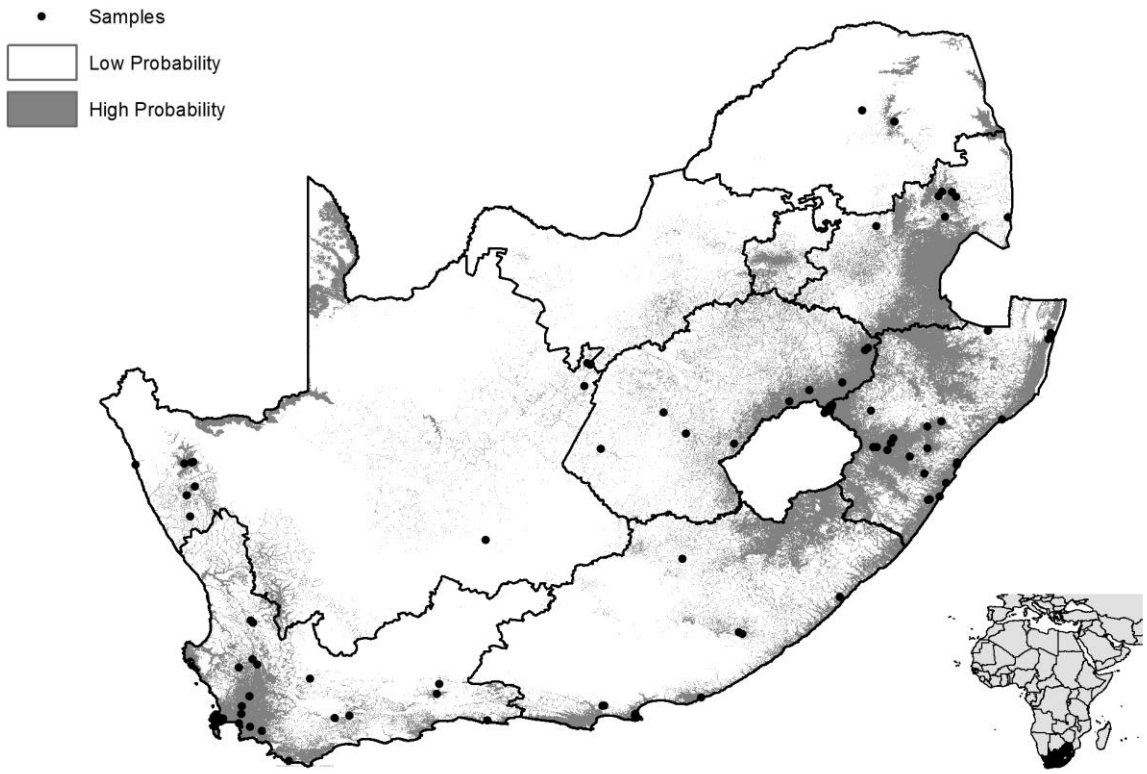

Figure 2.17. A map of South Africa indicating predicted occurrence of *B. dendrobatidis* by Tarrant *et al.* (2013). The area in grey indicates high probability of *B. dendrobatidis* prevalence and the areas in white indicates low probability. The black dots are samples that were collected during previous studies (Tarrant *et al*., 2013).

From a study comparing *B. dendrobatidis* infection between streams and ponds over a distance of 22 km, similar to this study's coverage, it was found that frogs had a significantly higher *B. dendrobatidis* prevalence within streams with flowing water than permanent ponds (Kriger & Hero, 2007). This coincides with the fact that most *B. dendrobatidis* related mass mortalities occur in streams with flowing water (Berger *et al.,* 1998; Lips *et al.,* 2006).

Another study that covered 31 sites along a 2,315 km stretch of Australia's east coast showed a significant difference in *B. dendrobatidis* prevalence between those sites located near the equator and those located farther away from the equator (Kriger *et al.,* 2007).

This study however only compared rivers with flowing water with one another. Each of the four rivers had incidences of *B. dendrobatidis* over the duration of the study, but no significant difference was found between them.

The popular hiking trail that involves the chain ladder runs along the Tugela River for about 1 km once the hiker has reached the plateau (Witsieshoek, 2016). The Tugela River, as well as the Vemvane River, are the most consistent river in terms of water flow. When rainfall is inadequate or drought conditions persist, the other two rivers (Bilanjil and Ribbon Falls) are often reduced to a few stagnant pools. The presence of hikers may be the reason why the *B. dendrobatidis* prevalence is the most constant in the Tugela River between the four sites as illustrated in Figure 2.6. They might be spreading the pathogen throughout the system. The Vemvane River is also relatively constant in terms of *B. dendrobatidis* infections (Figure 2.6) and is also listed as a popular destination for hikers in that area (Witsieshoek, 2016).

It is clear from Figure 2.7 that *B. dendrobatidis* prevalence has no statistical significance between sites but the different years do differ significantly from one another. Many environmental factors will contribute to these fluctuations. In this study different seasons and rainfall were examined as possible driving factors, although many other environmental factors such as temperature may have an influence too (Longcore *et al.,* 1999).

2.4.2. Seasonal effects on *B. dendrobatidis*

A study conducted in two streams in the north eastern Atlantic forest of Brazil tested the seasonal response on *B. dendrobatidis* (Valencia-Aguilar *et al.,* 2016). Three species, *Agalychnis granulosa, Aplastodiscus sibilatus,* and *Proceratophrys renalis*, were included in the study and showed an increase of *B. dendrobatidis* incidence in the summer, which is also the rainy season. The study also showed that *B. dendrobatidis* incidence was very low during the dry season. *B. dendrobatidis* prevalence in *A. sibilatus* decreased significantly and almost dissipated completely in

A. granulosa in the dry season (Valencia-Aguilar *et al.,* 2016). At high alpine habitats, climate change is assisting the spread of *B. dendrobatidis* according Bosch *et al*., (2007) as winter months then become shorter and milder. The pathogen is able to survive in water at temperatures as low as 4 °C (Piotrowski *et al*., 2004) and if water temperatures increase, *B. dendrobatidis* will grow faster on "overwintering larvae" allowing the pathogen to persist throughout the year regardless of the season (Bosch *et al*., 2007).

From this study there was no significant difference between seasonal surveys. Summer surveys which have higher rainfall tend to have a higher *B. dendrobatidis* incidence in most years which can be seen in Figure 2.8 and 2.9, but is still statistically insignificant. Summer surveys also show higher variation in *B. dendrobatidis* prevalence although the mean incidence values between summer and winter surveys are too close together to say anything substantial (Figure 2.10). This may again be attributed to a number of reasons, such as water temperature, water flow, population density or behaviour of *A. hymenopus* tadpoles.

2.4.3. Rainfall's influence on *B. dendrobatidis*

From the study conducted by Kriger *et al.,* (2007), where a number of environmental factors were examined to observe their effects on *B. dendrobatidis*, it was found that rainfall and thermal regimes have an influence on *B. dendrobatidis* prevalence and intensity. It was found that as the rainfall increased, so did the zoospore count. In another study it was found that drought reduces *B. dendrobatidis* infection intensities and that the mortalities from chytridiomycosis were reduced (Terrell *et al.,* 2014).

In the present study however, it was found that the drier months had a higher *B. dendrobatidis* incidence as can be seen in Figure 2.11 and that once the rainfall increases, the *B. dendrobatidis* prevalence decreased. From Figure 2.12 it can also be seen that there was a higher *B. dendrobatidis* prevalence in drier months. From the graph it can be seen that all the *B. dendrobatidis* prevalence data points that are

40 % and higher are below 100 mm rainfall. When the rainfall increases above 100 mm the *B. dendrobatidis* prevalence is below 40 %.

This may be due to the fact that when it rains the water flow becomes stronger and the chytrid zoospores become diluted and wash down the river more readily. When there are drought conditions, such as in December 2015 (Figure 2.11), on the plateau, the river forms pools that don't flow and the tadpoles are found in a higher density while the chytrid zoospores become trapped. The tadpoles are also in closer contact with one another and therefore the pathogen spreads easily. When the rainfall increases again the *B. dendrobatidis* prevalence drops as the zoospores are once again washed downstream, off the plateau.

2.4.4. Effects of growth and development of tadpoles on *B. dendrobatidis*

It has been found that tadpoles that are larger and further developed are more likely to become infected with *B. dendrobatidis*. A study conducted by Viera *et al.* (2013) shows a strong correlation between tadpole size and infection. From their study it is suggested that smaller tadpoles have lower prevalences because of the smaller area of keratinized cells and that species with long larval stages such as the bullfrog (*Lithobates catesbeianus*) are more exposed to *B. dendrobatidis* zoospores for a longer time period in the water. Another study from Smith *et al.* (2007) supports this finding in *A. hymenopus* tadpoles found in the Mont-aux-Sources area and adds that the development, in the other words, the Gosner stage of the tadpole is the driving factor for *B. dendrobatidis* infection. Therefore more developed tadpoles were more likely to be infected. A study by Valencia-Aguilar *et al.* (2016) on *Agalychnis granulosa* and *Aplastodiscus sibilatus* also supports the findings that as the size and development increases so does the probability of infection. Yet, they concluded that size (body length) is the driving factor for infection independent of the Gosner stage.

From this study there was both a very strong linear correlation between the *B. dendrobatidis* prevalence and Gosner stage (Figure 2.13) and *B. dendrobatidis* prevalence and body length (Figure 2.14). Statistically there is a stronger correlation between body length and *B. dendrobatidis* prevalence. To determine the driving factor of *B. dendrobatidis* for this study, Figure 2.15 was first drawn to observe variation between body length and Gosner stage. It can be seen from this Figure that they also have an interdependent relationship, as the one increases so does the other. It can also be seen that for every stage there is a lot of variation in size. In Figure 2.16 the growth ratio (Gosner stage/body length ratio) was then plotted against *B. dendrobatidis* prevalence, and from this it is evident that the tadpole size is the driving factor for infection. It should still be taken into account that these specific characteristics for each tadpole and life history traits are often related to species' susceptibility to infections and can vary from species to species.

Chapter 3

Microsatellite development

3.1. Introduction

The protection of biodiversity is of utmost importance in this day and age and conservation genetics is gaining popularity as one of the more effective research tools that can be applied towards avoiding extinction of endangered and threatened species (Oliveira *et al*., 2006). In the past "traditional" molecular markers were quite insufficient in terms of statistical significance and often inaccurate. Today the uses of microsatellites with highly variable loci have such a strong and reliable statistical significance, that it is entirely possible to determine the differentiation between species groups that may have a high risk of extinction (Hedrick, 2001).

Microsatellites are found widespread in eukaryotic and prokaryotic genomes and are also known as SSRs (simple sequence repeats) or STRs (short tandem repeats) (Field and Wills, 1998; Tóth *et al*., 2000). They consist of non-coding DNA regions made up of tandemly repeated DNA-motifs of one to six nucleotides, repeated five to 50 times. The sequence units consist of A (Adenine), C (Cytosine), G (Guanine) or T (Thymine). The sequence TATATA, for example, is known as a dinucleotide microsatellite and GTCGTCGTC is known as a trinucleotide microsatellite. If the units are repeated four times, they are known as tetranucleotides or if they are repeated five times, they are known as pentanucleotide motifs (Chistiakov *et al*., 2006).

There are four types of repeat sequences according to which microsatellites can be classified. The first is a perfect microsatellite in which the repeat sequence, for example: ACACACACACACAC, is not interrupted by a base that doesn't belong to the DNA-motif. The second is an imperfect microsatellite where there is a pair of bases, for example TG within ACACACACACTGACACACACAC, in between the repeated DNA-motifs that does not match the original sequence. The third is known as an interrupted microsatellite. It occurs when there is a small sequence, for example, TAGAG within ACACACACTAGAGACACACAC, that doesn't match the original DNA-motif sequence. The fourth microsatellite is known as a composite microsatellite as there are two adjacent distinctive sequence repeats, for example, GTGTGTGTGTGTATATATATATA (Oliveira *et al*., 2006). The difference between a homozygous microsatellite and a heterozygous microsatellite, is that the homozygous microsatellite locus has the same number of sequence repeats on both homologous chromosomes, whereas a heterozygous microsatellite locus, has a different number for each allele. This is beneficial because microsatellite markers are useful for discriminating different individuals, because at the same locus the population usually contains several alleles each with a different number of repeats. This is why microsatellites are convenient and important for use in population genetics and also forensic science (Oliveira *et al*., 2006).

For a growing interest microsatellite applications exist and applications include constructing genetic species maps, the study of human genetic diseases (O'Donnell & Warren, 2002), for population genetics and for use in genotyping and paternity testing (Wright & Bentzen, 1994; Schlötterer, 2000). Microsatellites were popularized during the 1990s and are now used in countless labs (Manel *et al*., 2003) Although these analyses were originally created for human research, it has become very useful for research on plants (Roa *et al*., 2000; Collevatti *et al*., 2001) and animals (Schlötterer *et al*., 1991), especially when investigating occurrences such as genetic drift and gene flow (Heywood & Irionda, 2003). Other examples where microsatellites have answered biological questions, include those focusing on speciation, taxonomy, effective population sizes and mating systems (Squirrell *et al*., 2003). From a study conducted by Newman & Squire (2001), microsatellites were used to investigate genetic population structure of a frog species. It was found that most wood frog

(*Rana sylvatica*) populations had very similar allele frequencies within about 5 km, suggesting a high gene flow among nearby populations.

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Except for Antarctica, amphibians are found on every continent of the world and about 5,700 species have been described up to date. Most have a limited distribution and mobility and many are easy to sample because they are found congregated at specific locations, for example, associated with rivers, which makes them popular subjects for population genetics (Beebee, 1996). Regrettably, as mentioned in Chapter 2, they are experiencing species extinctions and major population declines in recent decades, far more severe than birds or mammals (Stuart *et al*, 2004). Therefore the application for conservation genetics is needed and the development of molecular markers plays a vital role in conservation efforts (Jehle *et al*., 2005). Molecular markers offer more reliable results when studying genetic diversity and evolutionary relationships as morphology-based identification is often inaccurate. Species trait expressions are influenced by environmental factors and can cause individuals from the same species to look different, while the more dependable microsatellites use genetic relatedness (Chae *et al*., 2014).

Genes have the ability to affect interactions with other species and therefore effects are often seen at community level. This is why, even beyond its effect on populations, intraspecific genetic diversity is important. According to Johnson *et al*. (2006), Zytynska *et al*. (2011; 2012) and Whitham *et al*. (2012) dominant host species support more species-rich communities than genetically-poor communities. Molecular tools can also be used to show the stages of genetic interchange of entities between isolated or spatially separated populations, as many species have fragmented populations and are only connected through the movement of individuals (Hellberg *et al*., 2002). Environmental stressors can put a great deal of pressure on populations and the amount of isolation a population can handle has important implications for the population if it's to continue surviving. Populations that are less isolated can be repopulated by those populations that are geographically separated; therefore their recovery will take less time (Botsford *et al*. 2009; Cowen & Sponaugle 2009; Bernhardt & Leslie 2013).

This chapter describes the development and methodology of microsatellites in *Amietia hymenopus* as they greatly increased the understanding of animal and plant population genetics, wild and domestic, over the last few years (Balloux & Lugon-Moulin, 2002; Storfer *et al*., 2007). They have significantly improved our insight within areas as varied as population size, history and structure; even migrations patterns are better understood. Other advantages include individual identification and improved awareness about kin selection (Uller & Olsson, 2008). These markers are ideal for understanding developments such as previously mentioned gene flow, as well as understanding bottlenecks and local adaptation (Spencer *et al*., 2000; Waits *et al*., 2000; Rasmus, 2005). For this study microsatellites were developed to aid in population genetics to observe gene flow in *Amietia hymenopus* found on the plateau of the Drakensberg Mountains.

3.2. Methods and results

3.2.1. Site allocations

From the nine sites, five were selected (Site 2, 4, 6, 7 and 8) for microsatellite development (Figure 3.1). Site 9 did not have any *A. hymenopus* tadpoles, instead, one *A. umbraculata* sub-adult was found. Samples from Site 1 were omitted because the density of *A. hymenopus* was too small. Two further Sites, Site 3 and 5 were omitted because they were found to be in the same river as Site 2 and 6.

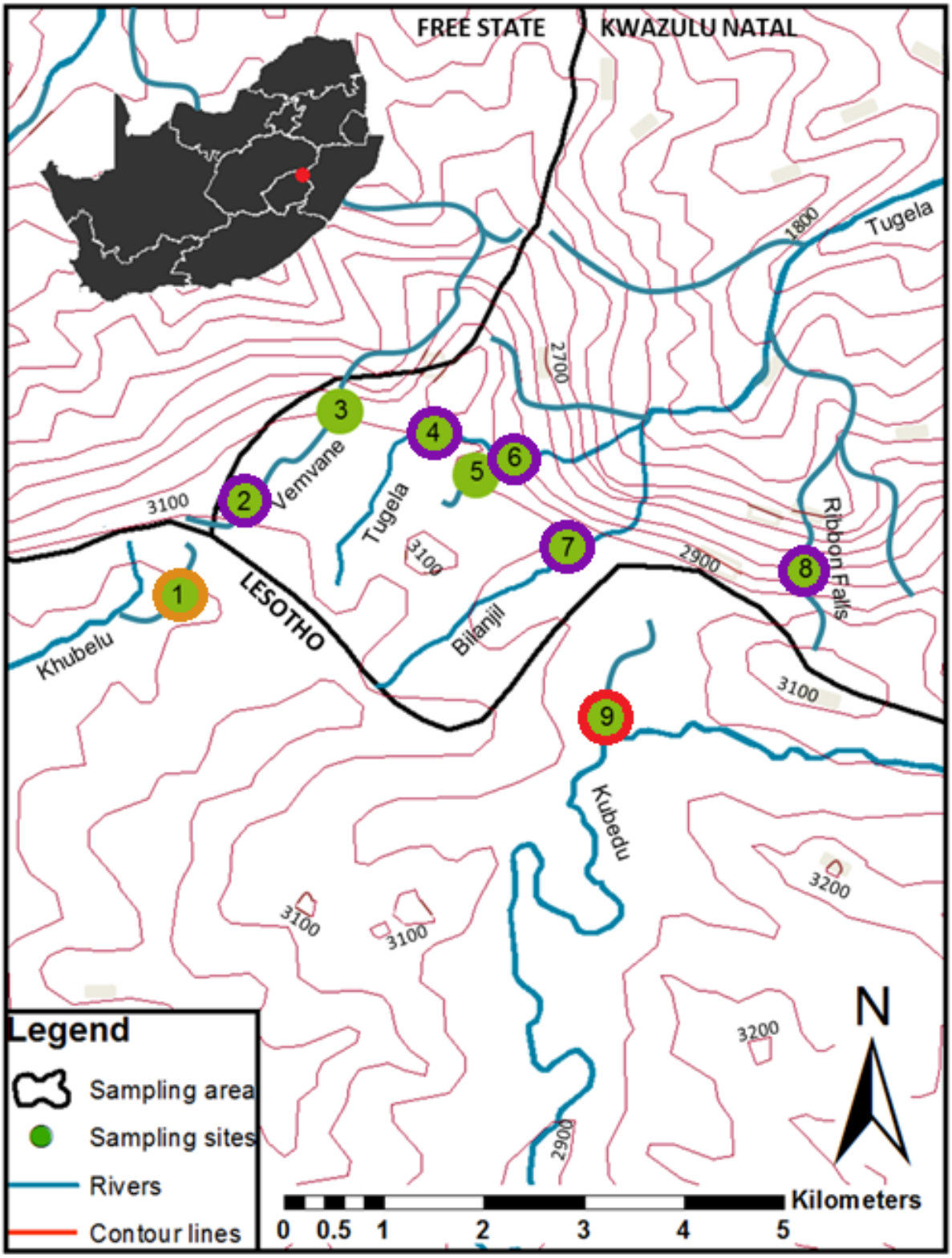

Figure 3.1. GIS map indicating topography, the sample area and sites selected for sampling tadpoles for microsatellite development. Purple borders indicate sites where sampling was done. The site with a red border had no *A. hymenopus* tadpoles and the site with an orange border had too few tadpoles than the required minimum for genetic analysis. The two sites with no borders were omitted from the study to prevent duplication within sites.

Nine sites were targeted in the Mont-aux-Sources area to establish genetic variation in *A. hymenopus* metapopulations. The sites were selected to be between 2 600 and 3 000 m above sea-level along the Amphitheatre escarpment and over the Namahadi pass into Lesotho. They are situated over a distance of about 20 to 25 km with ranging valleys and hills in-between and mostly within KwaZulu-Natal, with the exception of the two sites in Lesotho (Sites 1 and 9). Site 1 was named Khubnam and is situated in Lesotho north of Namahadi pass. Site 2 was named Nampolice, because it is situated near an abandoned police station just before the Nahadi pass although from Figure 3.1 it can be seen that it is the origin of the Vemvane River which is the name of Site 3. Site 4 was named Tugela 2 and Site 6 was named Tugela. In-between Site 4 and 6 is Tukela head which was Site 5. Site 7 was situated on the south side of another hill and was named Bilanjil. Five kilometres further south-east Site 8 was situated and named Ribbon Falls. Site 9 was the second site within Lesotho and was named Kubedu. All the sites were named after the rivers from where the sampling was done, except Nampolice.

3.2.2. Collection of tadpoles

At each site 30 tadpoles were collected under Ezemvelo Wildlife permit no: OP3781/2015 and OP526/2014 and Lesotho permit no. MTEC/NES/CONV/1. To ensure that tadpoles from the same cohort wouldn't be collected for each site sampling was spaced a few meters apart within each river. Tadpoles were collected by visually inspecting the river and identifying them based on morphological characteristics (Du Preez & Carruthers 2009). Tadpoles were then collected from the river using the dip-net method described by Shaffer *et al*., (2001) and euthanized using MS222 (Tricaine Methanesulfonate). They were then preserved in 70 % ethanol within a 20 ml polypropylene tube. Samples were transported to the lab at the North-West University, packaged and shipped to the University of Manchester U.K. for further analysis (DEFRA import permit IMP/GEN/2014/06).

3.2.3. Microsatellite development

3.2.3.1. Isolation of genomic DNA from tissues samples

Promega Wizard® SV 96 Genomic Purification System was used to extract DNA from the tadpoles' tail muscle samples. 20 mg of tissue was used, cut into two equally sized pieces and both pieces were placed into a 96-well, deep well plate. 275 µl of Digestion Solution Master Mix (Table 3.1) was added to each sample in the 96-well deep well plate. When it was sure that the sample was completely covered with the Digestion Solution Master Mix, the plate was covered with an adhesive seal. The plate was then placed in a water bath at 55 °C to be incubated overnight (approximately 16 to 18 hours).

Table 3.1. The composition of the Digestion Master Mix per sample volume.

Digestion solution Master Mix	**Volume per sample (µl)**	**Total volume for 96 samples (ml)**
Nuclei Lysis Solution	200	22.0
0.5M EDTA (pH 8.0)	50	5.5
Proteinase K, 20 mg/ml	20	2.2
RNase A Solution, 4 mg/ml	5	0.55
Total Volume	**275 µl**	**30.25 ml**

After the overnight incubation, the seal was removed and 250 µl of the Wizard® SV Lysis Buffer was dispensed into each well of the deep-well plates that contained lysate. The contents of each well were mixed well by pipetting a few times and lysate was processed soon after as the lysate had to warm. The vacuum was then prepared as illustrated in Figure 3.2. The Binding Plate was placed in the vacuum Manifold Base and the Binding Plate was orientated in the Vacuum Manifold with the

numerical column headers toward the vacuum port. The vacuum line was then attached to the vacuum port on the Manifold base.

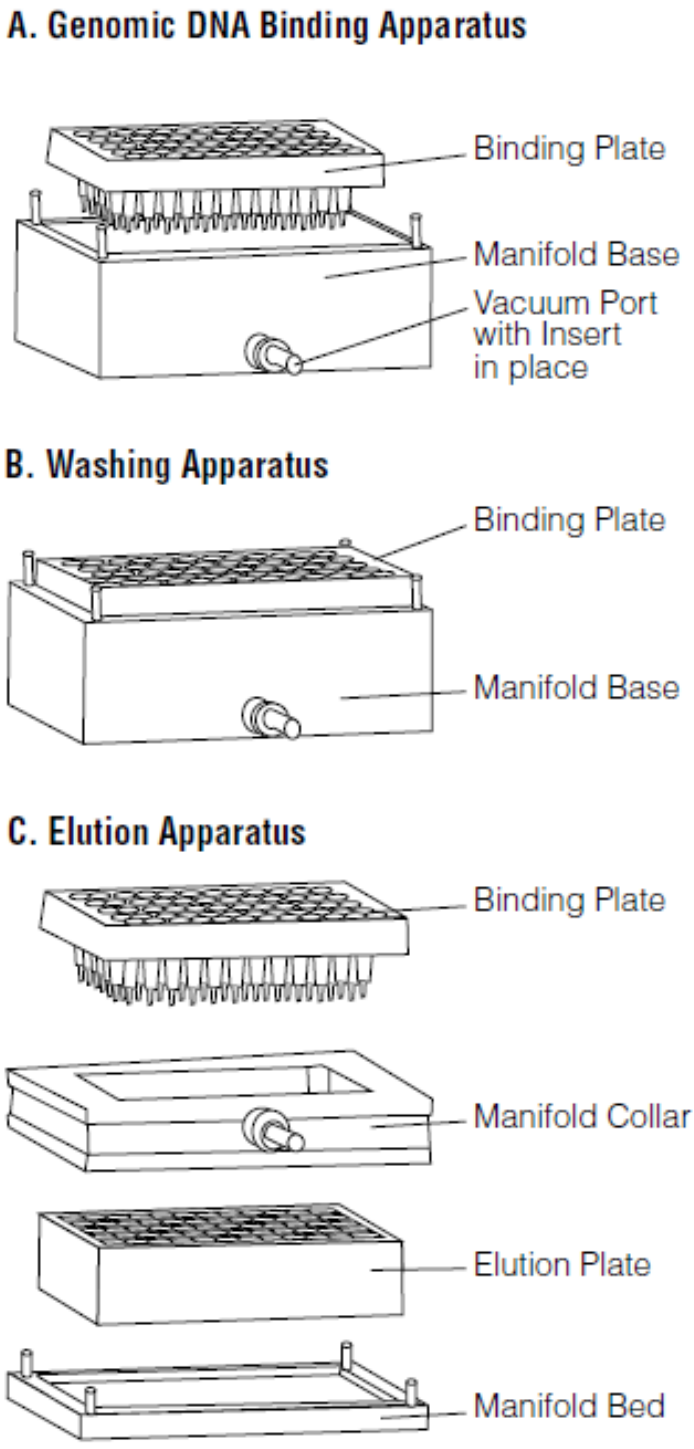

Figure 3.2. The Vac-Man® 96 Vacuum Manifold setup with panels A, B and C. Image taken from the Promega Wizard® SV 96 protocol.

The tissue lysates were then transferred to the wells of the Binding Plate and the vacuum was applied until all the lysate had passed through the Binding Plate. 1 ml of Column Wash Solution was then added to each well of the binding plate and vacuumed until the Column Wash Solution passed through the Binding Plate. This was repeated twice to ensure that the Binding Plate was washed three times by the Column Wash Solution. After the wells were emptied, the vacuum was applied for an additional six minutes that allowed the binding matrix to dry. Once the Binding plate was removed from the Manifold Base, it was tapped gently on a clean paper towel to remove the remaining ethanol. The 96-well Deep Well plate was then placed in the Manifold Bed and the Manifold Collar was positioned on top. The Binding Plate was then positioned on top of the Manifold Collar. The Manifold Collar containing the Binding Plate was then placed on top of the 96-well Deep Well Plate which was positioned on the Manifold Bed as is shown in Figure 3.2. It was ensured that the Binding Plate tips were centred on the Deep Well Plate wells.

250 µl of Nuclease-Free water at room temperature was then added to each well of the Binding Plate and then incubated for two minutes at room temperature. The vacuum was then applied to the Nuclease-Free Water until it had passed through the Binding Plate. Another 250 µl of Nuclease-Free water was added and incubated for two minutes and then vacuumed through the Binding Plate so that a total of 500 µl was eluded. The Vacuum was then released and the Binding Plate removed. The Manifold Collar was then removed ensuring that the Deep Well Plate remained on the Manifold bed. 440-450 µl eluate DNA remained and was stored at -20 °C, in a tightly covered plate sealer.

3.2.3.2. Sequencing of an *Amietia hymenopus* sample

A single sample was then sequenced from the Tugela River in one flowcell of an Illumina Miseq using a Nextera® kit using extracted DNA. This was done by adding 20 µl of genomic DNA at 2.5 ng/µl to each sample well of the 96-well plate. Then 25 µl of TD (Tagment DNA) Buffer was added to the wells that contained DNA followed

by another 5 µl of TDE1 (Tagment DNA Enzyme). By pipetting up and down a few times it was guaranteed that the samples were well mixed. The samples were then centrifuged at 280 x g at 20 °C for one minute. The 96-well plate was then placed in the Illumina Miseq thermal cycler after it had been preprogramed to preheat at 55 °C for five minutes and then held at 10 °C. This step used the Nextera transposome to tagment gDNA (genomic DNA), which is a process that fragments DNA and then tags the DNA with adapter sequences in a single step.

The next step purified the tagmented DNA from the Nextera transposome and was done as follows: 50 µl was transferred from the one 96-well plate after the cycle was complete to another 96-well plate and was pipetted up and down 10 times to ensure thorough mixing. The sample mixture was then transferred to a Zymo-Spin I-96 Plate and placed on a collection plate where it was centrifuged at 1300 x g at 20 °C for two minutes. The samples were washed twice by adding 300 µl of Zymo wash buffer from a trough to each sample well, centrifuging at 1300 x g at 20 °C for two minutes and then discarding the flow-through. The samples were then centrifuged again at 1300 x g for two minutes to ensure that there was no residual wash buffer. The Zymo-spin I-96 Plate was then placed on a third 96-well Plate and 25 µl of RSB (Resuspension Buffer) was added directly to the column matrix in each well. The samples were then incubated for two minutes at room temperature after which they were centrifuged at 1300 x g at 20 °C for two minutes.

The following step then amplified the purified tagment DNA and added index adapters by using a 5-cycle PCR program. The PCR step added Index 1 (i7) and Index 2 (i5), sequencing, and common adapters (P5 and P7) which were required for cluster generation and sequencing. The 96-well plate was then set up onto a Truseq Index Plate Fixture as illustrated in Figure 3.3.

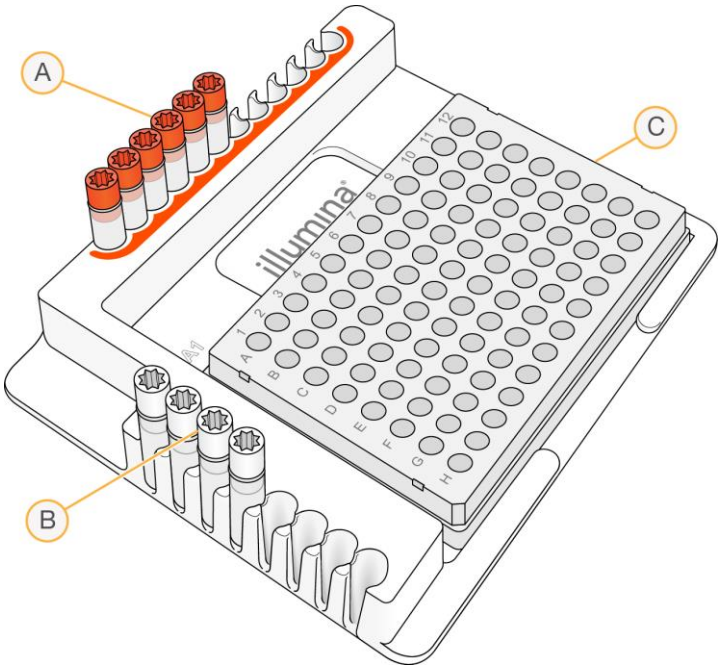

Figure 3.3. The Truseq Index Plate Fixture setup. A Columns 1–12: Index 1 (i7) adapters (orange caps), B Rows A–H: Index 2 (i5) adapters (white caps), C 96-well plate. Image copied from the Nextera® kit protocol.

5 µl of Index 1 (i7) adapter was then pipetted down each column and 5 µl of Index 2 (i5) adapter wad pipetted down across each row. Next another 15 µl of NPM (Nextera PCR Master Mix) and 5 µl of PPC (PCR Primer Cocktail) were added to each well. 20 µl of the previous 96-well plate was then added to the current plate and mixed well. The samples were then centrifuged for one minute at 280 x g at 20 °C. The plate was then placed in the Illumina Miseq thermal cycler to run the PCR AMP program and was preprogramed to preheat at 100 °C, 72 °C for three minutes, 98 °C for 30 seconds and then to run five cycles at 98 °C for 10 seconds, 63 °C for 30 seconds, 72 °C for three minutes and to then hold at 10 °C.

The next step made use of AMPure XP beads to purify the library DNA and provided a size selection step that removed short library fragments. The 96-well plate was centrifuged at 280 x g at 20 °C for one minute so that condensation was collected. The contents were then transferred to a new midi plate. AMPure XP beads were vortexed for 30 seconds and the appropriate volume of beads were added to a

trough. 30 µl AMPure XP beads were added to the midi plate. The samples were then shook at 1800 rpm for two minutes and incubated at room temperature for five minutes. The plate was then placed on a magnetic stand until the liquid had cleared and then the supernatant was removed. The samples were then washed twice by adding 200 µl of 80 % EtOH, incubating for 30 seconds until the supernatant appeared which was then removed. All residual EtOH was then removed from each well and the beads were air-dried for 15 minutes. The plate was then removed from the magnetic stand and 32.5 µl of RSB (Resuspension Buffer) was added. Again the samples were shook at 1800 rpm for two minutes and incubated at room temperature for two minutes. The samples were then placed back onto the magnetic stand until the liquid cleared and 30 µl supernatant was then transferred from the midi plate to the TCY plate.

The following step was done to pool libraries before sequencing. The NDP (Nextera Dilution Plate) barcode label was added to a new 96-well midi plate and the NPP (Nextera Pooled Plate) barcode was added to a new 96-well midi plate (for indexed libraries). Then 10 µl library was transferred from the TCY plate to the corresponding well of NDP. The concentration in each well was normalised to 2 nM using Tris-C1 10 mM, pH 8.5 with 0.1 % Tween 20. The samples were then shook at 1000 rpm for two minutes and centrifuged at 280 x g for 1 minute. Then 5 µl from each well in column 1 of NDP was transferred to column 1 of NPP and this was done for the remaining columns of NDP until samples were pooled in column 1 of NPP. Then the contents of each well of column 1 were combined into A2 of NPP and shook at 1800 rpm for two minutes. The libraries were then diluted to the loading concentration. The end result yielded 2 x 6,465,564 reads of 250 base pair (bp) length.

3.2.3.3. Primer design

Microsatellite primers were then designed and optimised for Illumina paired-end sequence data using the methods of Griffiths *et al.,* (2016). Reads were briefly exposed to quality filtering and trimming with Trimmomatic (Bolger *et al.,* 2014) yielding 2 x 3,756,407 reads (leading = 3, trailing = 3, sliding window – window size =

4bp, quality = 20; minlen = 50bp). The process is aimed at removing low-quality reads and is particularly designed for pair-end data. If either member of a pair does not pass user-specific quality thresholds, Trimmomatic discards both members. Pairs that survive the Trimmomatic process are then used for microsatellite identification and the design for PCR primers. Trimmomatic is then also used to eliminate adapter sequences from reads that are residual from the sequencing process (Griffiths *et al.,* 2016). Using PANDAseq, paired reads were then assembled where possible. Only within successfully assembled reads were microsatellite loci searched for to increase PCR success rates (Masella *et al.,* 2012).

3.2.3.4. Identifying amplifiable loci

In the assembled reads, potentially amplifiable microsatellites were identified using Pal_finder. The program (Pal_finder v.0.02.04) can identify sequences containing motifs of up to 6 base pairs and then analyses the flanking regions for aptness as PCR priming sites (Castoe *et al.,* 2012). Primer3 was then used to design primers suitable to the selected PCR priming sites. Files outputted by Primer3 then gave a list of all the loci found.

Pal_filter (Griffiths *et al.,* 2016) was used to select the optimal loci from Pal_finder to further test Primers for 36 tri- and tetra-nucleotide loci. This was done to filter out loci for which Primer3 could not design primers and to filter out loci with imperfect motifs. It also filters out loci in which the primer sequences occur multiple times in the total reads.

3.2.3.5. Primer design and testing

Potential microsatellite primers were identified by sequencing a single sample from the Tugela River on the Illumina platform according to Griffths *et al*. (2016), and a total of 30 possible primer pairs were identified.

Initial primer testing was then conducted on eight samples to confirm that the primers that were developed would amplify across all the potential genetic variation from the region. These samples came from across the Tugela Site. Nampolice Site (28°45'19.70"S, 28°52'2.60"E), Bilanjil Site (28°45'37.10"S, 28°53'50.40"E; 28°45'37.90"S, 28°53'53.20"E) and the Ribbon Falls Site (28°45'44.90"S 28°55'4.00"E).

Using the Qiagen Type-It® Microsatellite PCR kit the primers were first tested in singleplex PCR in 5 µl reactions using unlabeled primers. In preparation for using the kit, the master mix, template DNA, RNase-free water and the primer mix were first mixed to avoid localized concentrations of salts. The reaction mix was then prepared according to Table 3.2.

Table 3.2. Reaction components for PCR of microsatellites.

Component	**Volume/Reaction**	**Final Concentration**
Reaction mix		
2x Type-it Multiplex PCR Master Mix	12.5 µl	1x
10x primer mix, 2 µM each primer	2.5 µl	0.2 µM
RNase-free water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	200 ng DNA Start with 10 ng
Total volume	25 µl	

The reaction was mixed thoroughly by pipetting up and down a few times and dispensed into the PCR plate. The template DNA (200 ng /reaction) was then added to the individual PCR wells containing the reaction mix. The thermal cycler was then programmed with the following cycling conditions: 95°C for five minutes, 35 x (95°C for 30 seconds; 60°C for 90 seconds; 72°C for 30 seconds), 60°C for 30 minutes and the plate was placed in thermal cycler and started.

Gel electrophoresis was then run on a 1 % agarose gel to separate DNA by size (length of base pairs) to show successful amplification. This was done by measuring 1 g of agarose powder and adding it a flask containing 100 ml of 1X TAE buffer (Tris-Acetate-EDTA buffer). The TAE buffer was prepared as a 50X stock solution by dissolving 242 g Tris base in water, adding 57.1 ml of acetate (100 % acetic acid), then adding 100 ml of 0.5 M sodium EDTA (pH 8.0) and adding dH20 to fill the solution up to one litre. From the 50X TAE stock, 20 ml was diluted into 980 ml of DI water to make 1x TAE stock. The solution was then microwaved for 1-3 minutes until the agarose was completely dissolved and then let to cool down for about five minutes. 0.2-0.5 µg/mL of Ethidium bromide (EtBr) was then added to the final product which is a compound that binds to DNA and allows it to be visualized under ultraviolet (UV) light. The agarose and the comb were then placed in the gel tray and left to set at room temperature for 20-30 minutes until it was completely solidified. The loading buffer was then added to each sample. The solidified agarose gel was then placed in the electrophoresis unit and filled with 1X TAE buffer until the gel was covered. After which the DNA ladder was carefully loaded in the first lane of the gel followed by the samples in the additional wells of the gel. The gel was run at 80-150 V until the dye line was approximately 75-80 % of the way down and removed from the electrophoresis unit. The DNA was then observed with UV light for the presence of one or two DNA bands. Primer pairs that produced more than two bands or none at all were discarded from further testing.

3.2.3.6. Tagging primers

After completion, 27 loci gave acceptable results. Forward primers were then tagged with a universal tail sequence at their 5' end (GCCTCCCTCGCGCCA). A 6FAM florophore was then tagged to the tail sequence at the 5' end and was added to the PCR as third primer.

The PCR was run again, with the forward primer changed to a concentration of 0.5 µM. The PCR products were then checked again on a 1 % agarose gel to confirm the consistent amplification. The results were then sent for fragment length analysis on an ABI 3730 DNA Analyzer with the GeneScan™ LIZ1200 size standard at the University of Manchester Sequencing Facility.

3.2.3.7. Scoring alleles

Using Genemapper v3.7, a program that transfers annotations from a well noted genome to other genomes (Applied Biosystems), the alleles were scored and binned using MsatAllele v1.02 (Alberto, 2009). The process of binning assisted in the visualization of raw allele size variations, which revealed peculiar bin distributions and detected potential scoring errors or unforeseen spacing between bins. It also assisted with the binning of raw fragment sizes and writing up summary statistics for each locus, as well as aiding with the exported genotype files (Alberto, 2009). Loci that formed more than two alleles per locus, which were problematic to score or did not amplify consistently, were thrown out of further analysis.

3.2.3.8. Conducting multiplex PCRs

In five multiplex reactions, similar to the singleplex reaction done previously, the remaining 17 loci were combined using three universal tail and florophore combinations (Blacket *et al.,* 2012; Culley *et al.,* 2013), which can be seen in Table 3.3 for combinations of primers in multiplexes.

Table 3.3. Combinations of primers in multiplexes.

Locus name	Motif	Primer sequence (5'→ 3')	Genbank accession no.
AHym8	ATT	F: TCTGGGACTGGGATTGACCTAGTGG R: GCAGTAAAGCAGAGCAGCCAATTCC	KX772181
AHym12	AATC	F: ACACATGGCATTTTAACATGTTGGG R: TAAATGAAATGTGATGGCGTGTCGG	KX772185
AHym7	ATT	F: GCTTGATGTACATTAGCGTGATCAATAGG R: CGGATCCTTCTGAGGTTCTAAGGGG	KX772176
AHym25	ATCT	F: AGGTGTCCCTCTTTTCCCTCTCAGG R: TGCACCAACCCATTTATATATCCTGACC	KX772179
AHym19	ATCT	F: TATTTCGGGGACCAGGGGATGC R: CCTGAACTGACCAAGTGTCTGGACC	KX772177
AHym22	ATT	F: GCTGATGTACCGATTGTCTGCATCC R: GGATACTTAGCTTAACAAAACCATGCAGC	KX772178
AHym26	TCTG	F: ATCTCACGGTCCCTCAGACTGC R: CAGTGCCAATAAGACATGCCAGTCC	KX772180
AHym11	AAAG	F: GGCAGGAGTGAGAGAAAGAAAGG R: AGCATGCTCTACATACAGCACCACC	KX772184
AHym23	AAAG	F: ATAAATACACCCCAATGCCCTGTCC R: AGCAATGCTTTTGGAGAGGTAGAGC	KX772183
AHym24	TCTG	F: AGGAATGGTGTCTGGAAAACAGGG R: GAGTGCTGATAGTCTGCCTGCC	KX772182
AHym27	ATCT	F: TGAGGGTACCTGCTCTCGTATTGGG R: TCAGAAAGCCTACAGGCAGTGATG	KX772186

PCR conditions for multiplex were then modified from the previously run singleplex PCR after optimization. The annealing temperature was changed to 66 °C for multiplex 1, 2 and 3. The cycle number was decreased to 32 for multiplex 5 and the annealing temperature for multiplex 4 was changed to 62 °C with a cycle number of 30.

3.2.3.9. Genotyping loci

From the Tugela River, 22 individuals were genotyped at all loci. Loci were expected to be heterozygous and were therefore analysed for the probability of departure from the Hardy-Weinberg Equilibrium (HWE) with Genodive (Meirmans & Van Tienderen, 2004), as the principal states that allele frequency in a population or metapopulation will continue to be constant from generation to generation if there are no other evolutionary influences such as genetic drift. The disequlibrium linkage between loci was then assessed with Genepop on the web which is a population genetics software program. The program was used for the Hardy-Weinberg equilibrium estimation and to convert the input Genepop file to formats that can be used by other programs, allowing communication between them (Raymond & Rousset, 1995; Rousset, 2008). The B-Y FDR (false discovery rate) was then computerized (Benjamini & Yekutieli, 2001; Narum, 2006). Followed by the determining of the null alleles with FreeNA (Chapuis & Estoup, 2007). To eliminate significant linkage disequilibrium, six loci were discarded from samples AHym 14, 15 4, 21, 9, 22 or 1.

3.3. Conclusion

In conclusion, all loci were polymorphic ranging from three to 16 alleles per locus. Observed heterozygosity ranged between 0.455 and 0.941, while there was a significant deviation from the HWE at three loci. At low frequencies, null alleles were present in some loci, with the highest frequency of 0.166 found in AHym11 and AHym23. This could be the reason for the departure from the HWE if correlated with heterozygote deficiencies at these loci. Although null alleles can't create structure if there isn't any, they will still be valuable for assessing population structure for this species as the study conducted by Newman & Squire (2001) that assessed migration of wood frogs through population genetic structure. *Amietia hymenopus* though, is an aquatic species and less migration will be expected. The FST values can also be corrected for the presence of null alleles (Chapuis and Estoup 2007). Finally the accomplishment of using three-primer labeling system in these markers

accompanied by multiplexing, decreases costs and increases genotyping efficiency within the lab.

Chapter 4

General conclusion and recommendations

4.1. Disease monitoring of *Amietia hymenopus*

About 500 amphibian species around the world are known to be infected with *Batrachochytrium dendrobatidis* (Aanensen, 2016). The pathogen is widespread and persistent in all of the rivers in the Mont-aux-Sources region that flow towards South Africa. Statistical analysis of the infection profile of *Amietia hymenopus* in the Drakensberg Mountains, was done in accordance with the first objective of this study: to determine how environmental factors influence disease dynamics of this species. This was achieved by analysing data that was accumulated over 10 years in the area.

It was found that the different sites did not differ statistically in pathogen prevalence, although the two sites (Tugela and Vemvane) with the least variation in *B. dendrobatidis* prevalence, cross the path of popular tourist hiking trails. Pathogen spread along or between these rivers could constantly be facilitated by hikers, for example, contaminated mud on hiking boots.

Recommendation: *hikers should restrict direct contact with water sources while hiking in the mountains, or clean their boots before crossing water sources.*

It was expected that there would be variation between seasonal surveys as winters in the study are well below the optimal growth range of *B. dendrobatidis* (Longcore *et*

al., 1999). It was probable that the *B. dendrobatidis* prevalence would be less than in the summer. From the analysis it was found that there was no statistical significant difference in infection prevalence between seasons. This uniform infection pattern could be explained by the fact that the variation in climate in this region has such a narrow range that it did not inhibit or augment zoospore production to the extent that its effect was visible in prevalence levels of the host population. Tadpoles are also known to be reservoirs for the pathogen; therefore it will always be present in the system, winter or summer. Temperature could be one of many environmental factors that influenced the host-pathogen relationship.

Recommendation: *other factors should be included in the monitoring plan, such as the influence of rainfall on river flow rates and how it affects the host/pathogen dynamics. It is also recommended that the surveys are done more consistently, for example the same time each year and if possible more regularly, although regular monitoring is expensive. An advantage to proceeding with monitoring is the opportunity it grants to train a new workforce as this is an area with an apparent endemic* B. dendrobatidis *source*.

Rainfall was found to have an influence on the pathogen. As rainfall increases, the prevalence of *B. dendrobatidis* decreases. It affects tadpole density, as higher water flow and more water overall within the river causes tadpoles to be dispersed more across the river. As *B. dendrobatidis* zoospores only swim about 2 cm and do not live for a long period of time (Berger *et al*., 2005; Kilpatrick *et al*., 2009), the likelihood of encountering a susceptible host is reduced. The opposite was observed as a very high *B. dendrobatidis* prevalence was seen in drought conditions, the more condensed the tadpoles are, the more individuals become infected. From this study it was found that if there is an average rainfall of more than 100 mm per month, the *B. dendrobatidis* prevalence was below 40 %. However, if it rains less than 100 mm average per month, the *B. dendrobatidis* prevalence may reach up to 100 %.

Recommendation: *more detailed measurement should be used when observing the influences of rainfall for instance collect rainfall data at more regular time intervals for more accurate interpretation of results.*

A very clear correlation was seen in the size and development of the tadpoles in accordance to *B. dendrobatidis* prevalence. As the tadpoles became bigger and more developed, their chance of being infected increased. The tadpoles for this species are known to have a slow development, often more than one cohort is found in the same system, and for this reason they spend several months in the water (Van Dijk, 1996), providing more time for the pathogen to infect them. From the study it was determined that tadpole's body size is the driving factor for infection. This is due to the size of the tadpole being more closely correlated to *B. dendrobatidis* infection as the tadpole's size increases compared to the developmental stages. Tadpoles with such a long larval stage are the main reason why *B. dendrobatidis* is particularly persistent in these systems as they remain reservoirs for the pathogen (Daszak *et al.,* 1999, 2003).

Recommendation: *pathogen dynamics should be observed in adult frogs to determine if the infection pattern is similar to that of tadpoles or how it differs, because ultimately frog-to-frog transmission between rivers is the result of migrating adults. It is important to note that this species is predominantly aquatic (Lambris, 1988) therefore it has a high likelihood of frequently coming into contact with zoospores. A marked difference from the larval stage is that the density of adult frogs is much lower than the tadpole density.*

4.2. Microsatellite development for *A. hymenopus*

Microsatellites of *A. hymenopus* were developed to aid in the completion of the second objective. Although the development of microsatellites was successful, time and opportunity did not allow me to perform population genetics on the host species. However, it is vital to complete this step if one is to understand if and to which extent gene flow takes place between populations in close proximity, but seemingly geographically isolated as the fogs are not known to move over land (Mouton, 1996). In addition it will provide insight into the influence of a pathogen in the context of population genetic diversity. Furthermore it will be useful in the conservation *of A. hymenopus* as it can be used for the study of genetic drift, population bottlenecks or inbreeding. Most importantly it identifies isolated populations that may become threatened or even extinct.

References

AANENSEN, D.M. 2016. Bd-maps. http://www.bd-maps.net. Date of access: 26 Sep 2016.

ALBERTO, F. 2009. MsatAllele_1. 0: an R package to visualize the binning of microsatellite alleles. *Journal of Heredity*. 100:394-397.

ALEXANDER, M. 2016. Drakensberg: barrier of spears. http://www.southafrica.info/about/geography/drakensberg-050705.htm#.WAkRDcneKwJ. Date of access: 12 Oct 2016.

ALFORD, R. A. & RICHARDS, S.J. 1999. Global amphibian declines: a problem in applied ecology. *Annual Review of Ecology and Systematics*. 30:133-165.

APLIN, K. & KIRKPATRICK, P. 1999. Progress report on investigations into chytrid fungal outbreak in Western Australia. Western Australia Museum. Perth.

BALLOUX, F. & LUGON-MOULIN, N. 2002. The estimation of population differentiation with microsatellite markers. *Molecular Ecology*. 11:155-165.

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

BATAILLE, A., FONG, J.J., CHA, M. WOGAN, G.O.U., BAEK, H.J., LEE, H., MIN, M. & WALDMAN, B. 2013. Genetic Evidence for a high diversity and wide distribution of endemic strains of the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* in wild Asian amphibians. *Molecular Ecology*. 22:4196-4209.

BATES, M.F. 2002. The distribution of Amietia vertebralis (Hewitt 1927) (Anura:Ranidae), with comments on its taxonomic and conservation status. Navorsing van die Nasionale Museum. Bloemfontein. 18:77-94.

BEEBEE, T.J.C. 1996. Ecology and Conservation of Amphibians. Chapman & Hall. London.

BENJAMINI, Y. & YEKUTIELI, D. 2001. The control of the false discovery rate in multiple testing under dependency. *Annals of statistics*. 1165-1188.

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

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.

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

BERGER,L., SPEARE, R. & HYATT,A.D. 2000. Chytrid fungi and amphibian declines: Overview, implication and future directions. Campbell, A., ed. Declines and Disappearances of Ausralia n frogs. Environmental Australia, Canberra.

BERNHARDT, J.R. & LESLIE, H.M. 2013. Resilience to climate change in coastal marine ecosystems. *Annual Review of Marine Science*. 5:371-92.

BLACKET, M.J., ROBIN, C., GOOD, R.T., LEE, S.F. AND MILLER, A.D. 2012. Universal primers for fluorescent labelling of PCR fragments—an efficient and cost-effective approach to genotyping by fluorescence. *Molecular Ecology Resources*. 12:456-463.

BLAUSTEIN, A. R., HOKIT, D.G. O'HARA, R.K. & HOLT. R.D. 1994.Pathogenic fungus contributes to amphibian losses in the Pacific Northwest. *Biological Conservation.* 67:251-254.

BLAUSTEIN, A.R. & JOHNSON, P.T.J. 2003. The complexity of deformed amphibians. *Frontiers in Ecology and the Environment* 1:87-94.

BLAUSTEIN, A.R., ROMANSIC, J.M., SCHEESSELE, E.A., HAN, B.A., PESSIER, A.P. & LONGCORE, J.E. 2004. Interspecific Variation in Susceptibility of FrogTadpoles to the Pathogenic Fungus *Batrachochytrium dendrobatidis* . *Conservation Biology*. 19:1460-1468.

BOLGER, A.M., LOHSE, M. & USADEL, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*. 30: 2114-2120.

BORDOLOI, S., BORAH, M.M., CHAKRAVORTY, P. & SINHA, B. 2001. First record of the Ranid frog *Paa annandalii* (Boulenger 1920) from north-eastern region (Arunachal Pradesh) of India with a note on its larval stages. *Current Science*. 80:1219-1222.

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

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.

BOTSFORD, L.W., WHITE, J.W., COFFROTH, M.A., PARIS, C.B., PLANES, S., SHEARER, T.L., THORROLD, S.R. & JONES, G.P. 2009. Connectivity and resilience of coral reef metapopulations in marine protected areas: matching empirical efforts to predictive needs. *Coral Reefs*. 28:327-337.

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.

BRANCH, W.R. 1988. South African Red Data Book – Reptiles and Amphibians. South African National Scientific Programmes Report no. 151. CSIR, Pretoria.

BRISTOW, D. 2003. Best walks of the Drakensberg. Struik. Cape Town.

CALIFORNIA CENTER FOR AMPHIBIAN DISEASE CONTROL. 2007. Testing for*Batrachochytrium dendrobatidis* (“Bd”). www.ccadc.us/docs/BdTesting.pdf. Date of access: 02 Aug 2016.

CASTOE, T., POOLE, A. & DE KONING, A. 2012. Rapid microsatellite identification from Illumina paired-end genomic sequencing in two birds and a snake. *PLoS One*. 7:e3095.

CHAE, W.B., HONG, S.J., GIFFORD, J.M., RAYBURN, A.L., SACKS, E.J. & JUVIK, J.A. 2014. Plant morphology, genome size, and SSR markers differentiate five distinct taxonomic groups among accessions in the genus Miscanthus. GCB *Bioenergy*. 6:646-660.

CHAPUIS, M.P. & ESTOUP, A. 2007. Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution*. 24:621-631.

CHISTIAKOV, D.A., HELLEMANS, B. & VOLCKAERT, F.A. 2006. Microsatellites and their genomic distribution, evolution, function and applications: a review with special reference to fish genetics. *Aquaculture*. 255:1-29.

CHITTENDEN, H. 2009. Roberts vöelgids, ‘n volledige veldgids tot meer as 950 vöelspesies in Suider-Afrika. John Voelker Vöelboekfonds. Cape Town.

COLLEVATTI, R.G., GRATTAPAGLIA, D. & HAY, J.D. 2001. Population genetic structure of the endangered tropical tree species *Caryocar brasiliense*, based on variability at microsatellite loci. *Molecular Ecology*. 10:349-356.

COWEN, R. & SPONAUGLE, S. 2009. Larval dispersal and marine population connectivity. *Annual Review of Marine Science*. 1:443-466.

CREGAN 1999

CRAWFORD, A. J., LIPS, K. R. & BERMINGHAM, E. 2010. Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. *Proceedings of the National Academy of Sciences*. 107:13777-13782.

CULLEY, T.M., STAMPER, T.I., STOKES, R.L., BRZYSKI, J.R., HARDIMAN, N.A., KLOOSTER, M.R. & MERRITT, B.J. 2013. An efficient technique for primer development and application that integrates fluorescent labeling and multiplex PCR. *Applications in Plant Sciences*. 1.

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*. 5:735-748.

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

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.

DRURY, S.E.N., GOUGH, R.E. &. CUNNINGHAM, A.A. 1995. Isolationof an iridovirus-like agent from common frogs (*Rana temporaria*). *Veterinary Record.* 137:72-73.

DU PREEZ, L. H. & CARRUTHERS, V. 2009. A complete guide to the frogs of southern Africa. South Africa, Struik. Cape Town.

DUNN, O.J. 1964. Multiple comparisons using rank sums. *Technometrics*, 6:241-252.

ELDRIDGE, N. 1998. Life in the balance: humanity and the biodiversitycrisis. Princeton University Press, Princeton, New Jersey.

FARRER, R.A., WEINERTA, L.A., BIELBY, J., GARNER, T.W.J., BALLOUX, F., CLAREA, F., BOSCH, J., CUNNINGHAM, A.A., WELDON, C., DU PREEZ, L.H., ANDERSON, L., POND, S.L.K., SHAHAR-GOLAN, R., HENK, D.A. & FISHER, M.C. 2011. Multiple emergences of genetically diverse amphibianinfecting chytrids include a globalized hypervirulent recombinant lineage. *PNAS*. 108: 18732–18736.

FIELD, D. & WILLS, C. 1998. Long polymorphic microsatellites in simple organisms. *Proceeding of the Royal Society of London, Series B: Biological Sciences.* 263:209-215.

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 Review*. 21:2-9.

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. 2009a. 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., WALKER, S. F. 2009b. Global Emergence of *Batrachochytrium dendrobatidis* and Amphibian Chytridiomycosis in Space, Time, and Host. *Annual Review of Microbiology*. 63:291-310.

FONG, J.J., CHENG, T.L., BATAILLE, A., PESSIER, A.P., WALDMAN, B. & VREDENBURG, V.T. 2015. Early 1900s Detection of *Batrachochytrium dendrobatidis* in Korean Amphibians. *PLoS One*. 10:e0115656.

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.

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.

GHOSH, P. & FISHER, M.C. 2016. Dr Jekyll and Mrs Hyde: Risky hybrid sex by amphibian-parasitizing chytrids in the Brazilian Atlantic Forests. *Molecular Ecology. 25:2961-2963.*

GOSNER, K.L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*. 16:183-190.

GRIFFITHS, S.M., FOX, G., BRIGGS, P.J., DONALDSON, I.J., HOOD, S., RICHARDSON, P., LEAVER, G.W., TRUELOVE, N.K. & PREZIOSI, R.F. 2016. A Galaxy-based bioinformatics pipeline for optimised, streamlined microsatellite development from Illumina next-generation sequencing data. *Conservation Genetics Resources.*1-6.

HEDRICK, W. 2001. Conservation genetics: Where are we now? *Trends in Ecology and Evolution*. 16:629-636.

HELLBERG, M., BURTON, R., NEIGEL, J. & PALUMBI, S.R. 2002. Genetic assessment of connectivity among marine populations. *Bulletin of Marine*. 70:273-290.

HEYWOOD, V.H. & IRIONDO, J.M. 2003. Plant conservation: Old problems, new perspectives. *Biological Conservation*. 113:321-335.

HOULAHAN, J.E., FINDLAY, C.S., SCMIDT, B.R., MYER, A.H. &. KUZMIN, S. L. 2000. Quantitative evidence for global amphibian population declines. *Nature*. 404:752-755.

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 stebbensi*. *Diseases of Aquatic Organisms*. 31:161-167.

JEHLE, R., WILSON, G.A., ARNTZEN, J.W. & BURKE, T. 2005. Contemporary gene flow and the spatio-temporal genetic structure of subdivided newt populations (*Triturus cristatus, T. marmoratus*). *Journal of Evolutionary Biology*. 18: 619-628.

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

JOHNSON, M., LAJEUNESSE, M. & AGRAWAL, A. 2006. Additive and interactive effects of plant genotypic diversity on arthropod communities and plant fitness. *Ecology Letters*. 9:24-34.

KIESECKER, J. M. & BLAUSTEIN, A.R. 1995. Synergism between UVB radiation and a pathogen magnifies amphibian embryo mortality in nature. *Proceedings of the National Academy of Science U.S.A.* 92:11049-11052.

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.

KNAPP, R.A., BRIGGS, C.J., SMITH. T.C. & MAURER, J.R. 2011. Nowhere to hide: impact of a temperature-sensitive amphibian pathogen along an elevation gradient in the temperate zone. *Ecosphere*. 2:1-26.

KRIGER, K.M., PEREOGLOU, F. & HERO, J. 2007. Latitudinal Variation in the Prevalence and Intensity of Chytrid (*Batrachochytrium dendrobatidis*) Infection in Eastern Australia. *Conservation Biology*. 21:1280-1290.

KRIGER, M.K. & HERO, J. 2007. The chytrid fungus *Batrachochytrium dendrobatidis* is non-randomly distributed across amphibian breeding habitats. *Diversity and Distributions.*13:781-788.

KRUGER, D.J., WELDON, C. & DU PREEZ, L.H. 2011. Resolving the confusion: Amietia vertebralis and A. umbraculata tadpole morphology. *African Zoology*. 46:309-319.

KRUSKAL, W.H. & WALLIS, W.A. 1953. Errata: Use of Ranks in One-Criterion Variance Analysis. *Journal of the American Statistical Association*. 47: 583-621.

LA MARCA, E., LIPS, K.R., LÖTTERS, S., PUSCHENDORF, R., IBÁÑEZ, R., RUEDA-ALMONACID, J.V., SCHULTE, R., MARTY, C., CASTRO, F., MANZANILLA-PUPPO, J. & GARCÍA-PÉREZ, J.E. 2005. Catastrophic population declines and extinctions in Neotropical harlequin frogs (*Bufonidae: Atelopus*). *Biotropica*. 37:190-201.

LAMBRIS, A.J.L. 1988. Rana Vertebralis: Species account. W.R., ed. South Arican Red Data Book – Reptiles and Amphibians. South African National Scientific Programmes Report no. 151. CSIR, Pretoria.

LAMBRIS, A.J.L. 1989. A review of the amphibians of Natal. *Lammergeyer*. 39:1-212.

LINSTROM, W. 1981. Drakensberg Geological Survey. Government Printing Works Pretoria.

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 USA*. 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.

MANEL, S., SCHWARTZ, M.K., LUIKART, G. & TABERLET, P. 2003. Landscape genetics: combining landscape ecology and population genetics. *Trends in Ecology & Evolution.* 18:189-197.

MANN, H.B. & WHITNEY, D.R. 1947. On a test of whether one of two random variables is stochastically larger than the other. *The annals of mathematical statistics*. 1: 50-60.

MARAIS, J. 2004. Snakes of Southern Africa. Struik. Cape Town.

MARAIS, J & ALEXANDER, A. 2008. A Guide to the Reptiles of Southern Africa. Struik. Cape Town.

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.

MASELLA, A.P., BARTRAM, A.K., TRUSZKOWSKI, J.M., BROWN, D.G. & NEUFELD, J.D. 2012. PANDAseq: paired-end assembler for Illumina sequences. *BMC Bioinformatics*. 13:31.

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

MCCARTHY, T. & RUBIGE, B. 2005. The Story of Earth and Life. Struik. Cape Town.

MEIRMANS, P.G. & VAN TIENDEREN P.H. 2004. Genotype And Genodive: two programs for the analysis of genetic diversity of asexual organisms. *Molecular Ecolology Notes.* 4:792-794.

MINTER, L., BURGER, M., HARRISON, J., BRAACK, H., BISHOP, P. & KLOEPFER, D. 2004. Atlas and Red Data Book of the Frogs of South Africa, Lesotho and Swaziland. Smithsonian Institution Monitoring and Assessment of Biodiversity Program. Avian Demography Unit, University of Cape Town.

MORGAN, J.A.T., VRENDENBURG, V.T., RACHOWICZ, L.J., KNAPP, R.A., STICE, M.J., TUNSATLL, T., BINGHAM, R.E., PARKER, J.M., LONGCORE, J.E., MORITZ, C., BRIGGS, C.J. & TAYLOR, J.W. 2007. Population genetics of the frog-killing fungus *Batrachochytrium dendrobatidis*. *Proceedings of the National Academy of Sciences of the USA*. 104:13845-13850.

MOUTON, P. LE.F.N. 1996. Amphibians and reptiles. Lesotho Highlands Water Project final report. Contact no. 1008, Baseline Biology Survey and Reserve Development, Phase1B. Volume 3 – Fauna. AfriDev Consultants (for Lesotho Highlands Development Authority), Darling, South Africa.

MUTHS, E., PILLIOD, D.S. & LIVO, L.J. 2008. Distribution and environmental limitations of an amphibian pathogen in the Rocky Mountains, USA. *Biology Conservation*. 141:1484-1492.

NARUM, S.R. 2006. Beyond Bonferroni: Less conservative analyses for conservation genetics. *Conservation. Genetics*. 7:783-787.

NEL, W. & SUMNER, P.D. 2008. Rainfall and temperature attributes on the Lesotho-Drakensberg escarpment edge, Southern Africa. *Georgrafiska Annaler*. 90: 97–108.

NEWMAN, R. A. & SQUIRE, T. 2001. Microsatellite variation and fine-scale population structure in the wood frog (Rana sylvatica). *Molecular Ecology*. 10 1087-1100.

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

O'DONNELL, W.T. & WARREN, S.T. 2002. A decade of molecular studies of fragile X syndrome. *Neurosciences*. 25:315-338.

O'GRADY, J. 1998. Dead frogs in a dry 'berg. Natal Witness 23 (September): 24.

OLIVEIRA, E.J., PÁDUA, J.G., ZUCCHI, M.I., VENCOVSKY, R. & VIEIRA, M.L.C. 2006. Origin, evolution and genome distribution of microsatellites. Genetics and *Molecular Biology*. 29:294-307.

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

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

PARRIS, M. J., BAUD, D. R. & QUATTRO, J. M. 2004. Interactive Effects of a Heavy Metal and Chytridiomycosis on Gray Treefrog Larvae (*Hyla chrysoscelis*). *Copeia*, 2:344-350.

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.

PILLIOD, D.S., MUTHS, E., SCHERER, R.D., BARTELT, P.E., CORN, P.S., HOSSACK, B.R., LAMBERT, B.A., MCCAFFERY, R. & GAUGHAN, C. 2010. Effects of amphibian chytrid fungus on individual survival probability in wild boreal toads. *Conservation Biology*. 24:1259-1267.

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.

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

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.

RASMUS, N. 2005. Molecular Signatures of Natural Selection. *Annual Review of Genetics.* 39:197-218.

RAYMOND, M. & ROUSSET, F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*. 86:248-249.

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.

RICHARDS, S. R., MCDONALD, K. R. & ALFORD, R. A. 1993. Declines in populations of Australia's endemic tropical rainforest frogs. *Pacific.Conservation*. *Biology*. 1:66-77.

ROA, A.C., CHAVARRIAGA-AGUIRRE, P., DUQUE, M.C., MAYA, M.M., BONIERBALE, M.W., IGLESIAS, C. & TOHME, J. 2000. Cross species amplification of cassava (*Manihot esculenta*) (Euphorbiaceae) microsatellites: Allelic polymorphism and degree of relationship. *American Journal of Botany*. 87:1647-1655.

RODRÍGUEZ-BRENES, S., RODRIGUEZ, D., IBÁÑEZ, R. & RYAN, M.J. 2016. Spread of Amphibian Chytrid Fungus across Lowland Populations of Túngara Frogs in Panamá. *PloS One*. 11:e0155745.

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.

ROUSSET, F. 2008. genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources*. 8:103-106.

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

SAWS (SOUTH AFRICAN WEATHER SERVICE). 2016. Historical rain maps. http://www.weathersa.co.za/climate/historical-rain-maps. Date of access: 26 Sep 2016.

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.

SCHLÖTTERER, C. & HARR, B. 2000. Drosophila virilis has long and highly polymorphic microsatellites. *Molecular Biology and Evolution*. 17:1641-1646.

SCHLÖTTERER, C., AMOS, B. & TAUTZ, D. 1991. Conservation of polymorphic simple sequence loci in cetacean species. *Nature*. 354:63-65.

SCHOCK, D.M., RUTHIG, G.R., COLLINS, J.P., KUTZ, S.J., CARRIÈRE, S, GAU, R.J., VEITCH, A.M, LARTER, N.C., TATE, D.P., GUTHRIE, G. 2010. Amphibian chytrid fungus and ranaviruses in the Northwest Territories, Canada. *Diseases of Aquatic Organisms.* 92:231-240.

SEIMON, T.A., SEIMON, A., DASZAK. P., HALLOY, S.R.P., SCHLOEGEL, L.M., AGUILAR, C.A., SOWELL, P., HYATT, A.D., KONECKY, B. & SIMMONS, J.E. 2007. Upward range extension of Andean anurans and chytridiomycosis to extreme elevations in response to tropical deglaciation. *Global Change Biology*. 13:288-299.

SHAFFER, H.B., ALFORD, R.A., WOODWARD, B.D., RICHARDS, S.J., ALTIG, R.G. & GASCON, G. 2001. Quantitative sampling of amphibian larvae. In: HEYER, W., DONNELLY, M., MCDIARMID, R., HAYEK, L. & FOSTER, M. (eds) Measuring and Monitoring Biological Diversity: Standard Methods for Amphibians. Smithsonian Institution Press, Washington, D. C.

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.

SKERRATT, L.F., BERGER L., SPEARE, R., CASHINS, S., MCDONALD, K.R., PHILLOTT, A.D., HINES, H.B., KENYON, N. 2007. Spread of Chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth*. 4:125-134.

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. *Diseases of Aquatatic Organisms*. 74:159-164.

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.

SOUCHON, D. 2005. Serpent Spires. Jonathan Ball Publishers. Jeppestown.

SPENCER, C.C., NEIGEL, J.E. & LEBERG, P.L. 2000. Experimental evaluation of the usefulness of microsatellite DNA for detecting demographic bottlenecks. *Molecular Ecology*. 9:1517-1528.

SQUIRRELL, J., HOLLINGSWORTH, P.M., WOODHEAD, M., RUSSELL, J., LOWE, A.J., GIBBY, M. & POWELL, W. 2003. How much effort is required to isolate nuclear microsatellites from plants? *Molecular ecology*. 12:1339-1348.

STORFER, A., MURPHY, M.A., EVANS, J.S., GOLDBERG, C.S., ROBINSON, S., SPEAR, S.F., DEZZANI, R., DELMELLE, E., VIERLING, L. & WAITS, L.P. 2007. Putting the 'landscape'in landscape genetics. *Heredity*. 98:128-142.

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*. 306:1783-86.

SUCHET, R. 2006. A backpackers guide to Lesotho. Russel Suchet. Underberg.

TALLEY, B.L., MULETZ, C.R., VREDENBURG, V.T., FLEISCHER, R.C. & LIPS, K.R. 2015. A century of *Batrachochytrium dendrobatidis* in Illinois amphibians (1888–1989). *Biological Conservation*. 182:254-261.

TARRANT, J., CILLIERS, D., DU PREEZ, .LH. & WELDON, C. 2013. Spatial Assessment of Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) in South Africa Confirms Endemic and Widespread Infection. *PLoS One*. 8: e69591.

TERRELL, V.C.K., ENGBRECHT, N.J., PESSIER, A.P. & LANNOO,M.J. 2014. Drought reduces chytrid fungus (*Batrachochytrium dendrobatidis*) infection intensity and mortality but not prevalence in adult crawfish frogs (*Lithobates areolatus*). *Journal of Wildlife Diseases*. 50:56-62.

THUILER, W. 2000. Climate change and the ecologist. *Nature*. 448:550-552.

TÓTH, G., GÁSPARI, Z. & JURKA, J. 2000. Microsatellites in different eukaryotic genomes: Survey and analysis. *Genome Research*. 10:967-981.

ULLER, T. & OLSSON, M. 2008. Multiple paternity in reptiles: patterns and processes. *Molecular Ecology*. 17:2566-2580.

UNESCO. 2016. Maloti-Drakensberg Park. http://whc.unesco.org/en/list/985. Date of access: 14 June 2016.

VALENCIA-AGUILAR, A., TOLEDO, L.F., VITAL, M.V.C. & MOTT, T. 2016. Seasonality, Environmental Factors, and Host Behavior Linked to Disease Risk in Stream-Dwelling Tadpoles. *Herpetologica*. 72:98-106.

VAN DIJK, D.E. 1996. Anuran fauna of Lesotho Highlands in the Khatse Dam catchment area and Jorodane River region. *Koedoe*. 39:56-57.

VIEIRA, C.A., TOLEDO, L.F., LONGCORE, J.E. &. LONGCORE, J.R. 2013. Body length of Hylodes cf. ornatus and Lithobates catesbeianus tadpoles, depigmentation of mouthparts, and presence of *Batrachochytrium dendrobatidis* are related. *Brazilian Journal of Biology*. 73:195-199.

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., KNAPP, R.A., TUNSTALL, T.S. & BRIGGS, C.J. 2010. Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proceeds of the National Academy of Science*. 107:9689-9694.

WAITS, L., TABERLET, P., SWENSON, J.E., SANDEGREN, F. & FRANZEN, R. 2000. Nuclear DNA microsatellite analysis of genetic diversity and gene flow in the Scandinavian brown bear (Ursus arctos). *Molecular Ecology*. 9:421-431.

WALKER, S.F., BOSCH, J. JAMES, T.Y., LITVINTSEVA, A.P., VALLS, J.A.O., PIÑA, S., GARCÍA, G., ROSA, G.A., CUNNINGHAM, A.A., HOLE, S., GRIFFITHS, R. & FISHER, M.C. 2008. Invasive pathogens threaten species recovery programs. *Current Biology*. 18:853-854.

WELDON, C., DE VILLIERS, A.L. & DU PREEZ, L.H. 2007. Quantification of the trade in Xenopus laevis from South Africa, with implications for biodiversity conservation. *African Journal of Herpetology*. 56:77-83.

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: 2100-2105.

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.

WHITHAM, T.G., GEHRING, C.A., LAMIT, L.J., WOJTOWICZ, T., EVANS, L.M., KEITH, A.R. & SMITH, D.S. 2012. Community specificity: life and afterlife effects of genes. *Trends in plant science.* 17:271-281.

WITSIESHOEK. 2016. Chain Ladder Walk/Climb. Avalible: http://www.witsieshoek.co.za/activities/hiking-trails/chain-ladder-via-sentinel. Date of access: 28 Sep 2016.

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.

WRIGHT, J.M. & BENTZEN, P. 1994. Microsatellites: Genetic markers of the future. *Reviews in Fish Biology and Fisheries*. 4:384-388.

YOUNG, B.E., LIPS, K.R., REASER, J.K., IBÁÑEZ, R., SALAS, A.W., CEDEÑO, J.R., COLOMA, L.A., RON, S., LA MARCA, E., MEYER, J.R. & MUÑOZ, A. 2001. Population declines and priorities for amphibian conservation in Latin America. *Conservation. Biology*. 15:1213-23.

ZYTYNSKA, S.E., FAY, M.F., PENNEY, D. & PREZIOSI, R.F. 2011. Genetic variation in a tropical tree species influences the associated epiphytic plant and invertebrate communities in a complex forest ecosystem. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*. 366:1329-36.

ZYTYNSKA, S.E., KHUDR, M.S., HARRIS, E. & PREZIOSI, R.F. 2012. Genetic effects of tank-forming bromeliads on the associated invertebrate community in a tropical forest ecosystem. *Oecologia*. 170:467-75.