Spatial epidemiology of amphibian chytridiomycosis in the Orange River system of South Africa

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Nature always finds a way to realign the cogs when humans throw a wrench in the works, if only humans are able to see it

Dedicated to my family, because without them I would not have been the person I am today – warts and all.

ABSTRACT

South Africa not only hosts a large number of amphibians, but also two different lineages of *Batrachochytrium dendrobatidis,* the postulated endemic *Bd*CAPE and the Global Panzootic Lineage (*Bd*GPL). The Orange River is one of the largest river systems in South Africa, stretching 2500km and encompassing various climates and areas within South Africa. With more than one lineage being present in South Africa, and similar genotypes isolated from Europe, the possibility exists for new lineages to be introduced to either naïve individuals within a population or into a population where another lineage occurs, due to connectivity and anthropogenic activity within a river system. The Orange River was surveyed, not only to establish which lineages are present in this system, but also to collect isolates for whole genome sequencing. The competition and virulence capabilities of the lineages occurring in South Africa were tested by use of experiments on a South African host – *Sclerophrys gutturalis.* Because it is important to know the ability of a pathogen to spread through a population, a second experiment was conducted to test the transmission capability to negative individuals and to individuals infected with a different genotype of the same geographical origin. Sampling of the Orange River was done at approximate intervals of 100 km between sites stretching from Vioolsdrif to Aliwal North. Swabbing and toe-clipping were done on all adult frogs and toads encountered and tadpoles of *Amietia delalandii* were also collected for attempted amphibian chytrid isolation. The first experiment was done to determine the virulence capabilities of South African and European lineages. Competition trials consisted of animals being exposed sequentially to the different genotypes. During this experiment the influence of the different lineages on the rate of mortality and the infection intensities on the animals were compared between the different geographical origins. The transmission experiment was conducted using only local lineages and individuals of different disease status were co-housed after the dosing stage completed. Trials consisted of an individual infected with either lineage being co-housed with a naïve individual and a trial where two individuals infected with different lineages were co-housed.

The Orange River survey yielded 27 cultures in total, from both toe-clips (adults) and tadpoles. These cultures were obtained from four different sites. The swab data showed that amphibian chytrid is widespread throughout the Orange River system. Both *Bd*CAPE and *Bd*GPL lineages are present within this system. Although amphibian

chytrid was found throughout the entire system, overall prevalence and infection intensities were low. When concerned with the experiments it was seen that the expat lineages showed a significant increase in rate of mortality dependent on dosage, while the same was not seen for the local lineages. In terms of competition between lineages on a single individual, it was seen that both expat lineages managed to establish an infection on a single host, while the local lineages were not as successful in co-infecting the same host. The transmission experiment showed that both local lineages are successful in being transmitted to a naïve individual just by being housed in the same enclosure, with *Bd*CAPE infecting more naïve individuals than *Bd*GPL. The latter being more lethal towards the infected individuals.

The competition capabilities of the different lineages, as well as their ability to transmit to other individuals is especially important in a corridor habitat such as the Orange River. Habitat characteristics may change as well as host population composition progressing through the system, and thus the pathogen's capabilities to spread to the available individuals and establish infections needs to be known, even when just marginally exposed to potentially naïve hosts.

Key terms: Orange River, *Batrachochytrium dendrobatidis*, transmission capability, corridor habitat, disease competition, epidemiology.

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To my parents, Mom and Dad, thank you for your support and always being there to listen, and for growing an interest in amphibians as much as I have.

Lastly, thank you Lord for giving me the strength and opportunities that I receive every day.

DECLARATION

I, Jean Ruhan Verster, declare that this work is my own, unless otherwise acknowledged. This dissertation is only submitted at the North-West University for the MSc. degree in Environmental Sciences and has not been submitted to any other university.

J.R. Verster

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CHAPTER 1: GENERAL INTRODUCTION

To an amphibian the world is full of dangers, infectious diseases being one among the multitudes. Infectious diseases is a term used to describe an entity consisting of microorganisms or parasites that alter the behaviour and physiology of the host (WHO, 2015). Infectious diseases can have a variety of traits that influence the host (Cohen & Williamson, 1991). Infections that influence the human body are often easier to find due to the fact that humans have the ability to communicate the symptoms to a physician who can ultimately effect a change in the behaviour of the infected individual. Animals, on the other hand, do not always have this ability and scientists only realise or treat the disease after a major change in the population size, or the behaviour of the animal has taken place. A major infectious disease that is currently influencing many amphibian populations around the globe is Chytridiomycosis (Berger *et al.*, 1998; Daszak *et al.*, 1999; Bosch *et al.*, 2001; Garner *et al.*, 2011). The amphibian chytrid fungus is widespread across the globe, in South Africa positive frogs were sampled in five out of nine provinces, and the oldest recorded case of an infected individual in South Africa is a *Xenopus laevis* from the Western Cape, collected in 1938 (Weldon *et al.*, 2004). There are older positive specimens that have been sampled such as a *Rana* (*Lithobates*) *sphenocephala* individual sampled in 1888 from Illinois (USA) (Talley *et al.*, 2015), three positive individuals, two *Fejervarya limnocharis* and one *Bufo gargarizans,* all sampled in 1933 from China (Zhu *et al.*, 2014), a *Lithobates catesbeianus* individual collected in 1928 from California (USA) (Huss *et al.*, 2013) and a *Xenopus borealis* individual collected in 1934 from Kenya (Vredenburg *et al.*, 2013). This shows that chytridiomycosis has been widespread across the globe for roughly a century.

1.1 Epidemiology

Disease studies need to consider the geographical spread of the causing agents, as the distribution may provide ways to determine where the pathogen originated, as well as showing which populations of hosts may be at risk (Rothermel *et al.*, 2008). It is also true that diseases may be seasonal in their prevalence, showing increased prevalence in some seasons while seemingly absent in others (Rothermel *et al.*, 2008; Meyer, 2009; Conradie *et al.*, 2011). Often disease prevalence studies are aimed at finding “patient zero”, in this case referring to the oldest confirmed positive case, but seeing

that the oldest individuals positively identified using modern molecular technologies confirm that *Batrachochytrium dendrobatidis* was present on multiple continents in the late 1800s and early 1900s, finding "patient zero" is highly unlikely (Huss *et al.*, 2013; Vredenburg *et al.*, 2013; Zhu *et al.*, 2014; Talley *et al.*, 2015). Although these studies identified the historical widespread presence of *Batrachochytrium dendrobatidis*, the mapping of the different lineages may yield an estimate of the region of origin for different lineages and thus shed light on the reason for the adverse effects seen in populations upon introduction of a novel lineage.

Chytridiomycosis is caused by a pathogenic amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Longcore *et al*., 1999). The lifecycle of *B. dendrobatidis* consists of a motile zoospore, which swims in water, encysts on suitable growth media and enlarges to form the sporangia that ultimately produce more zoospores (Longcore *et al.*, 1999). *B. dendrobatidis* may show a slight variation in morphology depending on the growth substrate, for example, nutrient agar vs. on the amphibian host skin (Voyles, 2011).

The amphibian trade has been strongly implicated as at least a contributing factor to the distribution of *Batrachochytrium dendrobatidis* worldwide, which is also a hypothesis on how this pathogen could have been spread globally (Weldon *et al.*, 2004; Fisher & Garner, 2007; Weldon *et al.*, 2007; Schloegel *et al.*, 2012; Liu *et al.*, 2013). *Batrachochytrium dendrobatidis* also comprise different, genetically distinct lineages, of which some can be found on multiple continents (Farrer *et al.*, 2011; Schloegel *et al.*, 2012; Bataille *et al.*, 2013). In South Africa different lineages of the disease are present, and thus different areas can be subjected to different forms of the pathogen, eliciting different reactions from the hosts (Fisher *et al.*, 2009a; Farrer *et al.*, 2011). South Africa also boasts a wide range of climatic regions, all affecting the amphibian population present in that area in different ways, as well as the type of pathogens that may be present in that niche (Fisher *et al.*, 2009b). To acquire any distribution data pertaining to amphibian chytrid necessitates a study of the interaction between the amphibian populations and the climatic, as well as anthropogenic factors. The specific disease dynamics within a species also needs to be known, to be able to conduct a successful survey of the disease in that region (Imasuen *et al.*, 2011).

Global climate change is a well-known phenomenon (Ficke *et al.*, 2007) and a rise in temperature may not be beneficial to the growth of amphibian chytrid, as this fungus

exhibits a disability to grow at temperatures of 28°C and above, climate change could thus potentially negatively affect the ecology of amphibian chytrid (Lawrence, 2008). A temperature rise may, in essence, cause *B. dendrobatidis* prevalence to be limited to disjunct, micro-climatic niches (Kilpatrick *et al.*, 2010). A rise in temperature may also place the amphibian populations under pressure or may prove to increase their vulnerability, or toleration, to diseases such as *B. dendrobatidis.* This increased vulnerability to *B. dendrobatidis* may be due to the host's development or seasonal cycle being altered, such as later breeding seasons (Ficke *et al.*, 2007). Amphibian chytrid related extinctions may thus become especially prominent when the amphibian populations are already vulnerable due to other factors adversely impacting them (Laurance, 2008; Ribas *et al.*, 2009).

The ability of the host to tolerate the infection also plays an integral role in the disease's proliferation. Ribas *et al*. (2009) found that during the experiments they performed, the frogs from the higher temperature groups could tolerate the infection and recover from it, and they also found that the defence of the host against *B. dendrobatidis* infections would show a peak and then decline with a continuing rise in temperature, thus possibly explaining the reason for *B. dendrobatidis* seasonality in different climates. It does remain a fact that the capacity for a species to survive a *B. dendrobatidis* infection, is a species specific characteristic (Rowley & Alford, 2007).

1.2 The Orange River

The Orange River is the largest river system in South Africa (Ramollo, 2014). It stretches approximately 2500km, with a total catchment area of approximately 1 000 000 km², as shown in Figure 1-1 (DWA, 2012). This catchment area includes four countries: Lesotho, where it originates in the Maluti Mountains at an altitude of approximately 3400 m above sea level (Mahasa *et al.*, 2015), South Africa, Botswana and Namibia, where it delimits the Namibian southern border. Although it originates in Lesotho, South Africa is responsible for 97% of the total water withdrawal from the Orange River.

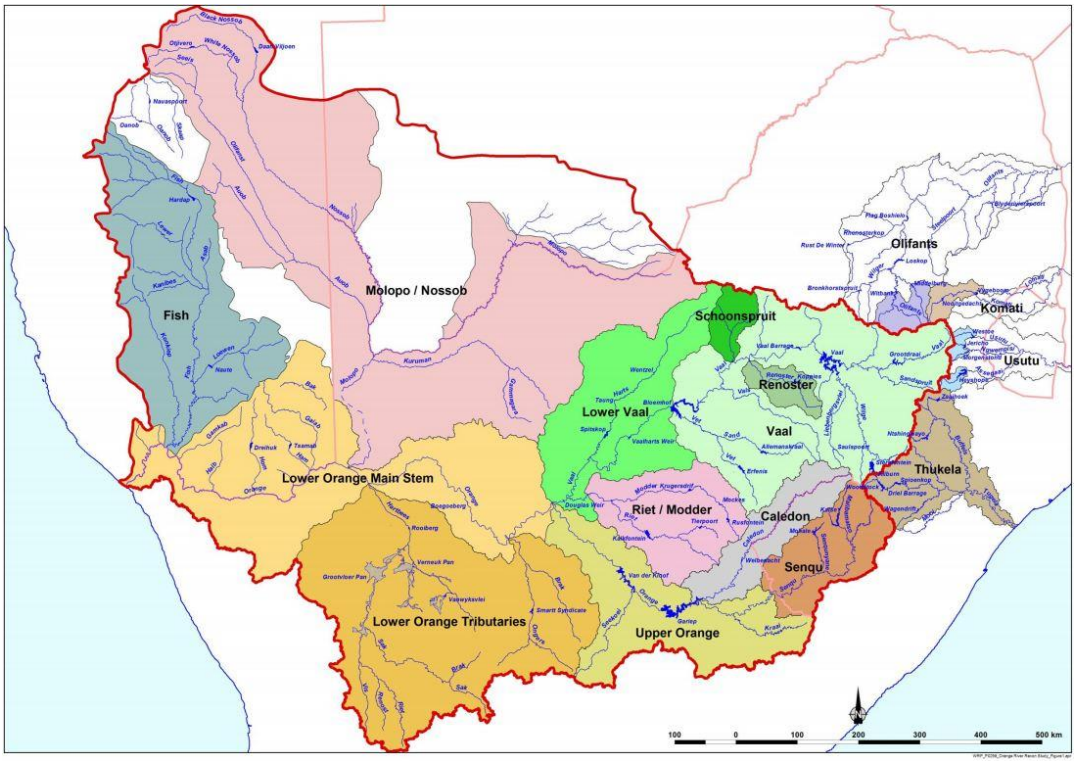

Figure 1-1: Catchment area of the Orange River (DWA, 2012)

There are a number of major storage dams that regulate the flow of the river, the largest being Gariep Dam and Vanderkloof Dam, the latter being 1400 km from the mouth of the river on the West coast of South Africa (Fair, 2003). The Orange River supplies other systems as well with inter-basin transfer schemes such as the Lesotho Highlands Water Project, supplementing the Vaal River system and the Orange-Fish tunnel which supplements the Fish River system (Fair, 2003). Although these schemes are situated high upriver they still link the Orange River with other systems in South Africa, thus potentially allowing for any influence from the Orange to extend to other systems.

1.3 Sampling

To mount a system-wide survey for a disease not only necessitates the consideration of sites and areas to be targeted but also an evaluation of the influence the sampling may have on the population being sampled at any particular site, especially when individuals are euthanized. The number of samples at each site required to yield statistically valuable data is also an issue. A pathogen study needs to incorporate the prevalence of the pathogen and the chance of opportunistically encountering said pathogen (Amos,

1985). Thus to determine the ideal sample size per site requires the consideration of the presumed pathogen prevalence, the population size (which is also often estimated or unknown) and the relative confidence of detecting the pathogen (Amos, 1985). It is argued that when doing a survey for ranavirus, for example, the sites and individuals sampled, need to be randomly selected (Gray *et al.*, 2015). This random selection of individuals eliminates the bias that a surveyor may incur by selecting individuals showing obvious signs of disease. A selective approach (non-random) cannot be used to determine prevalence, as mostly positive animals will be sampled, but in the case of chytridiomycosis, symptoms are often not easy to detect, especially at low-level infections (Voyles *et al.*, 2009). One study determined that in order to detect at least one positive individual in a population with a minimum prevalence of 5%, at least 60 individuals need to be sampled (Skerratt *et al.*, 2008). This capture of a defined number of individuals is more commonly used during continual surveillance projects, while for an initial study, it is more prudent to sample all the individuals encountered. Opportunistic sampling needs to aim at a minimum number of animals to allow for the detection of the pathogen, seeing as the prevalence of the disease in the system is currently unknown. The species that will be used during each phase of this project will be addressed in the following chapters. Owing to the fact that chytridiomycosis has been detected in archived specimens from all over South Africa (Weldon *et al.*, 2004) and the small population sizes of amphibians in the arid western part of the country, a sample size of 30 animals may be a more feasible option. This would also prevent oversampling of the sites.

1.4 Objectives

1.4.1 Determining the presence and distribution of *Batrachochytrium dendrobatidis* in the Orange River system

From an amphibian perspective, chytridiomycosis is just one of the possible threats to survival. The arid nature of the Northern Cape, which covers the larger part of the Orange River system, limits amphibians to sources of water that are available throughout the year, thus irrigation dams, drainage canals and side streams from the main river. This also places them in close proximity to water systems with a high rate of flow, or exchange with other sources (Volschenk *et al.*, 2005). This rate of exchange, as

well as the interconnectivity of the system with other major river systems in South Africa, requires the investigation into the prevalence and identified genotype of *B. dendrobatidis* in this system when concerned with the natural biota. Amphibian chytrid, presenting two different lineages in South Africa with different characteristics and pathogenicity for each lineage (Farrer *et al.*, 2011), already warrants an investigation of this system, as one lineage may easily be introduced into a new environment, or population, with possible adverse effects (Fisher *et al.*, 2009b). The relative quick expansion in the range of *B. dendrobatidis* to be virtually globally present suggests that it has the capacity to evolve rapidly, and this may also allow it to survive beyond the previously described environmental parameters, allowing it to spread into currently uninfected areas (Fisher *et al.*, 2009b). This ability to evolve can best be gauged when the current distribution or identity of the pathogen is known. The focus on the Orange River and the relative distribution of the disease along this linear system may then enlighten future conservation planners on the ability of the disease to spread over vast distances along linear corridor habitats, and thus incorporate this in conservation and mitigation planning.

Experimental phases

The isolates of *B. dendrobatidis* obtained from South Africa before the commencement of this study indicated that more than one lineage occurs and thus the Orange River with its expansive range of habitat and climate niches may provide the necessary environmental requirements for more than one lineage of *B. dendrobatidis* to occur within this system. If this is the case then there also exists the probability that one or more transition zones occur along the river where the conditions are conducive for the survival of both lineages. If this should be the case, then that amphibian community could potentially be under threat from increased disease pressure due to multiple lineage presence. Another potential effect may be the disruption of the amphibian community composition due to the competitive exclusion of certain species due to vulnerability, leading to a new equilibrium among amphibian species in these habitats (Begon *et al.*, 2006). The effect that the presence of more than one lineage would have on the amphibian community is currently unknown, but these conditions could be possible to duplicate to some extent in the laboratory.

1.4.2 Determining the virulence and competition capability of different *Batrachochytrium dendrobatidis* lineages and linking it to spatial origins

The same lineages of *B. dendrobatidis* that can be found in South Africa have also been isolated in other parts of the world, such as Europe (Farrer *et al.*, 2011). To determine the virulence of each strain, we will compare costs incurred by a local frog species when exposed to South African and European isolates of genetically similar lineages. Using a South African host and a non-native pathogen will reflect the potential effect an invasive form of amphibian chytrid will have on naïve hosts since the experimental animals will be naïve to all amphibian chytrid fungi. Although it might be unreasonable to think that a "continental jump" is possible for a pathogen, anthropogenic movement of biota, water and other products, especially with trade, may aid in the spread of pathogens into new areas with unknown consequences for local populations. The amphibian chytrid fungus has been found in *Xenopus gilli* individuals exported to a Spanish breeding facility from South Africa. These individuals were found to be infected and this led to the decimation of the captive populations of *Alytes muletensis* (Mallorcan midwife toad) housed in the same breeding facility, that was to be reintroduced into their natural habitats (Walker *et al.*, 2008). Invasive species, non-intentionally introduced, also pose the risk of carrying this disease (Miaud *et al.*, 2016). It is thus a possibility that a non-native pathogen may find its way into South Africa, either by way of import or accidental release and establishment of invasive species, especially seeing as the South African climate allows several invasive species to thrive (Pimentel, 2011). This experiment will thus attempt to determine the virulence of the South African chytrid lineages and compare these to similar treatments with potentially invasive lineages, allowing predictions to be made regarding their effect on wild amphibian populations.

1.4.3 Transmission and displacement of native indigenous lineages with invasive (previously indigenous) lineages

A large-scale linear system ranging in climate and habitat type may present the possibility for two different lineages of amphibian chytrid to survive in close proximity to each other, maybe even the same population. The second experiment of this project

aims to replicate the conditions that would exist if two lineages of amphibian chytrid were to be present in the same population of amphibians. Thus, what is the potential for transmission of a pathogen between two individuals? For an epidemic to occur, a limited number of infected individuals are needed, if the pathogen is hyper-virulent and has the potential to infect the naïve population (Miaud *et al.*, 2016). The two lineages present in South Africa were shown to have different virulence characteristics, in terms of the time till hosts becoming symptomatic (Farrer *et al.*, 2011), yet the ability to displace each other, or for one to prevent infection of the host with another, remains to be assessed. In South Africa, although both genotypes may not be endemic, they may be endemic to different populations and thus a convergence of two populations with different endemic genotypes is possible, especially in a linear corridor of habitat such as the Orange River. This could potentially affect the populations adversely, or the populations could have acquired an immunological or behavioural response to cope with the pathogen by previously being exposed to a similar pathogen, which could result in less adverse effects.

This experiment will thus be designed to test the probability of transmission between an infected individual and a naïve individual, which had no prior exposure to the pathogen. But seeing as that is not the only scenario possible, another test will be conducted, whether an animal already infected with one genotype (*Bd*GPL) of the pathogen, can become infected with another genotype (*Bd*CAPE). Thus, not only will this experiment allow the determination of the probability of transmission if an infected individual is inadvertently introduced into a naïve population, but it will also allow the prediction to be made as to possible consequences at convergence zones where more than one lineage of *B. dendrobatidis* are present in a population of amphibians.

1.5 Breakdown of chapters

Chapter 1 provides the basic rationale and background of this project and provides the various study objectives. Chapters 2 and 3 have been compiled as stand-alone chapters, each dealing with the research questions of the various objectives. Chapter 2 is about the amphibian chytrid survey within the Orange River system and subsequent isolation of *B. dendrobatidis*. Chapter 3 deals with the design, execution and the results obtained from the competition and virulence determination experiment. Chapter 3 also

contains the transmission and displacement potential experiment and thus elaborates on the design and execution thereof. Each chapter will, however, be presented as a potential manuscript for submission for the purpose of publication in peer-reviewed journals. Chapter 4 is the general discussion and implication of all the different phases of the project as a whole and attempts to discuss the relevance of each section to the improvement of our knowledge of a pathogen that is known to be present, but of which the distribution and capabilities are not fully known. In this chapter the potential influences, or threats, to amphibian populations already limited by both climate and development are also emphasized. Chapter 4 concludes by providing a summary and remarks of all findings.

CHAPTER 2: SPATIAL AND TAXONOMIC MAPPING OF AMPHIBIAN CHYTRIDS ASSOCIATED WITH THE ORANGE RIVER SYSTEM OF SOUTH AFRICA

2.1 Introduction

South Africa has a number of river systems, the largest being the Orange River, that stretches approximately 2500 km (Ramollo, 2014). The Orange River is influenced by many tributaries and the anthropogenic activities associated with it. The river cuts through a range of landscape types and climate zones from cooler highland regions in and around Lesotho to the arid regions of western South Africa (Tooth & McCarthy, 2004). Consequently the river acts as a lifeline that provides habitable environments for certain animal groups in an otherwise harsh environment, especially in the Northern Cape. Species distribution maps indicate that amphibians are among those organisms that have a higher concentration, in terms of species diversity and number of individuals, along the river than in the surrounding environment (Minter *et al.*, 2004). The agricultural dependency on the Orange River has resulted in the construction of numerous irrigation schemes that provide suitable habitat for a number of frog species and possible pathogens, with relative security from predatory fish (DWA, 2012; Fair, 2003). The main watercourse is not an ideal habitat for amphibians due to a high flow rate, numerous predators and daily fluctuations in water levels depending on the site's proximity to dams and other high volume release activities (Fair, 2003). The two major dams in this system, the Vanderkloof and Gariep Dams, are both used as hydro-electric power stations that increase the flow at night, thereby altering the availability of habitats (Fair, 2003). The connectivity of isolated habitats created by man-made structures such as canals, facilitates the movement of biota and therefore the spread of associated pathogens. Transmission may be compounded by the high density of amphibian hosts found in and around these canals.

Tarrant *et al*. (2013) hypothesized that these same conditions created by the Orange River will also favour the occurrence of the amphibian pathogen *Batrachochytrium dendrobatidis* (further referred to as amphibian chytrid and abbreviated as *Bd*) responsible for chytridiomycosis. *Bd* tends to be more prevalent in temperate and tropical regions, although limited to cooler months in the tropics due to its inability to survive high temperatures and desiccation (Piotrowski *et al.*, 2004; Kriger *et al.*, 2007).

Bd is also widespread in South Africa with a recorded overall prevalence across hosts, time and space of 14% (Tarrant *et al.*, 2013). Historically, *Bd* has been present in South Africa since the early 1930s (Weldon *et al.*, 2004) and very little evidence for chytridiomycosis is available, resembling an endemic infection on the sub-continent. It was seen that at least two distinct lineages of amphibian chytrid are present in South Africa (Farrer *et al.*, 2011; Schloegel *et al.*, 2012), yet their extent of occurrence is not known. Five different lineages of *Bd* can be found around the world (Farrer *et al.*, 2011; Bataille *et al.*, 2013), but the Tarrant *et al*. prevalence data do not take lineage identity into account. It was found that *Bd*GPL can invade areas where endemic *Bd*-lineages were previously prevalent (Jenkinson *et al.*, 2016), thus it is reasonable to suggest that *Bd*GPL could displace endemic *Bd*CAPE in the South Africa or vice versa, especially in a restricted corridor such as the Orange River and assuming *Bd*GPL acts as an invasive pathogen in the South African landscape.

*Bd*GPL was seen to be the lineage responsible for most epizootic events, but it was recently stated that the opportunity for hybrid lineages to be formed due to recombination, may further increase the danger towards amphibian populations (Morgan *et al.*, 2007; Jenkinson *et al.*, 2016). The potential for detecting contact zones have previously been a cumbersome task as the lineage identification depended on successful isolation and culturing, and it was impossible to determine the lineage without genome sequencing. However, in 2014 lineage specific real-time-PCR primers were developed for *Bd*CAPE and *Bd*GPL, allowing for the distinction to be made using this diagnostic tool in swabs taken from adult animals (Ghosh, 2014). This method opened the possibility to detect different lineages on individuals in a population using swabs, removing the dependency on isolation success and genome sequencing. It also allows for contact zones to be identified, where two distinct lineages may overlap in their distribution, and thus the areas with the highest potential for generating hybrid genotypes.

We assume that if *Bd* is present in the Orange River system that the infected populations will have a high prevalence due to habitat limitation within the corridor. We aim to identify and characterise the amphibian chytrids present in this system. By doing so, we will have a better understanding of the known distribution of amphibian chytrid in South Africa. We also hypothesize that due to multi-lineage presence in South Africa

the possibility exists for the Orange River to contain more than one lineage, resulting in opportunities for hybrid lineages to be formed at possible transition zones.

2.2 Materials and methods

2.2.1 Site selection

Identifying appropriate collection sites along the Orange River provide a number of challenges. Not only does the river stretch about 2500 km, but continuous access to the main body of water by vehicle is largely limited due to private ownership of adjacent land and thick reed beds that stretch for kilometres on end.

Twelve sites were selected at approximately 100 km intervals along the main watercourse of the Orange River as far as practically possible (Figure 2-1). This consideration was made to prevent oversampling and to obtain a holistic view of the entire river while limiting the biased expenditure of resources at a single site. Precise sampling intervals depended on access to the river itself and the availability of lodging that could serve as a field station. These twelve sites and their respective coordinates are listed in Table 2-1 and hereon referred to by the names of the nearest town. Two sites, Springbok and Petrusville, are not directly adjacent to the main watercourse, but due to the highly connective nature of aquatic habitats in these regions, and the limitation to amphibian survival elsewhere, these sites are a valuable contribution to the study of amphibian chytrid in the Orange River system.

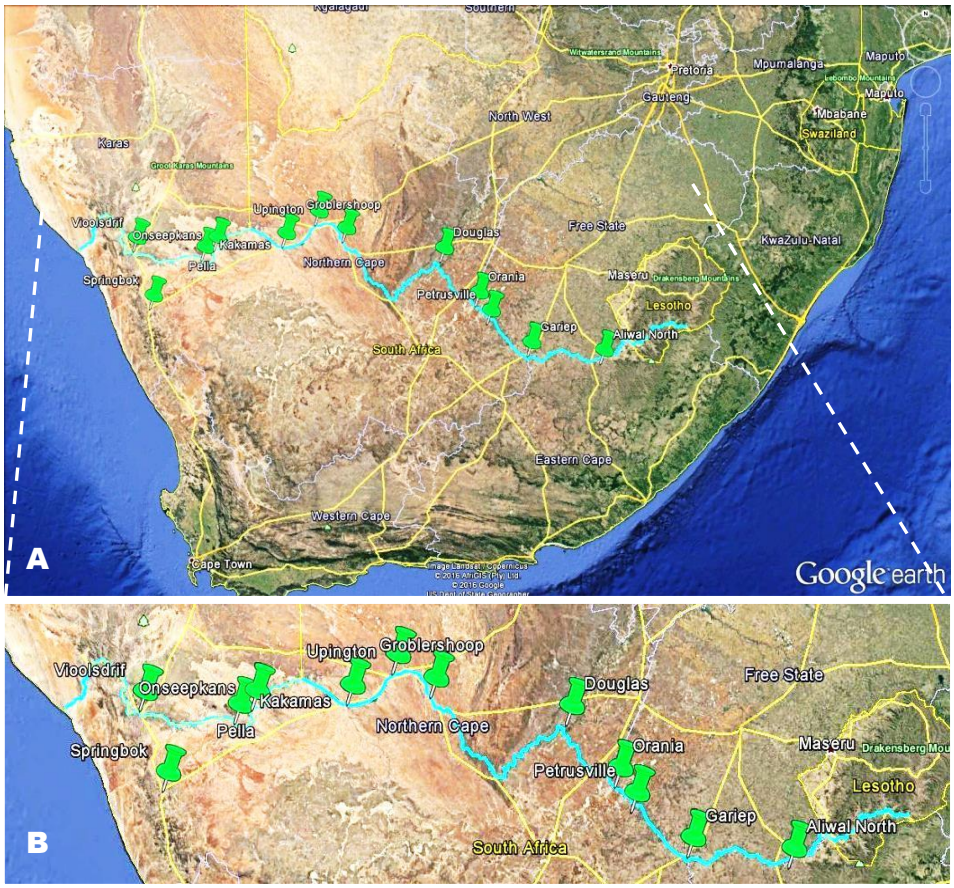

Figure 2-1: The location of sampled sites along the Orange River (A- Location within South Africa; B- Distribution of sites within the system)

Table 2-1: The specifications of sample sites listed in order from West to East

Local Name	Coordinates	Distance to River (line of sight)	Site type
Vioolsdrif	S28.6960; E17.5974	<1 km	Side stream (Main river)
Springbok	S29.6827; E17.8872	89 km	Ponds (Natural)
Pella	S28.9288; E19.0011	<1 km	Irrigation drainage ditch
Onseepkans	S28.7804; E19.2574	<1 km	Canal (Gravel 3m canal)
Kakamas	S28.7675; E20.6395	<1 km	Irrigation drainage ditch
Upington	S28.4275; E21.2971	1.6 km	Ponds (Man-made)
Groblershoop	S28.7189; E21.8311	<1 km	Irrigation drainage ditch
Douglas	S29.0708; E23.7387	<1 km	Ponds (Natural)
Orania	S29.7996; E24.4205	<1 km	Ponds (Man-made)
Petrusville	S30.0909; E24.6541	10.9 km	Temporary Ponds (Natural)

Gariep Dam	S30.6123; E25.4577	<1km	Side stream
Aliwal North	S30.7221; E26.9065	5.5 km	Ponds

2.2.2 Timing of fieldwork

Three field trips were conducted spanning over 14 months (February 2015 - April 2016). The eight most eastern sites fall within the predominantly summer rainfall region (field trips 1 and 3), while the western four sites receive winter rainfall (field trip 2). The sampling trips were thus planned according to the predicted rain season to maximize chances of encountering frogs.

2.2.3 Sampling and isolation of *Batrachochytrium dendrobatidis*

Tadpoles were collected with dipnets and kept in watertight containers containing water from the source at which they were collected. Tadpoles that were collected during the day were kept inside a cooler box to insulate them from thermal variation.

Adult frogs were located at the same sites after sunset by sight and torchlight, as well as by listening to their calls. All adult individuals encountered were caught while wearing disposable, sterile, non-powdered, nitrile gloves. Frogs were placed in separate, single-use, plastic bags containing 50ml of habitat-source water. Following species identification, the frogs were swabbed and toe-clipped.

During handling the frogs were gripped firmly by their thighs ensuring that no harm or pain was caused if animals tried to escape. A sterile throat swab was then used to swab all the ventral surfaces, as these are the areas that are most likely infected in adult frogs. The feet, thighs and the belly are of particular importance and the whole swabbing procedure took about 30 seconds per animal, gently swabbing every area five times (Crottini *et al.*, 2014). After swabbing, the individuals were toe-clipped in an attempt to isolate chytrid. The tip of the fourth digit (from the inside) was clipped using surgical scissors submerged in 70% Ethanol and heat sterilised. These tissues were then placed on an agar plate and grouped according to species and locality. All adult individuals were released at the site of capture. The initial agar plate was only used to

keep the samples viable during transit, when cleaning of the tissues in the field was not possible.

Cleaning of the tissue samples consisted of dragging the tissue through agar containing antibiotics with a sterile needle to mechanically remove transient bacteria and other dirt. The tissue was then placed in a 1.5 ml centrifuge tube containing nutrient broth (tryptone, gelatine hydrolosate and lactose) as well as antibiotics (Penicillin G and Streptomycin Sulphate). To prevent any potential loss of samples due to extreme temperature variation, these tubes were then stored at 4°C during the field trip and only incubated at 20°C upon arrival at the lab. In no instance were any of the samples stored at 4°C for longer than eight days.

All tadpoles were euthanised no more than eight hours after being collected. Euthanasia was performed by submersion of tadpoles in MS-222 solution (tricaine methane sulfonate; Kaplan, 1969). This solution causes minimal discomfort and is fast-acting (Arena & Richardson, 1990; Wright & Whitaker, 2001), while not causing the death of chytrid fungi (Webb *et al.*, 2005). The solution was made up to a concentration of 2g/l and the animals were submerged in it for 2-5 minutes. Mouthparts (labial tooth-rows and the keratinized jaw sheaths) of the tadpoles were excised using sterilised surgical scissors. These tissues were then cleaned in the same mechanical fashion as with the toe-clips and placed in the same nutrient broth-tubes. Larger tissue samples were split into several pieces, and stored in separate tubes.

All apparatus exposed to water or frogs were sterilized after sampling was concluded at a site. Sterilization was done by submerging all equipment that had contact with source water in a diluted bleach solution for 20 minutes, after which everything was sundried. This was done as to prevent the possible spread of *Bd* by the researcher.

Upon returning to the laboratory at the NWU Potchefstroom campus, the samples were incubated at 20°C and checked daily. If the media turned cloudy (murky) from fungal or bacterial contamination these samples were discarded in biohazard collection boxes. After approximately seven days the contents of the remaining tubes were emptied into 24-well plates and wells filled with nutrient broth (1% tryptone broth) without antibiotics. Plates were sealed using Parafilm® to prevent unwanted or accidental opening and contamination. The plates were inspected using an inverted light microscope on a daily basis. If the sample contained active growing *Bd*, it was allowed to grow for three to four

more days, until a viable colony was established, at which time the sample was transferred to a culture-flask. When a satisfactory sporangia density was reached, the flasks were transferred to a 4°C fridge for storage and checked weekly for viability. A duplicate of successful isolates were subjected to DNA extraction for genome sequencing. Cultures duplicated for sequencing were grown in 50ml Nalgene Nunc™ culture flasks for a maximum of two weeks at 20°C. These cultures were then moved to 50ml centrifuge tubes, where the spun down pellet was used to extract DNA from using the MasterPure™ Yeast DNA purification kit. Quantification was done using Tapestation™ 2200 and Quibt™2.0 Fluorimeter. The Illumina HiSeq™2000 was used to sequence the DNA using the high output V4 chemistry, resulting in 125+125 base-pair paired-end sequences (O'Hanlon & Fisher, 2017).

The swabs obtained from the animals were analysed using real-time qPCR at the laboratory of Imperial College's Department of Infectious Disease Epidemiology. The DNA extractions were subjected to an ITS-PCR (Boyle *et al.*, 2004), with Bovine Serum Albumin added to prevent PCR inhibition by phenolic compounds (Garland *et al.*, 2010). The system was set to run 60 cycles. This resulted in infection intensity being determined in genomic equivalents (GE) for *Bd* in general. Prevalence was calculated as the percentage of animals positive per site using the ITS-PCR data. The samples that tested positive using the ITS-PCR were then subjected to another real-time qPCR procedure using lineage-specific probes with TaqMan Fast Advanced Master Mix (Applied Biosystems) and only 50 cycles (last two stages being 95°C for 15 seconds and 62°C for 10 minutes), as to determine the lineage present in the swabs taken from adult frogs (Ghosh, 2014). Kolmogorov-Smirnov tests were used to compare the climatic regions in terms of infection intensity, and this was specifically chosen as the data obtained had a non-normal distribution.

2.2.4 Ethics and permits

Collection permits were obtained from the Northern Cape provincial government for Fauna - Non-bioprospecting - Research (Permit number FAUNA 0539/2016). This permit was issued in terms of the provisions of the Northern Cape Nature Conservation Act, 9 of 2009. This project also obtained ethical clearance from the Animal Research Ethics Committee (AREC-130913-015) as part of a larger study project (Larger project

title: Spatial epidemiology and characterization of panzootic amphibian chytrid fungus in Southern Africa. NWU ethics number NWU-00015-16-S5).

2.3 Results

A total of 426 amphibians were sampled during the Orange River surveys. The majority of individuals were adults (266), while 160 tadpoles were also sampled (Table 2-2). These samples represented nine amphibian species from six genera (Table 2-3).

Table 2-2: Prevalence (%) of *Bd* in adult individuals across species per site

Site	Number of adults positive	Prevalence (%)
Springbok	17	58.8 %
Vioolsdrif	0	-
Pella	15	100 %
Onseepkans	7	85.7 %
Kakamas	26	0 %
Upington	66	16.7 %
Groblershoop	51	15.7 %
Douglas	58	25.9 %
Orania	10	10 %
Petrusville	4	0 %
Gariep Dam	0	-
Aliwal North	12	8.3 %
	Total = 266	**Average = 30.7 %**

The qPCR analyses of the swabs obtained from the 266 adults revealed that 67 were positive. *Bd* prevalence at the various sites ranged from completely absent to 100 % infected, although small sample sizes were obtained.

The highest prevalence was seen in Pella, a site in the winter rainfall region, where 100% of adults tested positive for *Bd;* however, only 15 individuals were sampled (Table 2-2).

Table 2-3: Prevalence (%) across sites per species

Species	Number of positive adult individuals per site											Prevalence (%) per species
	Vioolsdrif	Springbok	Pella	Onseepkans	Kakamas	Upington	Groblershoop	Douglas	Orania	Petrusville	Gariep Dam	Aliwal North	
Amietia delalandii		2	15	6		11	8	13		0		1	28.87
Amietia fuscigula									1				12.50
Sclerophrys capensis													0.00
Sclerophrys gutturalis													0.00
Sclerophrys poweri								1					12.50
Tomopterna cryptotis								1					9.09
Vandijkophrynus gariepensis		6											66.67
Vandijkophrynus robinsonii		2											28.57
Xenopus laevis													0.00

Table 2-3 reflected the nine species sampled during the survey, and showed that *Amietia delalandii* were the most abundant, positive species across most sites. Although these individuals were sampled throughout the entire system, including the sites furthest apart, only 28.87 % of the individuals sampled from this species tested positive for *Bd.* Other species that tested positive were localized to single sites in the system, and *Vandijkophrynus gariepensis* also showed a high prevalence of *Bd* but the only six positive individuals sampled originated from Springbok.

Two lineages, *Bd*GPL and *Bd*CAPE were detected in the Orange River system (Figure 2-2). In the majority of cases individuals were only infected with one lineage, but six individuals did test positive for both lineages of *Bd* currently known for South Africa. These individuals were restricted to three sites; four individuals in Upington, one individual in Douglas and one individual in Orania.

Seeing that the total sampling period spanned over a year, the central sites of the river, receiving rain in summer or winter depending on the prevailing climatic conditions, were sampled twice, thus the reason for the higher number of samples at the sites from Kakamas to Douglas.

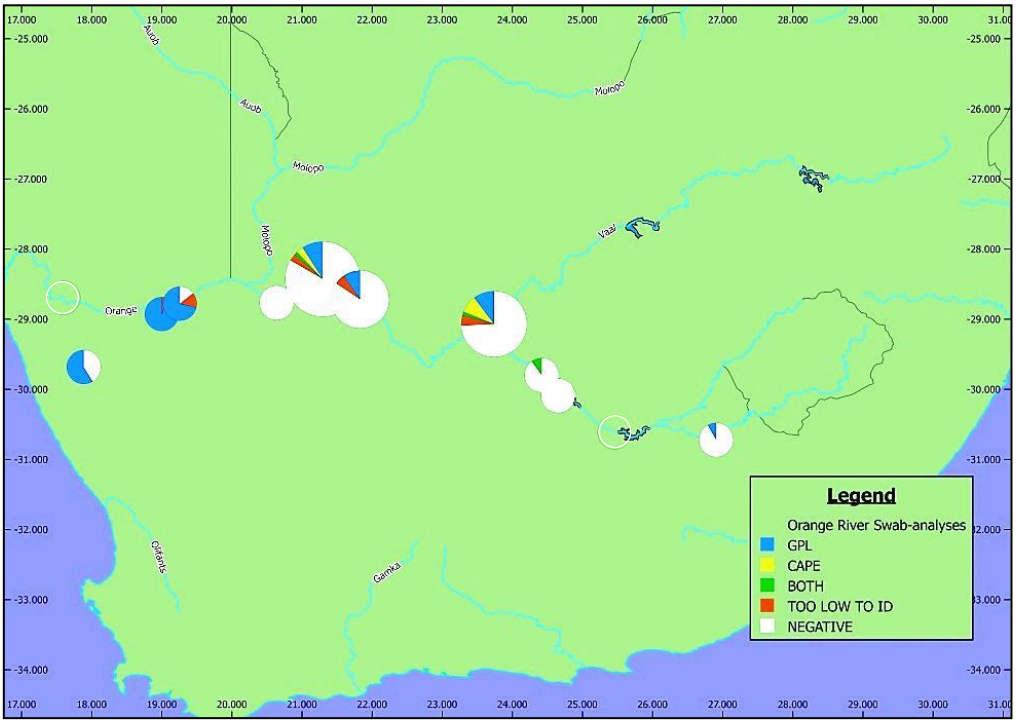

Figure 2-2: GIS map of the Orange River with inserts reflecting the composition of positives per site according to lineage. Relative sizes of pie-charts reflect sample sizes (Empty circles mark sites where no adults were sampled).

Figure 2-2 indicates that *Bd*GPL is in fact found as far east and west as the sampling sites allowed along the river. Although it shows that Aliwal North was infected with *Bd*GPL, there was only one positive individual in a sample of 12 animals that were tested, thus the prevalence at this site was low.

Throughout the entire survey region, *Bd*GPL was the predominant lineage, representing 70% of the total number of positive adults encountered, while 9% were *Bd*CAPE. The lineage for 12% of the positive samples could not be identified (indicated as Too Low to ID in Figure 2-2) due to the decreased sensitivity of the lineage-specific probes and thus the lower potential to detect low-intensity infections (Ghosh, 2014).

The average infection intensity for the entire river system was 8.44±3.24 (GE±SE). However, this varied from site to site and the highest average was detected in Pella with an average genomic equivalent count of 78.93±48.29, and the lowest positive site being Aliwal North with a genomic equivalent count of 0.07±0.07 (only one individual tested positive). The winter rainfall region, which consisted of the sites from Vioolsdrif to Kakamas (four westernmost sites) had an average genomic equivalent count of 39.214±22.914 and the summer rainfall region (eight remaining sites eastwards) had an average of 28.79±12.37 GE±SE. When site data are arranged linearly from West to East a clear decrease in infection intensity is evident (Figure 2-3). A Kolmogorov-Smirnov test of the data showed that the summer and winter rainfall regions had significantly different infection intensities ($p<0.0001$; $\alpha=0.05$; K-S $D=0.5376$) when the samples for the two major climatic conditions were pooled.

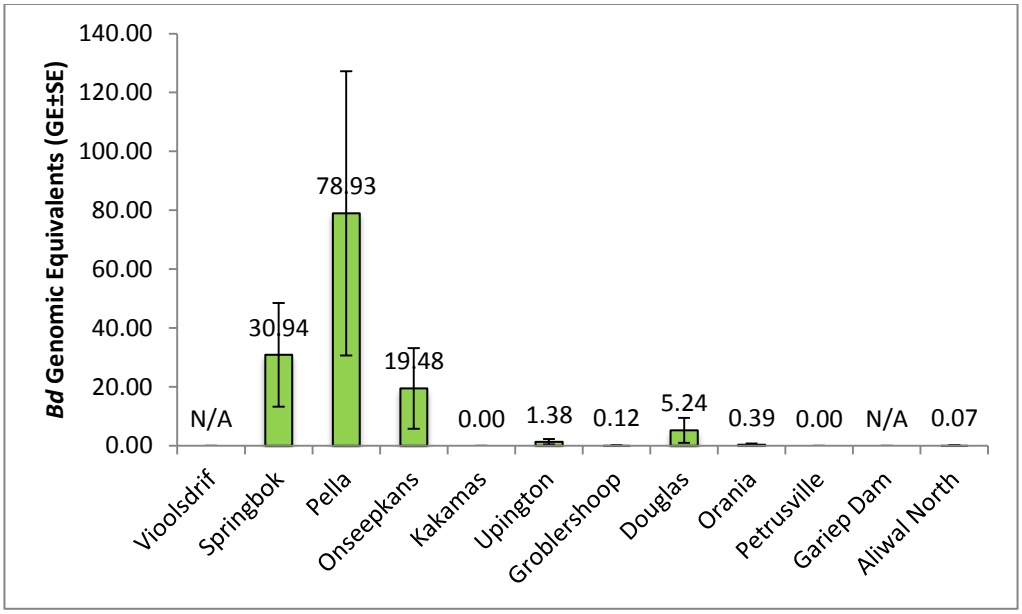

Figure 2-3: Average *Bd* infection intensity (GE±SE) per site from West to Eastern sites.

As seen from Figure 2-2 and Figure 2-3, two from 12 sites were completely negative, but these sites were separated by nearly 1500 km of the river (Kakamas and Petrusville), and thus negative sites are not clustered in a single area. At Gariep Dam no frogs or tadpoles could be found in spite of intensive surveying and at Vioolsdrif only *A. delalandii* tadpoles could be found. Figure 2-4 shows that the prevalence of positive,

adult individuals encountered followed a similar pattern as infection intensity, declining from West to East.

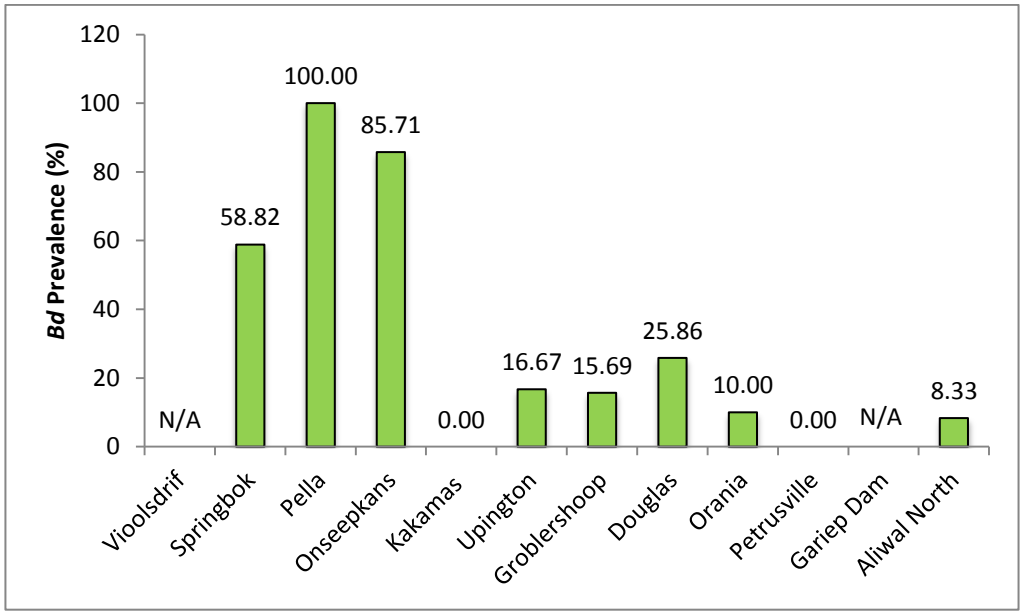

Figure 2-4: Average *Bd* prevalence (%) of adult frogs for the various sample sites ordered from the most Western to the most Eastern location.

Isolation attempts during three separate fieldtrips culminated in 27 cultures from four different sites (27 cultures from 426 attempts; Table 2-4). 18 were obtained from toe-clips taken from adult individuals and nine from the excised mouthparts of tadpoles. Cultures were obtained from four out of the 12 sites, three of them in the western reaches of the river and one from the most eastern site that was surveyed. All cultures were obtained from a single host species, the Common River Frog: *Amietia delalandii*. This species has a prolonged tadpole stage, which could be up to 12 months and tadpoles can grow to large sizes when environmental conditions permit (Du Preez *et al.*, 2009). This was also the one species that was found at 11 out of the 12 sites, with the only exception being Orania, where the related *Amietia fuscigula* was found. Seeing as 195 adult *A. delalandii* were sampled, an isolation success rate of 9.2 % was accomplished. Tadpoles only showed a success rate of 5.6%. Differences in isolation success can be seen within the sites between using tadpole tissue versus toe-clip tissue, although this may be a site-dependent variable.

Table 2-4: Species collected and respective isolation success for adult and tadpole samples

Site	Host species	% Isolation success (adult:tadpole)
Vioolsdrif	*Amietia delalandii*	0 : 15
Springbok	*Amietia delalandii*	0 : 0
	Vandijkophrynus gariepensis	0 : 0
	Vandijkophrynus robinsonii	0 : 0
Pella	*Amietia delalandii*	60 : 17.39
Onseepkans	*Amietia delalandii*	28.57 : 0
Kakamas	*Amietia delalandii*	0 : 0
	Sclerophrys gutturalis	0 : 0
	Sclerophrys poweri	0 : 0
	Tomopterna cryptotis	0 : 0
Upington	*Amietia delalandii*	0 : 0
	Sclerophrys capensis	0 : 0
	Sclerophrys poweri	0 : 0
	Xenopus laevis	0 : 0
Groblershoop	*Amietia delalandii*	0 : 0
	Sclerophrys poweri	0 : 0
Douglas	*Amietia delalandii*	0 : 0
	Sclerophrys capensis	0 : 0
	Sclerophrys gutturalis	0 : 0
	Sclerophrys poweri	0 : 0
	Tomopterna cryptotis	0 : 0
	Vandijkophrynus gariepensis	0 : 0
Orania	*Amietia fuscigula*	0 : 0
	Xenopus laevis	0 : 0
Petrusville	*Amietia delalandii*	0 : 0
	Xenopus laevis	0 : 0
Gariep Dam	No individuals	0 : 0
Aliwal North	*Amietia delalandii*	87.5 : 8.33
	Sclerophrys capensis	0 : 0
	Isolation success:	**58.69 : 13.57**

The 27 cultures obtained during the attempts were reduced to 14 that were accessioned with the African Amphibian Conservation Research Group (AACRG) collection at North-

West University (Table 2-5). When comparing the cultures isolated to the PCR results of these sites (Figure 2-2 vs. Table 2-5) it can be seen that they correspond, meaning *Bd*GPL was isolated where individuals tested positive for *Bd*GPL. There is one exception; sequence data of the culture from Aliwal North indicated that it was a hybrid (O'Hanlon & Fisher, 2017), but no single individual testing positive, by swabbing, showed reactivity to the *Bd*CAPE probe. This mismatch could be due to the fact that not all the frogs from which *Bd* was successfully isolated tested positive with qPCR.

Table 2-5: Details of successful *Bd* cultures

Isolate number	Collection number	Collection Date	Locality	Coordinates	Host species	Lineage
NCJ 137.1	SA-EC 4	Apr-16	Aliwal North, EC	S30.7221; E26.9065	*Amietia delalandii*	GPL
NCJ 138.2	SA-EC 5	Apr-16	Aliwal North, EC	S30.7221; E26.9065	*Amietia delalandii*	HYBRID
NCJ140.2	SA-EC 6	Apr-16	Aliwal North, EC	S30.7221; E26.9065	*Amietia delalandii*	GPL
NCJ 143.2	SA-EC 7	Apr-16	Aliwal North, EC	S30.7221; E26.9065	*Amietia delalandii*	GPL
NC 77.2	SA-NC 1	Aug-2015	Pella, NC	S28.9288; E19.0011	*Amietia delalandii*	GPL
NC 82.1	SA-NC 2	Aug-2015	Pella, NC	S28.9288; E19.0011	*Amietia delalandii*	GPL
NC 91.1	SA-NC 3	Aug-2015	Pella, NC	S28.9288; E19.0011	*Amietia delalandii*	GPL
NC 91	SA-NC 4	Aug-2015	Pella, NC	S28.9288; E19.0011	*Amietia delalandii*	GPL
NC 110	SA-NC 5	Aug-2015	Pella, NC	S28.9288; E19.0011	*Amietia delalandii*	GPL
NC 15.1	SA-NC 6	Aug-2015	Onseepkans, NC	S28.7804; E19.2574	*Amietia delalandii*	GPL
NC 16.1	SA-NC 7	Aug-2015	Onseepkans, NC	S28.7804; E19.2574	*Amietia delalandii*	GPL
NC 67	SA-NC 8	Aug-2015	Vioolsdrif, NC	S28.6960; E17.5974	*Amietia delalandii*	GPL
NC 68	SA-NC 9	Aug-2015	Vioolsdrif, NC	S28.6960; E17.5974	*Amietia delalandii*	GPL
NC 75	SA-NC 10	Aug-2015	Vioolsdrif, NC	S28.6960; E17.5974	*Amietia delalandii*	GPL

2.4 Discussion

Pathogen modelling is dependent on a multitude of factors, but the basis of all models is the understanding of the basic requirements of a pathogen or study organism, in terms of host and environment (Wollan *et al.*, 2008; Rohr *et al.*, 2011). A *Bd* prediction model

for South Africa showed that most of the Orange River has a high probability for it to survive, given its environmental preferences, constructed from actual distribution data in the country (Tarrant *et al.*, 2013). Our prevalence data for *Bd* confirmed the model prediction for the Orange River system. Not only is *Bd* present, but more than one lineage occurs within this system. This is the first confirmed lineage data gained for this wide ranging river system, and identifying two different lineages within the same system simultaneously, is a rarity.

Very little previous knowledge surrounding amphibian chytrid is known for the Orange River, thus this study provides a baseline for the greater part of the river. During this survey, sampling in consecutive years of the river-section between Kakamas and Douglas indicated that not all the host species are detectable in any one survey. This may be due to measured differences in the amount of rain between subsequent years (South African Weather Service, 2016) affecting the behaviour of the species and thus the potential of encountering them. Amphibians were concentrated around waterbodies and the generally arid nature of the survey region allows for only a few species to survive in this area, resulting in nine species being sampled, out of a possible 22 in total for the Orange River (Du Preez *et al*., 2009).

Of the nine species sampled, six were found to be positive, including the semi-aquatic *Amietia delalandii* - the most widely distributed of the species. The frog species are more likely to become infected than the toads, as they are, for the most part, associated with surface water, and hence more readily exposed to waterborne transmission of the fungus. The majority of waterbodies present apart from the river, are man-made waterbodies with permanent water. The concentration of amphibians around the permanent waterbodies increases the risk of pathogen transmission (Kriger & Hero, 2007). The semi-aquatic species formed a larger part of the adult sample size, they were more abundant and of all the individuals of all species sampled, *A. delalandii* formed the larger portion of sampled individuals. The fact remains that the habitats found along the river, consisting of drainage ditches and irrigation dams, are more suitable for the reproduction of river frogs than it is for the reproduction of toad species, due to very few standing puddles, needed for most toads to breed. Because of the arid nature of this region, temporary waterbodies (e.g. that form following thundershowers) are unlikely to last long enough to sustain tadpoles that lack rapid development. These temporary waterbodies may be the ideal habitat for different explosive breeding species.

Isolation success was seen to vary between tadpoles and adult individuals. There is a multitude of factors that may influence this. Tadpoles are dependent on water for survival, while adult individuals have the ability to move between waterbodies, potentially escaping infected sources. The tadpole's dependency on water also causes different microorganisms to grow on their mouthparts, resulting in difficulty when trying to isolate *Bd* from these tissues. When concerned with the isolation success from adults, it is possible to miss the infected tissue when taking a sample. The swabbing process covers the entire ventral surface of the amphibian, while tissue sampling is localised to a single toe tip. This may result in individuals testing positive with swabbing, but not resulting in cultures upon isolation attempts.

When modelling the distribution of *Batrachochytrium dendrobatidis,* as with any other organism, exceptions are bound to occur, which could be overcome by refinement and the increase of specificity of the model for the study area (Ron, 2005; Wollan *et al.*, 2008). Some animals, due to their behavioural adaptations and ecological traits, are easier to sample and may thus provide a larger part of the data collected, as with *A. delalandii* in our study. However, when the swabs from adult individuals were analysed, it was seen that the overall prevalence, not only low overall, also decreased form West to East, showing a significant difference between the winter rainfall region (West) and summer rainfall region (East). Interpreting the prevalence in a system of this size as a whole is challenging as the diversity of the system itself is vast, and the number of samples available differ greatly from one site to another.

Known data indicated that both *Bd*GPL and *Bd*CAPE lineages are known to be present in South Africa (Farrer *et al.*, 2011; Schloegel *et al.*, 2012), and both have been found to also be present along the Orange River. The Tarrant *et al*. (2013) showed that the species used to create the potential distribution model of *Bd* showed an average prevalence of 26.6% and in the entire sample obtained during this study the average prevalence across species was 25.2% (adults only), thus being relatively on par with the previously seen countrywide average. However, the two lineages are not equally prevalent, with almost 70% of infected animals displaying *Bd*GPL. It was seen that in few other locations, Brazil being one of the few (Jenkinson *et al.*, 2016), more than one lineage can be present in a single system and evidence of lineage interaction can be found. If two definite point introductions of the different lineages in the system were the case, or if one was present throughout the system and the second was displacing it,

then the expected distribution would show one area completely positive for one lineage, progressing into an interchange zone where one lineage would give way to another, and finally another homogeneously infected population with the other lineage. That is if total displacement of one lineage by the other is not yet the case. Upon swab analysis it was seen that this was not the case along the Orange River. Here it was seen that the winter rainfall region consisted of *Bd*GPL, but the summer rainfall region showed the presence of *Bd*GPL and *Bd*CAPE, with a significant reduction in the infection intensity. This multi-lineage presence was punctuated by the last eastern site only testing positive for a single lineage, *Bd*GPL (in terms of swab data), again. Thus neither a uniform distribution nor the expected distribution of two homogeneous areas along the river interacting in a small zone where the lineage displacement would be expected, was seen. Significantly, some individuals in the Orange River were infected with both *Bd*GPL and *Bd*CAPE, a situation seldomly seen before in multi-lineage environments. Unfortunately in no instance was an attempt at isolating a culture from the individuals testing positive for both lineages, successful.

The areas where more than one lineage occur may be the result of the introduction of one of the lineages, possibly *Bd*GPL, as *Bd*CAPE is thought to be endemic (Farrer *et al.*, 2011). A multi-lineage environment may, as was seen by Jenkinson *et al*., lead to hybrid lineages, through recombination. These hybrid lineages may then have accentuated characteristics possibly showing them to be more of a danger to the amphibian population (Morgan *et al.*, 2007), as was the case in Brazil (Ghosh & Fisher, 2016; Jenkinson *et al.*, 2016). A hybrid lineage was isolated from the Orange River, more precisely Aliwal North, the easternmost site. Swab data only yielded one positive individual while isolation attempts resulted in a minimum of four cultures, of which three were sequenced and identified as being *Bd*GPL. The fourth showed that it was a hybrid, together with the Brazil hybrid (Jenkinson *et al*., 2016), being the only hybrids known as of yet. Genetic hybrids are possible in areas where more than one lineage is present in the environment and genetic exchange between the present lineages are possible, as postulated by Ghosh and Fisher (2016). Phenotypic variation within the hybrid lineages may be possible as it was seen that known *Bd* lineages can be phenotypically plastic (Lambertini *et al.*, 2016). This phenotypic plasticity, which may be inherited, could then serve the hybrid lineage in its capacity for adaptation or spread, possibly allowing more niches to be occupied.

It is possible that *Bd*GPL is displacing the native *Bd*CAPE in these areas and that in future all individuals will only be infected with the invasive lineage. The second option is that *Bd*GPL is invading a previously negative area, although then a reaction in the host population towards the new pathogen is to be expected. It has been seen that amphibian declines do not occur in Switzerland even though *Bd*CH is present, but if *Bd*GPL came into an area, declines in the host population were indeed seen (Garner, 2016). The presence of more than one lineage, as is the case in the central part of the river, from Douglas to Upington, may pose a threat to the amphibian community as these individuals may be exposed to a new lineage, with different characteristics from the native lineage. The phenotypic differences that are seen within a lineage may affect the host as well, potentially affecting the amount of damage caused to the skin of the host, as an example (Fisher *et al.*, 2009a). Some of these phenotypic plasticity traits increase the potential for host infection (Lambertini *et al.*, 2016), thus a phenotypically plastic, invasive lineage such as *Bd*GPL could have the potential of causing adverse effects on the host population. Whether this is through increased infection potential, or increased infection intensity, is yet to be known.

Overall, the number of total positives for *Bd*GPL amounts to only 46 and these are spread over eight sites. The habitat available to the frogs over much of the Orange River is limited to the corridor around the river and associated man-made habitats. Although the habitat may be limiting, the species found are not generally described as dense population species, even during mating season, with some toad species being the only ones where the males may form choruses (Du Preez *et al.*, 2009). The naturally low abundance of these species, although in close proximity to each other, may thus inherently limit the potential disease-transmission threat. Two sites showed no individuals with infections (Kakamas and Petrusville, Figures: 2-2, 2-3), yet it cannot be confirmed as being negative as the number of individuals sampled were too low to yield a definitive status for the site (DiGiacomo & Koepsell, 1986).

The sections of river that tested positive for both lineages are both higher in altitude and different in rain season (summer rainfall) than the section testing positive for only *Bd*GPL. Approximately a 750 km stretch of the river (Upington to Aliwal North) tested positive for both lineages and Aliwal North showed a hybrid lineage, indicating that interaction between these different lineages have taken place to such an extent that genetic recombination occurred. It could be postulated that the presence of more than

one lineage could be responsible for the decreased infection intensity in the corresponding region due to interlineage competition, but again the sample size limits a definitive deduction.

Displacement of lineages is an ever-present phenomenon, and the characteristics allowing one lineage to displace another are still under scrutiny. The Western Cape Province of South Africa was initially infected with *Bd*CAPE as evidenced by the emergence of this particular lineage coinciding with the introduction of animals originating from the Western Cape to Mallorca, Spain (Walker *et al.*, 2008; Farrer *et al.*, 2011) The cultures isolated from Silver Mine in the Western Cape in 2008 was *Bd*GPL. The introduction of *Bd*GPL may be from several sources as Cape Town is a large port city. Whatever the means of introduction may be, the area is still infected with *Bd*GPL where it was previously believed to have been *Bd*CAPE. The area around the West Coast of South Africa is a highly productive fisheries area and thus Alexander Bay, at the mouth of the Orange River, serves as a port to some of these activities (Ramollo, 2014), and also serves as a possible connection between a known positive area and the Orange River. Sequenced cultures from the Weldon Lab collection shows that the area surrounding the origin of the Orange River (Mont Aux Sources) is positive for *Bd*CAPE and the Tugela River yields positives for this specific lineage well into Kwazulu-Natal. The connection between these two lineages that resulted in the hybrid lineage found at Aliwal North is compounded by the Orange River acting as a natural corridor for the pathogen to spread (Hess, 1994; Van Dyke, 2008).

This same corridor that allowed for the distribution of different lineages of *Bd*, also allows the possibility for the spread of the hybrid lineages created in this system, and seeing as the hybrid lineage is currently in central South Africa, in the largest river system within its borders, the possibility for spread to other systems or areas outside the corridor may also be possible. The spread of a hybrid lineage may be detrimental to the amphibian populations, if the same situation is to follow as was the case in Brazil (Jenkinson *et al.*, 2016). A second scenario is also possible, in that the hybrid lineage could be present in the environment together with the already existing lineages, as is the case with the central section of the river, or potentially outcompeted by the already present lineages of *Bd*, thus resulting in only a temporary occurrence of the hybrid lineage. The effect of more than one lineage on a single host, or on different hosts within the same environment, requires experimental clarification.

Through a survey of most of the Orange River system, it was seen that prediction models that suggested the river is a corridor of suitable habitat for *Bd,* was proven to be correct. Prevalence in the system was low, although a number of different species tested positive. It was possible to isolate the amphibian chytrid present in the system, and in both swab and sequence data it was seen that not only is *Bd* present, but both *Bd*CAPE and *Bd*GPL occurs, sometimes even on the same individual. A large section of the river was seen to be positive for both lineages, larger than a single contact zone is thought to be in a corridor of suitable habitat. This requires future studies to be focussed on the mixed lineage zone, from Upington to Aliwal North as an example, due to the fact that these sites are the most probable to display hybrid genotypes or natural disease displacement dynamics between the lineages. Recent developments of lineage-specific PCR-analyses eliminate the need for isolation and genome sequencing of a culture before the predominant lineage in a region can be determined. It also allows for more effective detection, as isolation success is, on average, very low. Seasonal monitoring and analyses using the lineage-specific PCR is essential to determine the persistent status of amphibian chytrid (and possible hybrid lineages) within the system.

CHAPTER 3: VIRULENCE, COMPETITION AND TRANSMISSION OF GENETICALLY DISTINCT *BATRACHOCHYTRIUM DENDROBATIDIS* LINEAGES

3.1 Introduction

Any organism known for causing disease - whether it be parasitic, bacterial or viral – should be able to infect naïve hosts (Walker *et al.*, 1996) and maintain infection until the pathogen's life-cycle is completed and new hosts become infected. The pathogen would have an fitness advantage, as far as transmission opportunities go, if it is able to infect multiple hosts, as is the case with *Batrachochytrium dendrobatidis* (Fernández-Beaskoetxea *et al.*, 2016). Moreover, pathogens that infect some hosts without adversely affecting them, benefit from these hosts acting as natural reservoirs and vectors (Weldon *et al.*, 2004; Garner *et al.*, 2006; Rödder *et al.*, 2013). *Batrachochytrium dendrobatidis* (hereon referred to as *Bd* or amphibian chytrid) is genetically diverse and adversely affects amphibian populations around the globe, leading to enormous biodiversity loss in the amphibian community (Farrer *et al.*, 2011). The genetically diverse lineages of amphibian chytrid, some of which are endemic to specific areas, or even continents (Farrer *et al.*, 2011; Bataille *et al.*, 2013), display variability in their virulence. The biggest driver of amphibian declines is the Global Panzootic Lineage (*Bd*GPL) that has been associated with a number of enigmatic declines around the globe (Farrer *et al.*, 2011).

Endemic pathogens have the capability of becoming a danger to the endemic hosts due to the anthropogenic impact on the environments creating new opportunities for adaptation through evolution, or allowing for the introduction of novel pathogen lineages (Rachowicz *et al.*, 2005; Fisher *et al.*, 2012). Pathogens that are introduced to new environments and cause drastic declines in the host population, may be the result of the absence of co-evolution between the host population and the pathogen, also termed the Novel Pathogen Hypothesis (Rachowicz *et al.*, 2005). Another possibility is that a local (endemic) pathogen may accentuate its pathogenicity to become adverse to its host population, the Endemic Pathogen Hypothesis (Rachowicz *et al.*, 2005). Both *Bd*CAPE and *Bd*GPL occur in South Africa (Farrer *et al.*, 2011) and thus both the Endemic Pathogen and Novel Pathogen hypotheses can be adapted to represent the introduction of a novel lineage into a new, previously unexposed, habitat and the accentuation of the

endemic lineage to compete with the newly introduced lineage, possibly leading to detrimental effects in the host population. South Africa has a diversity of landscapes and habitat available for both the host (Minter *et al.*, 2004) and the pathogens (Tarrant *et al.*, 2013). This together with the mosaic distribution of *Bd* lineages in South Africa (Weldon *et al.*, unpublished data) allows for interaction between lineages in populations from contact zones between *Bd*GPL and *Bd*CAPE. This possibility illustrates the need to study the complex ecological processes in a multi-lineage environment.

From an epidemiological point of view, an infectious disease needs to be able to infect new hosts in order to spread (Willey *et al.*, 2013), whereas from an ecological point of view it also needs to spread to new hosts to utilize the available habitat to compensate for the potential loss thereof due to the deaths caused by the disease (Begon *et al.*, 2006). This necessitates the opportunity for the pathogen to transmit to a new host and attempt to establish a colony despite the natural fauna already present on these hosts. The capability of diseases to transmit is especially important when concerned with rehabilitation projects, or the potential, sometimes accidental, release of animals into a new environment, as it could have potentially devastating effects on the natural fauna (Bosch *et al.*, 2001; Fisher & Garner, 2007).

Genetic exchange between host populations can either increase or decrease the ability of the hosts to cope with infection. Examples include but are not limited to decreased weight and increased time to metamorphosis (Parris, 2004), reduction in the effectiveness of foraging (Hanlon *et al.*, 2015) or, in theory, challenges the defence mechanisms of the host, such as the production of antimicrobial peptides (Fites *et al.*, 2013; Holden *et al.*, 2015). High genetic flow rates within the host population (*Plantago lanceolata*) may also increase the host's ability to reduce and ultimately lose infection with the fungi *Podosphaera plantaginis* (Jousimo *et al*., 2014). However, it is not just the host population that may use connected habitats to exchange genetic material, as the associated pathogens also travel with these hosts. When more than one lineage of a disease is present within a system, then a number of possible outcomes may be apparent. Firstly, it would not be advantageous for the disease to extirpate their hosts, as the hosts play irrefutably important roles in the disease's lifecycle (Willey *et al.*, 2013), including that of *Bd*. Although the lineages present in South Africa are genetically distinct, the evidence exists that recombination is possible with *Bd*GPL (Jenkinson *et al.*, 2016). *Bd*GPL is also found to be hypervirulent when compared to *Bd*CAPE (Farrer

et al., 2011; Jenkinson *et al.*, 2016). How this hypervirulence relates to host reaction in a setting where both lineages may be present is unknown. Co-evolution of particular lineages with particular host species may serve to increase their genetic diversity as both the hosts and the pathogen/parasite have to adapt to the evolution of the other (Fisher *et al.*, 2012). This opportunity for co-evolution existed in several populations, as was apparent from a number of studies in which *Bd* was found in specimens from the late 1800s and early 1900s (Weldon *et al.*, 2004; Fong *et al.*, 2015; Talley *et al.*, 2015). This together with the recent discovery that interlineage hybridization is possible between *Bd*GPL and *Bd*BRAZIL (Jenkinson *et al.*, 2016), results in the possibility that the pathogen might outperform the host in terms of evolutionary advantage. In South Africa, with *Bd*GPL and *Bd*CAPE occurring in close proximity to each other, the impact of these lineages on the host model needs to be assessed. One host may be exposed to both lineages or different hosts, with different pathogen lineages, may be exposed to each other. The same competition or niche differentiation that can be seen between host populations, may also apply to different pathogen lineages occupying the same host. If the hypervirulent-*Bd*GPL and hypovirulent-*Bd*CAPE hypothesis applies (Farrer *et al.*, 2011), one possible outcome is that a dominance controlled population of the pathogen may be seen on the host (Begon *et al.*, 2006). The same two genetically distinct lineages that are found in South Africa can also be found in Europe. Isolates from both these geographical origins allow for the experimental testing of the effect non-native, yet genetically similar, pathogens have on a native South African host.

This comparison between native lineages of *B. dendrobatidis* and non-native, but genetically similar, lineages will allow for assumptions to be made regarding the evolutionary tolerance towards the pathogen that the native host may have evolved over time (Weldon *et al.*, 2004). The potential for a pathogen to be invasive and the influence it has on the native host compared to native pathogen lineages can also be judged experimentally in terms of infection intensity, while the differences between lineages from a different geographical origins can be tested simultaneously. For the purposes of this study, different lineages from the same geographical area will be compared to each other in terms of competition. The infection of a host with more than one lineage, is a representation of what is possible in the natural habitat of the host, a habitat where more than one lineage of *B. dendrobatidis* is present. Animals possibly subjected to double lineage infections may form part of the amphibian populations on the edges of

disjunctly distributed patches of hypovirulent *Bd*CAPE bordering currently hypervirulent *Bd*GPL-positive areas – forming transition zones.

In an environment such as the Orange River where different pathogen lineages have been found within a single area, it is necessary to assess the transmission potential of these different lineages of *Bd*, and the impact on the hosts. However, in this instance where converging pathogen populations are simulated, two separate individuals will be infected with the disease and housed together to discern some of the transmission potentials of amphibian chytrid in a multiple lineage habitat.

3.2 Material and methods

3.2.1 General: Animal collection and husbandry

Considering the field data gathered, it was seen that *Amietia delalandii* has a high susceptibility to *Bd* infection. This species is semi-aquatic and is mostly found on the banks of standing bodies of water or slow flowing rivers, and is highly mobile with well-developed hindlimbs. Tadpole development can range from nine months to two years, depending on the prevailing conditions (Du Preez *et al*., 2009). These characteristics make this specific species difficult to rear in the lab, especially since they are prone to injury due to a high flight response. The habitat requirement of free standing water also introduces the possibility of dilution of the dosed pathogen and potential bacterial contamination. *Sclerophrys gutturalis*, on the other hand, has a development cycle that can be completed within five weeks, lay large numbers of eggs and breed in warmer months, while *A. delalandii* breeds during colder months. The relative docile and terrestrial nature of *S. gutturalis*, make them the ideal experimental model seeing as the juveniles can be safely reared with minimal potential for injury and can be housed in damp tubs without freestanding water.

The egg-strings of *Sclerophrys gutturalis* were collected at known breeding sites in and around Potchefstroom the morning after breeding pairs were observed in amplexus at the field sites. Three egg-strings were carefully lifted from the ponds with gloved hands and separated so that roughly half of an egg-clutch remained in the pond. Upon arrival at NWU laboratory (AACRG laboratory), the egg-strings were housed in shallow plastic trays that contained a mixture of aged borehole water and filtered water (1:1 ratio) and

supplied with filtered air by means of an air stone. Once tadpoles hatched they were transferred to larger aquaria (30x50x30cm) with the same medium. Approximately 300 tadpoles were placed in each aquarium. Environmental temperature for the entire laboratory was kept at 20°C. The artificial lighting in the laboratory was also cycled on a 12 hour-12 hour day/night cycle. Tadpoles were fed with Tetra Tabimen™ daily (100mg in 100ml H_2O; ~200µl/ tadpole). Cleaning of the aquaria was done by replacing two-thirds of the water every third day and syphoning debris off the bottom. All individuals showing the successful development of all four limbs were removed from the remaining tadpoles and housed in slanted (10°) aquaria of the same size. The slanted aquaria contained only 1 litre of the mixed water, which allowed for a shallow pool to form, while the remainder of the tank formed a terrestrial habitat lined with damp tissue paper substrate and a sterile PVC half-pipe for shelter. After complete reabsorption of the tail, the juveniles were moved and separately housed in 500ml plastic tubs with wetted tissue paper for substrate. Juveniles were fed seven pinhead crickets (recently hatched) every second day, while tubs were sprayed daily with filtered water. Cleaning of the tubs were done every fourth day, at which time the tissue paper was replaced and any debris removed. All animals were rotated on a daily basis (left-to-right, back-to-front, top-to-bottom) to prevent biased compounding effects from the environment within the laboratory.

As a biosecurity precaution during experiments, animals from separate treatments were handled with different pairs of gloves to prevent any unwanted introduction of pathogens. The hygiene protocol followed included the disinfection (using 70% EtOH) of all surfaces that the animals might potentially come into contact with, and the cleaning, rinsing and drying of the tubs that the animals were housed in.

3.2.2 General: Culturing

The isolates used in this experiment were obtained from the AACRG collection at the North-West University. These cultures have been identified, using genome sequencing, as belonging to *Bd*CAPE or *Bd*GPL (Table 3-1). The lineages used and the culturing schedule is specific to each experiment and was thus planned accordingly.

Table 3-1: Isolates used in both experiments

Acronym	Used in	Isolate name	Location (year of isolation)	Host Species	Isolated by
EU GPL (also Expat GPL)	Exp 1	IA 2011	Ibon Acherito, Spain (2011)	*Alytes obstetricans*	Fisher Lab
EU CAPE (also Expat CAPE)	Exp 1	TF5A1	Mallorca, Spain (2005)	*Alytes muletensis*	Fisher Lab
ZA GPL (also local GPL)	Exp 1 & 2	MG 04	Silver Mine, Western Cape, South Africa (2008)	*Amietia fuscigula*	Weldon Lab
ZA CAPE (also local CAPE)	Exp 1 & 2	SA 4C	Greytown, Kwazulu-Natal, South Africa (2010)	*Amietia delalandii*	Weldon Lab

3.2.3 Experimental 1 – Virulence and competition testing

The aim of this experiment is to compare the infection intensity and mortality rate of European lineages against the same lineages from South Africa. Thus for the treatments where disease dynamics in multiple lineages were tested, lineages originating from the same geographical region were used. Both *Bd*GPL and *Bd*CAPE were used in these experiments, focussing on the effect these lineages have on a single host species. This was done due to the virulence differences that can be seen within a single genetic lineage and the potential for recombination (Morgan *et al.*, 2007; Schloegel *et al.*, 2012; Lambertini *et al.*, 2016).

3.2.3.1 Experimental 1: Design

This experiment consisted of 13 treatments of 20 animals each (260 animals in total; Table 3-2) and spanned 31 days. Treatments where a single individual was exposed to four doses of *Bd*CAPE followed by four doses of *Bd*GPL, and vice versa, were incorporated to determine the disease dynamics if a single individual is exposed to both lineages. Every individual was therefore exposed to eight doses of *Bd*. The geographic origin of the lineages used, will be accounted for by comparing *Bd*CAPE originating from Europe with *Bd*GPL also originating from Europe, and the same for the two lineages found in South Africa. The negative control group was exposed to eight doses of culture media not containing any viable cells. The zoospore concentration of the doses ranged from 10 000 to 50 000 zoospores per millilitre. Animals were randomly allocated to their respective treatments. Initial body weights (0.001g accuracy) were

also taken before any dosing started, and were taken again after completion of the experiment following euthanasia.

Table 3-2: Experiment 1 design

	Treatment	No. toads	Dose 1	Dose 2	Dose 3	Dose 4	Dose 5	Dose 6	Dose 7	Dose 8
Expat lineage Treatments	1	20	EU GPL	EU GPL	EU GPL	EU GPL	Sham	Sham	Sham	Sham
	2	20	EU GPL	EU GPL	EU GPL	EU GPL	EU GPL	EU GPL	EU GPL	EU GPL
	3	20	EU GPL	EU GPL	EU GPL	EU GPL	EU CAPE	EU CAPE	EU CAPE	EU CAPE
	4	20	EU CAPE	EU CAPE	EU CAPE	EU CAPE	Sham	Sham	Sham	Sham
	5	20	EU CAPE	EU CAPE	EU CAPE	EU CAPE	EU CAPE	EU CAPE	EU CAPE	EU CAPE
	6	20	EU CAPE	EU CAPE	EU CAPE	EU CAPE	EU GPL	EU GPL	EU GPL	EU GPL
Local Lineage Treatments	7	20	ZA GPL	ZA GPL	ZA GPL	ZA GPL	Sham	Sham	Sham	Sham
	8	20	ZA GPL	ZA GPL	ZA GPL	ZA GPL	ZA GPL	ZA GPL	ZA GPL	ZA GPL
	9	20	ZA GPL	ZA GPL	ZA GPL	ZA GPL	ZA CAPE	ZA CAPE	ZA CAPE	ZA CAPE
	10	20	ZA CAPE	ZA CAPE	ZA CAPE	ZA CAPE	Sham	Sham	Sham	Sham
	11	20	ZA CAPE	ZA CAPE	ZA CAPE	ZA CAPE	ZA CAPE	ZA CAPE	ZA CAPE	ZA CAPE
	12	20	ZA CAPE	ZA CAPE	ZA CAPE	ZA CAPE	ZA GPL	ZA GPL	ZA GPL	ZA GPL
	13	20	Sham	Sham	Sham	Sham	Sham	Sham	Sham	Sham

Data recording for this experiment included the beginning and end weights, time till the onset of symptoms (also termed time till mortality), and thus the humane endpoint, and the infection intensities determined after euthanasia by means of ITS-PCR. The time till mortality is analysed using Kaplan-Meyer curves, while the infection intensities of the different treatments is compared using Kolmogorov-Smirnov tests (Confidence set at 95%) using Graphpad PRISM 6.

3.2.4 Experimental 2 – Transmission capability of South African lineages

The premise of potential for transmission between individuals was tested under different scenarios. Firstly, the transmission capability of both ZA GPL and ZA CAPE to a naïve animal was tested, in separate treatments. The second scenario was designed to represent the situation that might occur in a contact-zone, thus where two animals infected with different lineages of *Bd* occupy the same habitat.

3.2.4.1 Experiment 2: Design

This experiment consisted of four different treatments, containing 40 animals each in 20 replicates, resulting in 160 animals (Table 3-3). The experimental design required 40 animals to be infected with *Bd*GPL, the same number of animals for *Bd*CAPE, and 80 animals receiving sham doses. This dosing was done before any co-housing commenced. The animals were photographed before the first dosage of *Bd*, in order to be able to identify the infected animals using unique dorsal skin patterns. The animals were weighed before any dosing commenced (0.001g accuracy) and randomly allocated to treatments. The zoospore concentration of the doses ranged from 110 000 to 145 000 zoospores per millilitre. Every animal received four doses over five days (two doses, one rest day, two doses), followed by a two-day resting period, after which they were paired up with their respective partners on the 8th day and housed in a single enclosure for 14 days. The feeding regime was kept constant during the entire period (seven crickets per individual every second day).

Table 3-3: Experiment 2 design

Treatment	**No. toads**	**4 doses**		**No. toads**	**4 doses**
1	20	ZA CAPE	co-housed with	20	Sham
2	17	ZA GPL	co-housed with	17	Sham
3	19	ZA GPL	co-housed with	19	ZA CAPE
4	19	Sham	co-housed with	19	Sham

The variation in the number of toads (per implication the number of replicates) used per treatment was caused by animals becoming symptomatic and requiring euthanasia during dosing, before any co-housing commenced. The reduction in the number of control animals was caused by the mortality of one individual due to unforeseen circumstances.

All animals that developed symptoms of chytridiomycosis were euthanized at the set humane endpoint indicated by the loss of righting reflex or the inability to feed properly. At that point the co-housed individual was also euthanized regardless of its health status. These animals were identified using the photographs taken before the experiment then fixed in 70% EtOH. Photographic identification could be done due to the individuals retaining their dorsal skin pattern for the duration of the experiment. Thus after euthanasia, the dorsal surface of every individual was compared to the two high resolution recorded photos of the pair to confirm identification.

3.2.5 General: Dosing and diagnosis of the animals

Dosing of the animals were done in petri-dishes. Each petri-dish contained 20ml of aged borehole water and was allocated to a single animal. The animals were placed in these petri-dishes directly before dosing. Cultures were prepared by determining the number of active zoospores in each culture on the day of dosing, using a haemocytometer (dilution factor of 10 000). All cultures were then diluted to the concentration of the culture with the lowest zoospore count with sterile culture media. After preparing isolates for dosing, 500µl were pipetted onto the animal in the petri-dish. The animals were left in the petri-dishes for three hours after which they were returned to their individual enclosures (Protocol adapted from Garner *et al.*, 2009; Luquet *et al.*, 2012). The health of all animals were evaluated daily for any signs of distress. If any individual showed the loss of righting reflex or ability to feed (classified as *symptomatic*), the individual was euthanized and fixed in 70% ethanol. In Experiment 2 the animal that did not show any symptoms were termed *asymptomatic*. Euthanasia was performed by submersion of the individual in MS-222 solution (Kaplan, 1969), as this method does not negatively influence the chytrid fungi infection (Webb *et al*., 2005) and is rapid with minimum discomfort (Arena & Richardson, 1990; Wright & Whitaker, 2001).

DNA extractions were done using tissue obtained from the left leg of the euthanized animals, using Prepman Ultra (Applied Biosystems) and the RACE protocol (Adapted from Boyle *et al.*, 2004). Quantitative polymerase chain reaction (qPCR) testing, with Bovine Serum Albumin (Garland *et al.*, 2010), lineage-specific primers as well as a cycle setup of 50-cycles (last two stages being 95°C for 15 seconds and 62°C for 10 minutes: Ghosh, 2014), was used to determine the pathogen load of the animal at euthanasia. This specific testing was used to determine the precise lineage of the dominating pathogen in the competition treatments. Infection intensity results of the lineage-specific qPCR were compared using Kolmogorov-Smirnov tests (confidence set at 95%) in the statistical software Graphpad PRISM 6.

3.2.6 General: Ethics and permits

These experiments were approved by the Animal Research Ethics Committee (AREC-130913-015) of the North-West University (Ethics number: NWU-00015-16-S5). All egg-string collection was done under the collection permit for the North-West province of South Africa (Permit number: HQ 18/05/16-088 NW; issued by Department: Rural, Environmental, and Agricultural Development).

3.3 Results

3.3.1 Virulence and competition between lineages

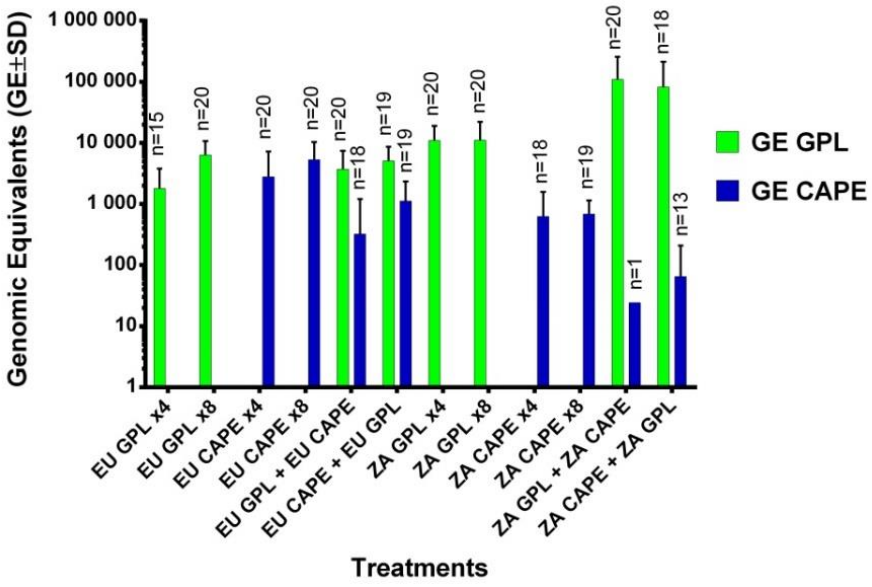

Figure 3-1: The infection intensities of *Batrachochytrium dendrobatidis* lineages (CAPE and GPL) each from South Africa (ZA) and Europe (EU).

Figure 3-1 shows that all treatments did indeed have infected individuals, but with varying mean infection intensities. In the treatments where animals were exposed to more than one lineage, both expat lineages managed to establish an infection on the animals independent of which lineage was first. Whereas with local lineages only one individual tested positive for both lineages when *Bd*GPL was the first to establish an infection; and 13 animals maintained their initial infection in the CAPE-first treatment, while a total of 18 animals tested positive for local-GPL from the same treatment. Incidentally the infection intensity of local-GPL in the competition treatments, were ten times higher compared to the normal local-GPL 4-dose treatment. The only difference between the treatments being that in the competition treatments, local-GPL would compete with local-CAPE, either after having established an infection or being introduced to an animal with an established infection of local-CAPE.

3.3.1.1 Effect of dose number on host survival

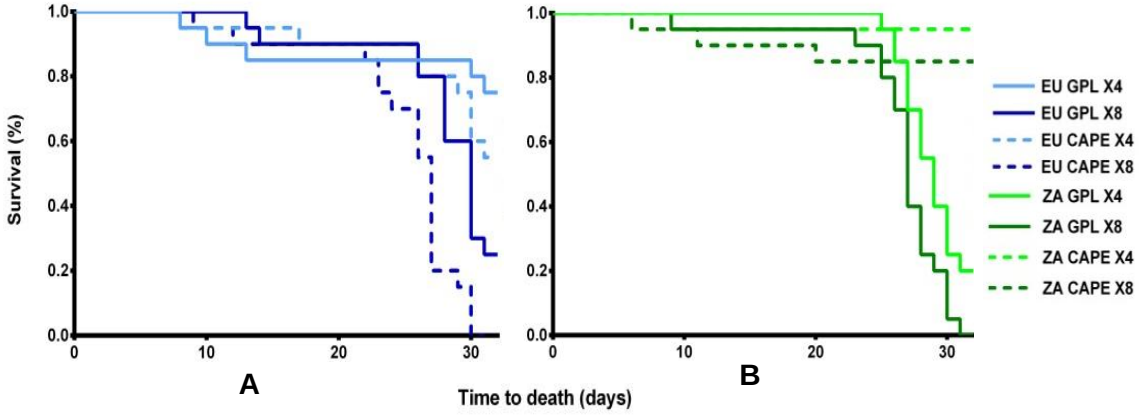

Figure 3-2: Kaplan-Meyer survival curves of the single lineage treatments (EU = expat-**A**, ZA = local-**B**)

The two treatments that had the highest mortality rate were the treatments that received eight doses of expat-CAPE (Figure 3-2 A) and local-GPL (Figure 3-2 B), respectively. These two treatments did not have any live animals left on the last day of the experiment. All treatments showed a decline over time in the number of individuals remaining asymptomatic except for the Control group. Treatment 4 (EU CAPE X8) had a mean infection intensity of 5296.28±5002.1 GE±SD (Figure 3-1). This was the second highest mean infection intensity for the expat lineages. The treatment with the highest infection intensity, from the single expat genotype exposure treatments, being treatment 2 (EU GPL X8) with 6340.61±4379.96 GE±SD (Figure 3-1). A comparison of infection intensity between the animals surviving until the end of the experiment and those that did not demonstrated that none of the single lineage exposures showed a significant difference except for the ZA CAPE X8 ($p=0.0018$; K-S $D=1.000$). The total number of diseased animals that reached the humane endpoints and had to be euthanized was 144 of 260. A total of 58 animals survived from the local lineage treatment groups, 38 from the expat lineage treatment groups, as well as the control group of 20.

Animals in the expat GPL treatments had a significant difference in the rate of mortality according to dose, showing that the eight-dose treatment had a significantly higher mortality rate than the four-dose treatment ($p=0.0135$; K-S $D=0.5000$). The expat-CAPE treatments also showed a significant difference with a p-value of 0.0015, with no animals surviving in the eight-dose treatment to day 30, compared to the four-dose that had 12 survivors by the end of the experiment.

When comparing the mortality rate of the different lineages originating from Europe, it was seen that for the four-dose treatment expat-GPL and expat-CAPE did not show a significant difference in the mortality rate ($p=0.8186$; K-S $D=0.2000$) and no significant difference in infection intensity was seen either ($p=0.7391$; K-S $D=0.2333$). However, the eight-dose treatments of these lineages did have a significant difference in survival ($p=0.0015$; K-S $D=0.6000$), with expat-CAPE requiring far less time to be lethal for the host than expat-GPL. However, no significant difference in infection intensities were seen for the eight-dose treatments of the expat lineages ($p=0.5596$; K-S $D=0.2500$).

No dosage-dependent difference in survival rate could be seen for either of the local lineages (local-GPL: $p=0.3291$, K-S $D=0.3000$; local-CAPE: $p>0.9999$, K-S $D=0.09737$). There was a significant difference in the infection intensity of local-CAPE between the four-dose and the eight-dose treatments ($p=0.0176$, K-S $D=0.5058$; Mean infection intensity 4-dose = 621.21±952.63 GE±SD (Figure 3-1), Mean infection intensity 8-dose = 682.66±461.93 GE±SD). Both the four-dose and eight-dose comparisons of local-GPL vs. local-CAPE yielded a significant difference in the rate of mortality of the individuals ($p=0.0002$, K-S $D=0.0002$ for four-dose; $p<0.0001$, K-S $D=0.8000$ for eight-dose), with local-GPL always requiring less time than its local-CAPE counterpart to be lethal for the individuals in question. The infection intensities of these treatments also showed a significant difference (local-GPL vs. local-CAPE four-dose: $p<0.0001$, K-S $D=0.8944$; local-GPL vs. local-CAPE eight-dose: $p<0.0001$, K-S $D=0.9000$) with local-GPL having very high mean genomic equivalent counts in all cases (ZA GPL X4 = 10978.4±7946.43 GE±SD and 11042.4±11069.4 GE±SD in the ZA GPL X8 versus ZA CAPE X4 mean at 621.21±952.63 GE±SD and 682.66±461.93 GE±SD in ZA CAPE X8; Figure 3-1).

3.3.1.2 Effect of lineage and geographical origin on host survival

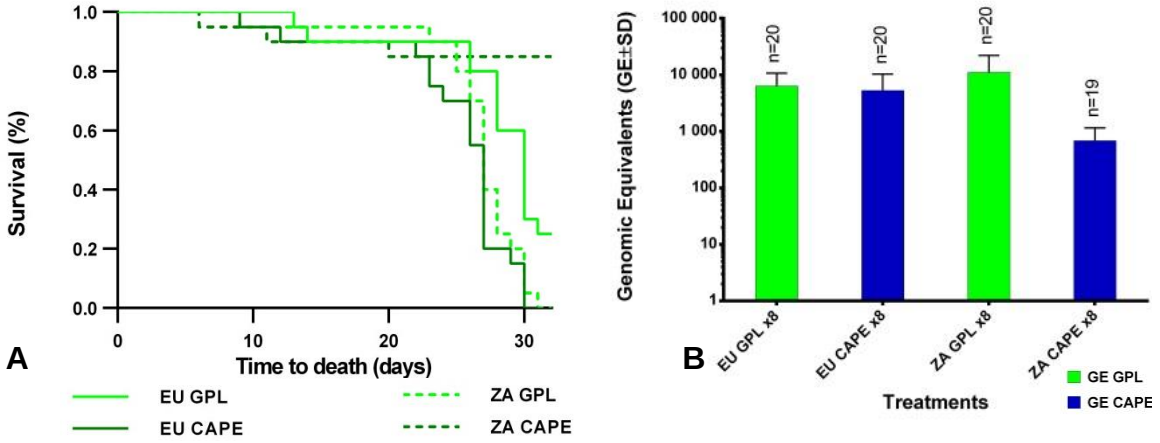

Figure 3-3: The Kaplan-Meyer survival curve (**A**) and infection intensities (**B**) of the eight-dose treatments

A significant difference in the mortality rate existed between the expat and local four-dose *Bd*GPLs, with the expat isolates being more virulent (p=0.0047, K-S D=0.5500). The EU GPL X4 vs. ZA GPL X4 treatments also showed a significant difference in infection intensities with a p-value of 0.0002 (K-S D=0.7333), local-GPL having the higher mean GE count (EU GPL X4 = 1789.40±1997.13 GE±SD, ZA GPL X4= 10978.4±7946.43 GE±SD). The 8-dose treatments of the *Bd*GPL comparison did not show the same difference in mortality rate (Figure 3-3 A; p=0.0815, K-S D=0.4000) and the infection intensity also did not show a significant difference (p=0.3291, K-S D=0.3000; EU GPL X8= 6340.61±4379.96 GE±SD and ZA GPL X8= 11042.4±11069.4 GE±SD). The comparison of the four-dose *Bd*CAPE treatments did not show a significant difference in time to mortality (p=0.1903, K-S D=0.3474) but the eight-dose did (p<0.0001, K-S D=0.8500) with the expat isolate being significantly more deadly. However, in both the 4-dose and 8-dose treatments, there was a significant difference between the infection intensity of expat-CAPE and local-CAPE (p=0.0028, K-S D=0.5889; p<0.0001, K-S D=0.7500, respectively). Expat-CAPE had a mean infection intensity of 5286.28±5002.10 GE±SD for the eight-dose treatment (2772.48±4437.71 GE±SD for four-dose) and local-CAPE had a mean infection intensity of 682.66±461.93 GE±SD for the eight-dose treatment (621.21±952.63 GE±SD for 4-dose).

3.3.1.3 Effect of pathogen competition on host survival

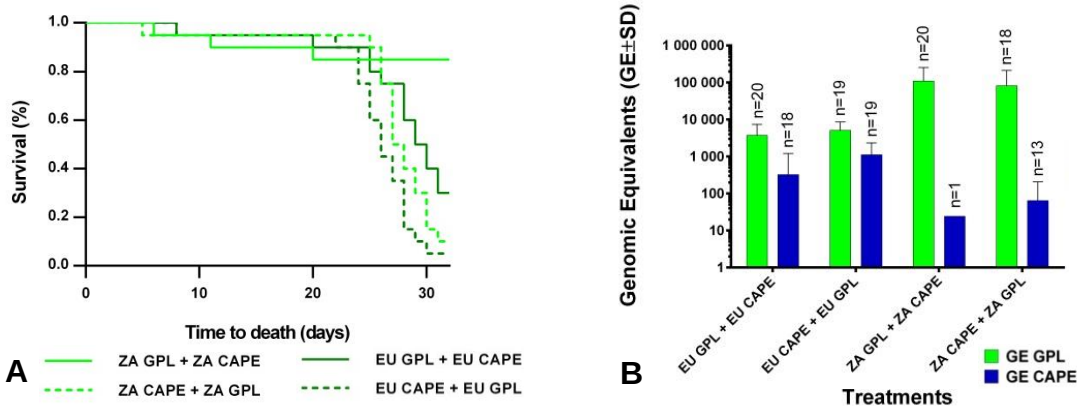

Figure 3-4: Kaplan-Meyer survival curves (**A**) and infection intensities (**B**) of lineage competition treatments.

The expat isolates showed a greater inclination towards co-infection with 90% and 95% becoming infected with both lineages for multiple lineage treatment groups. The mortality rate in the expat multiple lineage treatments showed a significant difference in the mortality rate of individuals between the different competition treatments (Figure 3-4 A; p=0.0348, K-S D=0.4500).

When comparing the infection intensities of expat-GPL on the animals at the end of the experimental period, it was seen that for the expat mixed lineage infections no significant difference (p=0.2265, K-S D=0.3342) could be seen between the treatment that received *Bd*GPL first and the treatment that received *Bd*CAPE first. However, *Bd*CAPE infection intensity was significantly higher (p=0.0052, K-S D=0.5673) in animals that received this lineage first (1121±1202 GE±SD) as opposed to animals that received *Bd*CAPE secondarily (322.7±885.6 GE±SD).

In the local isolate treatments, only 5% of the animals in the treatment that received *Bd*GPL first tested positive for both lineages and in the treatment that received *Bd*CAPE first, a total of 65%. Seeing as there is a vast difference in the successful co-infections, it is reasonable that the infections intensities of the *Bd*CAPE isolate originating from South Africa would differ significantly between these treatments, seeing as it was the lineage least often found in co-infections and a single co-infection in Treatment 11 can not be statistically compared to 13 in Treatment 12. Treatment 11 had a mean infection intensity of 24.07±0.000 GE±SD for *Bd*CAPE and Treatment 12 64.584±143.962

GE±SD. That having been said, it was seen that there was no significant difference in local-GPL infection intensities between the animals in the respective competition treatments (Figure 3-4 B; p=0.2110, K-S D=0.3444), with a mean infection of 109160±146599 GE±SD and 81853±130614 GE±SD, respectively.

When comparing the infection intensity of expat-GPL with the infection intensity of local-GPL in treatments 5 and 11, it was seen that they differed significantly with the local-GPL having much higher average infections (p<0.0001, K-S D=0.8500). The same significant difference was seen comparing the *Bd*GPL infection intensities of treatments 6 and 12 (p=0.0059, K-S D=0.5614). Comparing the *Bd*CAPE infection intensities could not be done due to the fact that so few animals showed a co-infection in the local isolates of the lineages.

No significant difference was seen in any of the treatments between the initial and the end weights upon termination of the experiment. The weights of the symptomatic animals also did not show any significant difference compared to those that remained asymptomatic until the experiment was terminated.

3.3.2 Transmission capability of South African lineages

This experiment was designed to simulate those conditions in nature when naïve individuals become exposed to infected individuals, or alternatively, two individuals infected with different genotypes of *Bd* become exposed to each other.

Table 3-4: Transmission capability of South African *Bd*GPL (#a = *Bd*GPL carriers of infection; #b = potential recipients of infection). Symptomatic individuals reached humane endpoints and required euthanasia.

Specimen #	Survival Time (days)	Symptomatic (1), Asymptomatic (0)	Genomic Equivalents
1a	6	1	52582.03
1b	6	0	91.10
2a	6	1	65894.84
2b	6	0	
3a	7	1	37534.39
3b	7	0	

4a	7	1	22290.59
4b	7	0	53.23
5a	7	1	162234.50
5b	7	0	
6a	8	1	22985.42
6b	8	0	423.73
7a	8	1	11632.60
7b	8	0	
8a	8	1	103417.2
8b	8	0	153.97
9a	9	1	36170.73
9b	9	0	76.66
10a	9	1	91309.33
10b	9	0	
11a	11	1	21993.35
11b	11	0	155.81
12a	11	1	11389.05
12b	11	0	
13a	12	1	24797.52
13b	12	0	943.06
14a	12	1	44951.71
14b	12	0	330.43
15a	13	1	9812.13
15b	13	0	369.37
16a	13	1	5219.75
16b	13	0	96.94
17a	13	1	165999.1
17b	13	0	138.70

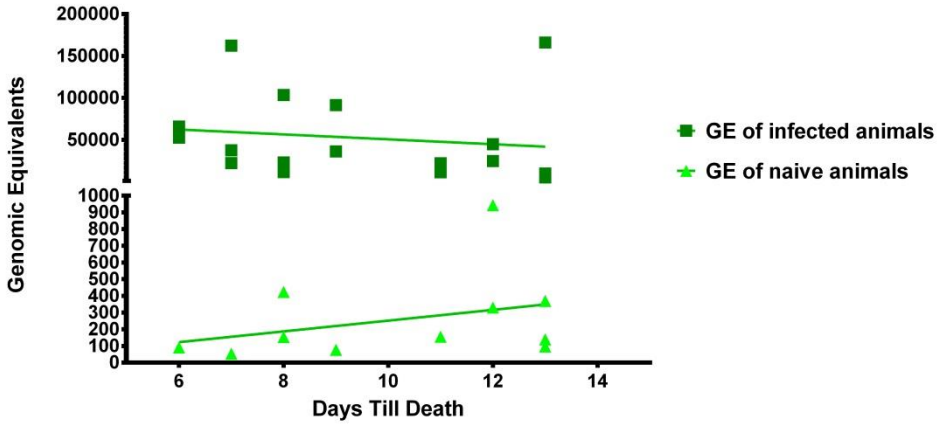

Figure 3-5: Scatter plot of the infection intensity correlated with time till displaying disease symptoms (humane endpoint).

In the *Bd*GPL transmission group none of the 17 pairs were alive until the anticipated end of the co-housing period. Invariably, the carriers were always the individuals becoming symptomatic and requiring euthanasia, resulting in the simultaneous euthanasia of the recipient animal (Table 3-4). 64.7% of the recipients developed an infection with *Bd*GPL. A significant difference ($p<0.0001$; K-S $D=1.000$) was seen between the carriers' and the recipient animals' infection intensities, recipient animals having had an infection intensity mean of 257.5±260.5 GE±SD as opposed to 52366±50404 GE±SD in the carriers (Figure 3-5).

Table 3-5: Transmission capability of South African *Bd*CAPE (#a = *Bd*CAPE carriers of infection; #b = potential recipients of infection). Symptomatic individuals reached humane endpoints and required euthanasia.

Specimen #	Survival Time (days)	Symptomatic (1), Asymptomatic (0)	Genomic Equivalents
1a	14	0	1294.16
1b	14	0	7.98
2a	14	0	927.40
2b	14	0	23.58
3a	14	0	914.93
3b	14	0	39.52
4a	14	0	843.22
4b	14	0	159.40

5a	14	0	911.92
5b	14	0	93.51
6a	14	0	2866.46
6b	14	0	23.94
7a	14	0	755.93
7b	14	0	9.67
8a	14	0	4965.145
8b	14	0	42.35
9a	14	0	491.58
9b	14	0	
10a	14	0	452.74
10b	14	0	
11a	14	0	645.98
11b	14	0	54.29
12a	14	0	335.50
12b	14	0	26.01
13a	14	0	223.49
13b	14	0	23.42
14a	14	0	316.3646
14b	14	0	2.08
15a	14	0	195.43
15b	14	0	13.10
16a	14	0	1029.305
16b	14	0	52.53
17a	14	0	9.999256
17b	14	0	625.75
18a	14	0	1395.64
18b	14	0	2.43
19a	14	0	178.95
19b	14	0	11.58
20a	14	0	1134.88
20b	14	0	

In the treatment where a recipient was co-housed with a *Bd*CAPE carrier, 85% of the recipients developed a *Bd*CAPE infection and all individuals, carriers and recipients, remained asymptomatic until the end of the experiment (Table 3-5). The infection intensity of recipients was significantly lower than those of *Bd*CAPE carriers, with a mean infection intensity of 994.5±1126 GE±SD for carriers and 71.24±147.2 GE±SD for recipients ($p<0.0001$, K-S $D=0.8912$).

Table 3-6: Inter-lineage competitive transmission of lineages originating from South Africa (#a=*Bd*GPL - carriers of infection; #b – *Bd*CAPE carriers of infection)

Specimen #	Survival Time (days)	Symptomatic (1), Asymptomatic (0)	*Bd*CAPE Genomic Equivalents	*Bd*GPL Genomic Equivalents
1a	6	1		13017.49
1b	6	0	720.69	
2a	6	1		9270.21
2b	6	0	454.29	
3a	7	1		204288.8
3b	7	0	25.57	2062.8
4a	7	1		7189.871
4b	7	0	347.03	
5a	8	1		19443.15
5b	8	0	357.62	
6a	8	1		8459.003
6b	8	0	305.21	
7a	8	1		4099.882
7b	8	0	756.00	
8a	8	1		23290.61
8b	8	0	352.14	
9a	9	1		20559.63
9b	9	0	535.85	
10a	9	1		239211.2
10b	9	0	1024.32	
11a	9	1		265177.1
11b	9	0	612.79	

12a	9	1		72339.84
12b	9	0	369.50	
13a	9	1		4396.853
13b	9	0	1170.69	
14a	10	1		37685.95
14b	10	0	572.22	
15a	11	1		103976.4
15b	11	0	1537.72	
16a	12	1		21727.56
16b	12	0	313.68	
17a	12	1		48472.74
17b	12	0	287.82	28.61009
18a	14	0		26784.34
18b	14	0	509.93	28.61817
19a	14	0		36733.61
19b	14	0	935.60	

In the treatment that paired the *Bd*CAPE carriers with *Bd*GPL carriers, only three of the animals that were *Bd*CAPE carriers developed an infection with *Bd*GPL, while retaining their initial *Bd*CAPE infection. None of the *Bd*GPL carriers developed a subsequent infection with *Bd*CAPE. Only two pairs of the animals in this treatment remained asymptomatic for the full-time span of the experiment (Table 3-6). There was, however, a significant difference in the infection intensities of initial *Bd*CAPE carriers and initial *Bd*GPL carriers ($p<0.0001$, K-S $D=1.000$). *Bd*CAPE carriers had a mean infection intensity of 588.9±365.3 GE±SD (for *Bd*CAPE) and *Bd*GPL 61375±82265 GE±SD (for *Bd*GPL). The *Bd*GPL infection established in the three *Bd*CAPE carriers also differed significantly from the *Bd*GPL carriers' infection intensities, with a mean infection intensity of 706.7±1174 GE±SD ($p=0.0006$, K-S $D=1.000$).

3.4 Discussion

Chytridiomycosis is one of those diseases often found in both major die-off events and in seemingly healthy populations (Farrer *et al.*, 2011). It has been postulated that it may be due to certain factors that are habitat-specific, thus environmental factors (Walker *et al.*, 2010), which could either be limiting to the disease or providing the hosts with a greater capacity to co-exist with the disease. *Bd*GPL has been denoted as the causative agent for a number of mass mortalities and extinctions of amphibians around the globe, even in communities that had an endemic, stable amphibian chytrid infection with another (possibly endemic) lineage (Farrer *et al.*, 2011). Thus *Bd*GPL has been described as being hypervirulent when compared to some endemic lineages (Farrer *et al.*, 2011; Jenkinson *et al.*, 2016; Lambertini *et al.*, 2016).

Where any disease is concerned two things are always of the utmost importance, viz. the capability of the disease to spread, and its possible impact on the survival of a population. To understand the potential impact a disease can have in a natural environment often requires the use of experiments. These experiments aid in the prediction of the outcome a disease may have in a natural environment, should the simulated condition in the laboratory study be representative of a feasible occurrence in nature. We simulated a number of variables that are possible in nature, for example, magnitude of exposure (dosage number) and multiple lineage presence, as well as the impact a genetically similar genotype originating elsewhere (invasive) may have compared to native genotypes.

Although it is known that antifungal bacteria present on amphibian skin can inhibit the growth of *Bd* (Lam *et al.*, 2010; Park *et al.*, 2014; Yasumiba *et al.*, 2016), it is not known whether these bacteria, or any bacteria having similar properties, are present on *S. gutturalis* reared in captivity. Seeing as all animals are reared together with the egg-string material initially present, it is reasonable to assume that some microorganisms may be present on the host's skin, although not tested as a variable in this experiment. The presence of these bacteria and a persistent infection with *Bd* thus follows the pattern of secondary succession, where a new stable community may be reached within the microfauna on the frog skin itself (Begon *et al.*, 2006).

3.4.1 Virulence and competition among amphibian chytrid lineages

The experiments were designed to give insight into the possible situations that may result if a foreign pathogen is introduced into a new population of amphibians. There are a number of possible scenarios that may be played out. The one focused on in this instance, is whether the geographical origin of the pathogen has a significant influence on the host's susceptibility to it. The most basic influence any pathogen has on a natural population of hosts is to adversely impact their lifespan, and per implication their fecundity. It was seen from the comparisons of the time till mortality of the animals, that there were diverse results, with either no significant difference or the expat lineages taking less time to be lethal than the local counterparts. Thus, from the perspective of a potentially invasive lineage, it was shown that a genotype similar to the endemic lineages in South Africa, but originating from another continent, may be devastating to South African hosts.

The number of doses in the experiments only showed a significant difference in infection intensities in the expat-GPL and local-CAPE treatments with higher number dose treatments always displaying a higher infection intensity. Although dose is not controlled in the natural environment or uniformly dispersed within a population, it was apparent that even a low number of doses resulted in infections in the host. It was interesting that although the different lineages all lead to successful infections in most part, the expat lineages did not show a significant difference in the infection intensities (EU GPL X4 vs. EU CAPE X4, etc.), while local lineages did, with *Bd*CAPE displaying lower infection intensity compared to *Bd*GPL. This could be an indicator that local hosts may be more resistant to the local-CAPE and thus some evidence for *Bd*CAPE being endemic to South Africa is provided, and could explain why native hosts seldom appear symptomatic in nature (Tarrant *et al.*, 2013).

The mixed lineage treatments are representative of a novel pathogen being introduced into a population that has an established infection with a different genotype, such as was the case in Brazil (Jenkinson *et al.*, 2016). The expat lineages showed a high probability of animals becoming infected with both genotypes during our experiment and at least 90% of the animals tested positive for both lineages. If an infection with both expat-GPL and expat-CAPE were to persist over a longer time-span than the experiment allowed, remains to be answered. Where expat *Bd*GPL was present before expat *Bd*CAPE, the *Bd*CAPE expat genotype did not manage to establish the same

infection intensities as when EU CAPE settled first. This difference was especially prominent in the local lineage treatments as where local *Bd*GPL established first, only a single individual became infected with local *Bd*CAPE. Where the local *Bd*CAPE established an infection first, local *Bd*GPL managed to establish infections to a relative high degree, in comparison to the first simulation. The expat *Bd*GPL infection intensities did not differ significantly between the different competition treatments. This absence of compensation for the presence of another expat lineage by means of increased infection intensity supports the notion that *Bd*GPL may be a more aggressive invader or better able to establish an infection regardless of the previous exposure of the host to another expat lineage. This observed succession pattern of lineages shows that there was not an initial competitive exclusion, nor a founder-controlled community of *Bd* where the expat lineages were concerned. Rather *Bd*-population structure on the host reflected a *Bd*GPL dominance controlled community, which is often seen in space-limited communities in nature (Yodkis, 1943).

When concerned with the local version of the mixed lineage treatments, it was seen that the co-infection success was significantly lower. The highest number of co-infections was seen where local-CAPE was followed by local-GPL. Where the animals were exposed to local-GPL first, only one animal managed to display an infection with both local-GPL and -CAPE at the time of euthanasia. Therefore the structure of the local *Bd*-population on the hosts resembled a dominance controlled community, with competitive exclusion taking place (Begon *et al.*, 2006). In terms of dynamics within the pathogen population, it is true that when a new lineage of amphibian chytrid is introduced into an already positive population, then diversity of the pathogen increases and as competitive exclusion takes place, the diversity will decline again until a dominance controlled population is reached, which would, essentially, consist of the lineage with the best competitive ability (Begon *et al.*, 2006). This increase and decrease in diversity of the pathogen that occur as the competitive exclusion continues, reflect what could be the case in an environment where more than one lineage is present, where two lineages might be detected for a limited time and then the superior lineage will be detected as dominance is reached.

Due to the fact that South Africa has two distinct lineages of *Bd* and the potential for hybridization exists, especially where *Bd*GPL and an endemic lineage are involved (Schloegel *et al.*, 2012; Jenkinson *et al.*, 2016), and co-infection being experimentally

proven to be possible, recombination with the possibility of forming new hybrid lineages comes to light. In terms of host populations in, for example, the Orange River, this could show some danger especially as a previous infection with local-CAPE would not safeguard them. If these animals are, however, adapted to surviving the infection with either lineage or even both, it might provide some evidence for co-evolution of South African hosts with local *Bd*CAPE and subsequently to introduced *Bd*GPL, at some point in history. Seeing as ZA CAPE is stated to be endemic to South Africa and introduced into Europe (Weldon *et al.*, 2007), it is possible that the expat *Bd*CAPE lineage has accentuated its virulence and infectivity to such an extent that the adaptation local hosts have to the local lineage is no longer effective against the previously endemic expat. This change of the expat *Bd*CAPE lineage's ability to cause mortality in local hosts, allows it to possibly be a potent invasive in its previously native range. When concerned with *Bd*GPL as a lineage, differences in lethality towards local hosts is also evident from the experiment, although the difference seen in the infection intensity comparison between the expat and the local lineage is more noticeable. *Bd*GPL has been said to have recently spread to be virtually globally present and be the driver of amphibian declines (Fisher *et al.*, 2009b), yet it is known to have been present in South Africa for a number of years, alongside the local *Bd*CAPE, despite *Bd*GPL's apparent ability to displace endemic lineages (Jenkinson *et al.*, 2016). At some point in the past *Bd*GPL was thus introduced to South Africa, and it did not cause the major declines in natural amphibian populations, as is often seen elsewhere. It was still apparent that the expat *Bd*GPL was more deadly in the experiment than the local corresponding lineage, thus some accentuation of virulence must have taken place, to such an extent that the local hosts are not as resistant to the expat *Bd*GPL as to the local *Bd*GPL, the same as is the case with local versus expat *Bd*CAPE. A number of factors may have caused the expat lineages to evolve differently compared to the local lineages, habitat-specific and population-specific factors being the main drivers. The factors that may influence the accentuation of a lineage can be legion, but to name a few, they could be host species (Stockwell *et al.*, 2010), high salinity environments (Stockwell *et al.*, 2012; Stockwell *et al.*, 2015), hydrology and temperature (Bustamante *et al.*, 2010) and seasonality of environmental conditions (Spitzen-Van Der Sluijs *et al.*, 2014). The fact that *Bd*GPL has been seen to have established stable infections in South Africa's natural amphibian populations may have allowed the host to evolve some resistance to this lineage of amphibian chytrid just as it did towards the proposed endemic lineage.

3.4.2 Transmission capability of amphibian chytrid

The main result sought from a transmission experiment is to determine the potential for an infection to be transmitted from one individual to another, thus in this case, the possibility of frog-to-frog transmission. Similarly the mixed lineage treatment determined the outcome of infection after co-housing animals with different initial lineage infections.

Although this experiment tested the one-on-one transmission capability of *Bd* between individuals, this might not always be the case, as populations that extend their ranges may come into contact with one another, whether it be directly or indirectly, but not always in equal numbers. In South Africa, where two *Bd* lineages are present, any one of the two may be introduced into a naïve environment. The transmission from one individual to another was seen to result in low infection intensities in the previously naïve individuals, and thus these individuals could possibly, in nature, carry the infection for longer before becoming symptomatic, or possibly lose it altogether (Briggs *et al*., 2010). Successful transmission was seen with *Bd*GPL, but to a lesser extent compared to that of *Bd*CAPE. It could be that *Bd*GPL itself grows to a higher infection intensity on the carrier, resulting in the individual becoming symptomatic and requiring euthanasia, thus limiting the time of co-housing. This would consequently lead to shorter time for transmission to take place, between the carrier and recipient, possibly leading to the fewer recipients becoming infected with *Bd*GPL. It was seen that all the animals initially infected with *Bd*GPL were the first to become symptomatic and had a high infection intensity, while the *Bd*CAPE individuals appeared healthy even when infected in their single lineage treatment, possibly due to the lower infection intensity. Seeing that animals were only euthanized upon becoming symptomatic, and this was never seen in *Bd*CAPE animals, it led to all recipient animals being exposed to the carriers for the full time-span of the experiment.

Interaction zones where the populations are both infected, but with different genotypes of *Bd,* resulted in a different pattern of transmission. Although it was seen that both genotypes can be transmitted to a naïve individual, only *Bd*CAPE carriers gained an infection with *Bd*GPL. This was, however, limited to three animals out of a possible 19 individuals. No *Bd*GPL carriers developed an infection with *Bd*CAPE. Again, the *Bd*GPL carriers were the first to become symptomatic, thus the total time that the individuals were exposed to each other, differed per replicate. The *Bd*CAPE carriers in the mixed lineage treatment did not have a significantly different infection intensity (*Bd*CAPE GE)

compared to the *Bd*CAPE carriers paired with naïve recipients. Neither did the *Bd*GPL carriers in the mixed treatment have a significantly different infection intensity than the *Bd*GPL carriers paired with naïve recipients. This leads to the deduction that although co-infection is possible, the exposure of a *Bd*CAPE carrier to a *Bd*GPL carrier did not influence the infection intensity of the lineage it was initially infected with, this being true for both genotype carriers. This absence of reaction in the pathogen itself towards exposure with another is in contrast with what was seen in the first experiment where a single individual was dosed with both lineages. The fact that the *Bd*GPL carriers were not infected with *Bd*CAPE could have two possible explanations, the first being that the *Bd*CAPE infection on the respective carriers was too low to successfully infect animals already positive with *Bd*GPL and the second was that *Bd*GPL prevented the infection with *Bd*CAPE to some extent, thus providing further evidence for Gause's principle between South African lineages of *Bd*, also apparent in the competition experiment (Begon *et al.*, 2006).

To conclude, in the experiment concerning the virulence and competition of *Bd* the goal was to determine the differences that can be seen in the influence of *Bd* lineages originating from local and different geographical areas but being genetically similar, on a local host species. In this instance, the expat lineages reflected the potentially adverse impact that would be seen if those lineages were to become invasive in South African amphibian populations. Mortality was the focus of this experiment, due to it being the first sign of disease normally seen within a natural population of amphibians. However, it was seen that the genetically similar lineages did not react the same to a South African host, and an origin-dependent factor did indeed have an influence on rate of mortality. Thus both the origin and the genotype have an influence on the rate of mortality. This was simulating the situation where a naïve individual was exposed to an environmental source of the disease (habitat previously utilized by an infected animal). This is possible as it was previously seen that *Bd* can survive for up to three months without a biotic host, abiotic factors permitting (Walker *et al.*, 2007). Also, water and other animals such as waterfowl can act as vectors for the disease, although it would only be able to vector the disease for short distances as desiccation remains an important factor for the survival of *Bd* (Garmyn *et al.*, 2012). The result of this spread by vectors can lead to new habitats being exposed to *Bd*. But in South Africa, we have different populations of amphibians that are infected with different lineages of *Bd* that could either contaminate the habitat of other populations or come into contact with each

other directly. It is important to note that a population here does not necessarily just allude to the frogs themselves moving into a new environment, but the pathogen moving/extending its range into new amphibian populations by means of transmission from one individual to the next.

The transmission experiment showed the capacity for the different lineages in South Africa to move through a population of frogs, leading the pathogen to potentially come into contact with another pathogen positive frog population. Here it was seen that the *Bd*GPL carriers had a very low likelihood of becoming infected with *Bd*CAPE, while the *Bd*CAPE carriers showed the possibility of becoming infected with *Bd*GPL. In a mixed lineage, natural system such as the Orange River system, bearing in mind that only *Bd*CAPE carriers became infected with *Bd*GPL and not the reverse, it is possible to hypothesize that the original lineage present would then have been *Bd*CAPE and the other lineage is newly introduced. All the results showed that *Bd*GPL did not eliminate *Bd*CAPE if the animals were initially infected with it, within the experimental time-frame. It was seen to be very unlikely for *Bd*CAPE to invade a host population infected with *Bd*GPL, thus supporting the novel pathogen hypothesis (Rachowicz *et al.*, 2005). If co-infection does indeed occur, it is not yet known if it will remain the case, due to limitations in the experimental time-frame. It was also seen that co-infection usually resulted in low infection intensities for *Bd*CAPE, which means that with an extended time of infection, it may indeed be extirpated by *Bd*GPL on the animal itself, but still experimentally no extirpation or change in the infection of *Bd*CAPE was observed.

It was thus seen that the origin and lineage of *Bd* have an influence on the host's ability to survive with the infection. It was also seen that co-infections are possible, such as those seen along the Orange River; however, it is directional in the sense that *Bd*CAPE populations tend to become infected with *Bd*GPL, but the reverse is highly unlikely.

CHAPTER 4: GENERAL DISCUSSION

4.1 Infection in nature

South Africa with its multiple climate zones and corridors of suitable habitat for *Bd* (Tarrant *et al.*, 2013; Ramollo, 2014) provides a relatively unique situation for the distribution of *Bd* along a linear habitat and to discover the different patterns of distribution that form within the host populations. The Orange River encompasses 11 associated river systems (DWA, 2012), increasing the potential impact of any pathogen present within this system.

Knowing from the work of Tarrant *et al.* (2013) that the Orange River provides the environmental conditions needed for *Bd* to survive, and focussing on the sites selected for this study, it was confirmed that *Bd* has a wide distribution within the system. Not only was it found in the entirety of the region sampled. The presence of *Bd*CAPE and *Bd*GPL is to be expected in a wide-ranging system with its origin in a region where *Bd*CAPE is predominant. *Bd*GPL has a widespread occurrence in the rest of South Africa and both lineages were found in the system, with one of the possible outcomes that one lineage might be displacing the other. When the distribution of the different lineages was mapped, it was seen that there was a large section of the river in which the amphibian population hosted both of the lineages. Although prevalence and infection intensities were low across the system, the area between Aliwal North and Upington hosted both lineages and in some cases, the same individual was infected with both *Bd*GPL and *Bd*CAPE. Thus, in spite of low infection intensities and prevalence, an infection with both lineages was still noticeable. This large section hosting both lineages, with *Bd*GPL spanning the full distance between the furthest sampled sites and *Bd*CAPE co-occupying a central section of the river and occurring at its origin in the central eastern Drakensberg mountains, provides a unique multi-lineage study system (Weldon *et al.*, unpublished data). The only previously documented case was for *Bd*Brazil and *Bd*GPL in the Atlantic Forest, where the endemic lineage was largely restricted, but co-existed with the expanding *Bd*GPL (Jenkinson *et al.*, 2016). For a linear system such as the Orange River together with the postulated endemicity of *Bd*CAPE in South Africa, one could assume that this lineage occurred throughout the entire system at some point. Alternatively its distribution may have been restricted by climatological or geographical boundaries. In a wide ranging system it may simply be

the case that the micro-climatic differences between the areas where only *Bd*GPL is present and the areas where both lineages are present, allows for the survival of both lineages in the one area and not in the other as landscape fitness changes (Van As *et al.*, 2012). Upon introduction, *Bd*GPL may have spread through previously uninfected range or encountered *Bd*CAPE resulting in an interchange zone. Depending on the stage of range expansion and interspecies relationship *Bd*GPL may entirely displace the endemic lineage, co-occur or be outcompeted by *Bd*CAPE. Judging by the current distribution of chytrid in South Africa (results from this study; Farrer *et al.*, 2011; Weldon *et al.*, unpublished data), we postulate that *Bd*GPL has expanded its range from the Western Cape towards the interior and eventually displaced *Bd*CAPE where it previously occurred. Walker *et al*. (2008) demonstrated that *Bd*CAPE used to occur in the Western Cape, but a recent survey only detected *Bd*GPL (Weldon *et al.*, unpublished data). Climate niche modelling identified the Orange River valley as an isolated suitable habitat for chytrid in an otherwise hostile semi-desert environment (Tarrant *et al.*, 2013). This habitat belt likely functioned as a corridor facilitating the more recent eastward spread of *Bd*GPL resulting in the contact zone with *Bd*CAPE that we currently witness. Further towards the east at the origin of the Orange River *Bd*CAPE occurs exclusively (Weldon *et al.*, unpublished data), suggesting that *Bd*GPL has not yet spread to this region or that its distribution is limited by unknown environmental factors. While infection intensity is low for both lineages from the contact zone, the infection intensity and prevalence increases significantly where only a single lineage was detected (from Pella westwards).

Species diversity has been found to have an influence, experimentally, on the prevalence and intensity of *Bd* infections in tadpoles (Johnson *et al.*, 2015), and thus presents another factor to consider in terms of infection intensity. The possibility exists in nature for the host community composition to suppress or alter the infection intensity on the individuals and lead to low-level infections.

The lineage identity was confirmed using genome sequencing of the different cultures isolated from the different positive sites. These cultures showed that in the easternmost site, Aliwal North, a hybrid lineage is found. This hybrid lineage could only have formed if both the lineages were present within the same environment to such an extent of time that recombination could occur. The formation of hybrids that are possibly evolutionary superior to the parental lineage, may cause the extinction of the parental lineages, in

very much the same way that *Bd*GPL may be displacing *Bd*CAPE, in accordance with the Red Queen Hypothesis (Stenseth, 1979). Under such conditions the parental lineage may persist without evolving to the new threat posed by the presence of the hybrid lineage. The same hypothesis can be applied to the hosts as the evolution of the hosts might be necessitated by the evolution of the pathogen. Paterson *et al.* (2010) demonstrated that the molecular evolution of resistance increased in *Pseudomonas fluorescens* infected with the viral phage Φ2, when the viral phage was allowed to evolve its infection abilities. Thus when present in the same population, and allowed to evolve, the rate of evolution within these organisms increased due to antagonistic co-evolution. Host-pathogen co-evolution between *Bd* and amphibians is likely to have occurred, given the well-documented relationship from archived collections, between these organisms for about a century, not only in South Africa, but various countries around the globe (Huss *et al.*, 2013; Vredenburg *et al.*, 2013; Zhu *et al.*, 2014; Talley *et al.*, 2015).

4.2 The experimental situation

Two possible situations may be apparent in a corridor habitat, where organisms are forced to interact with each other. The two organisms may interact indirectly, in which case the one organism might need to occupy a space previously exposed to the other, or they may interact directly, where the simultaneous occupation of the same habitat takes place (Dugatkin, 2004). In the case of a disease such as chytridiomycosis a naïve animal can be exposed to a habitat where an individual infected with amphibian chytrid may have passed through, or a naïve individual may be exposed to the infected individual directly. In both cases, there is still a risk for the naïve individual to become infected. This risk exists as *Bd* can be transmitted through the water, due to the motile zoospore life stage (Longcore *et al.*, 1999), which would reflect the indirect interaction of individuals to each other. The second situation is created by the fact that *Bd* is able to infect more than one species of amphibian (Fernández-Beaskoetxea *et al.*, 2016), with potential transmission during breeding season congregation of individuals, as an example, and that some species may act as reservoirs for *Bd* itself (Weldon *et al.*, 2004; Garner *et al.*, 2006; Rödder *et al.*, 2013). The experiments were designed to not only provide more information in the interaction between the different genotypes of chytrid,

but also to represent some of the different interaction conditions that may occur in the natural habitat.

When faced with a river system/corridor habitat that has multiple lineages of *Bd* present, low prevalence, as well as apparently negative sites, it is feasible to envision a *Bd*-naïve individual coming into contact with either or both of the lineages present in the system. The first experiment was designed to replicate the conditions in which an organism may be environmentally exposed to the pathogen, such as would be the case after an infected individual recently travelled through the same habitat. However, this experiment tested not only whether the experimental model, *Sclerophrys gutturalis*, can become infected, but also what would happen when the same individual is sequentially exposed to more than one genotype of the same pathogen. Co-infection with more than one lineage was witnessed under controlled experimental conditions and in nature during our survey of the Orange River system. In this instance, the two pathogenic lineages would be in direct competition with each other on the single host's skin, in which case the lineage that established an infection first, would hypothetically have a dominance controlled advantage (Begon *et al.*, 2006). This was seen in the local context where *Bd*CAPE was only marginally successful in infecting *Bd*GPL hosts experimentally, while *Bd*GPL had a relatively high success rate in infecting *Bd*CAPE hosts. This could be explained by the increased infectivity of *Bd*GPL (Farrer *et al.*, 2011) that was seen in this experiment in the form of number of animals being co-infected and the increased local-*Bd*GPL infection intensity. When testing whether a naïve animal can become infected through environmental transmission, it was seen that both local-*Bd*CAPE and local-*Bd*GPL can infect the naïve host, although local-*Bd*GPL is more aggressive in terms of the time it took to be lethal to the host, as well as the infection intensities that the host had at the point of euthanasia. Local-*Bd*GPL also did not demonstrate a dosage-dependent effect, increasing the risk of the naïve individual developing a high infection intensity regardless of the initial exposure.

The experimental setup also allowed us to test the effect that an invasive pathogen of European origin would have on a South African host, although the pathogens are genetically closely related to the South African pathogens. Although both European lineages showed a dosage-dependent rate of mortality, expat-*Bd*CAPE was more lethal than expat-*Bd*GPL in the high dosage treatments, contrary to the comparative virulence of local-*Bd*. However, the lineages originating from Europe did not differ significantly in

infection intensity, which begs the question as to what caused expat-*Bd*CAPE to be more lethal? When considering the possibilities in nature and significant differences only seen in the higher dosage experimental treatments, it is unlikely that pathogen densities of this nature would be encountered by chance, without a noticeable catastrophic release of a heavily infected population, as could be the case with reintroduction of species using individuals reared in captivity.

In the Orange River, the reactions of the hosts may differ as environmental conditions have been shown to have an influence on the reaction of the host towards infection with the pathogen (Ribas *et al.*, 2009), while in the laboratory the environmental conditions are kept as constant and controlled as possible. The potential of some hosts to acquire resistance to the disease could explain why the same trend was not seen with the South African lineages rendering South African hosts less susceptible to chytridiomycosis acquired through local-*Bd* lineages (McMahon *et al.*, 2014).

Within a corridor consisting of more than one lineage of the pathogen, it is possible that individuals infected with different lineages of *Bd* could come into contact with each other, leading to co-infection. When this occurs the transmission capability of the disease plays a very important role (Willey *et al.*, 2013). We tested a number of combinations in which this could occur. Firstly, would it be possible for a negative animal to become infected with the lineage of the other individual if they were housed in the same enclosure, due to transmission within a population to the negative individuals. Transmission to negative individuals is possible where the disease is not 100% prevalent, or where new individuals entered the population. It was seen that both *Bd*GPL and *Bd*CAPE were transmitted and established an infection in the negative animal that the carriers were paired with. *Bd*CAPE had a much higher percentage of naïve animals becoming infected than did *Bd*GPL, even though the infection intensity of the *Bd*CAPE positive animals was much lower than that of the *Bd*GPL positive animals. This suggests that pathogenicity is not necessarily positively correlated with transmission potential. The *Bd*GPL animals were more prone to showing signs of disease, causing the euthanasia of replicate pairs before the termination of the experiment. This could potentially explain the lower percentage of transmission, as there was simply less time in the co-housing phase. But both these treatments showed successful transmission of the pathogen to the naïve individuals through co-housing only.

The infection intensities of *Bd*CAPE were not sufficient to cause any signs of disease in the animals, yet they still managed to transmit the disease to the naïve animals. The slower increase in infection intensity potentially allows the individual to transmit the disease to more than one individual in a natural habitat before reaching a lethal infection, or recovering from the infection. The first experiment also showed that local-*Bd*CAPE, independent of dose, never had an average infection intensity of more than a thousand genomic equivalents, while local-*Bd*GPL were more than ten thousand in both the four and eight dose treatments. However, because infection intensity of *Bd*GPL animals was higher, it is more likely for local-*Bd*GPL to transmit through the environment than local-*Bd*CAPE, due to a higher amount of zoospores infecting new hosts independent of the survival status of the source host. Local-*Bd*CAPE, on the other hand, may persist at lower infection intensities on the host and allow more time for the host to come into contact with other potential hosts; thus allowing for individual-to-individual transmission to take place.

Our treatment that consisted of one *Bd*CAPE individual and one *Bd*GPL individual, resulted in one *Bd*CAPE animal gaining an infection with *Bd*GPL, and none of the *Bd*GPL animals gaining *Bd*CAPE. When frog skin is considered as the habitat for amphibian chytrid, it appears that local-*Bd*CAPE is excluded from the *Bd*GPL individuals the same way that dominance-controlled populations are regulated (Begon *et al.*, 2006). This potential exclusion is evidently supported by the first experiment in the treatment where animals received local-*Bd*GPL first and then local-*Bd*CAPE. But in the treatment where the animals received local-*Bd*CAPE first, it was seen that *Bd*GPL still dominated in terms of infection intensity and prevalence. The first experiment showed that local-*Bd*GPL has the capacity to reach higher infection intensities and even when local-*Bd*CAPE was already present it still infected the individual and managed very high infection intensities. This shows that over time it is likely to outcompete local-*Bd*CAPE. Even in the transmission experiment, which would reflect the populations at the front edge of an epidemic wave, the dominance-controlled principle was evident and thus the potential exclusion of local-*Bd*CAPE over time. In a South African context, it was seen that *Bd*GPL performed as hypothesized in the experiments, in the sense that it is more lethal than *Bd*CAPE, and may prevent infections with *Bd*CAPE. However, it was seen that the dynamics of the different lineages in terms of infection intensity obtained within the time span of the experiment were different. A possibility is that for European *Bd*CAPE to be able to compete with *Bd*GPL it had to evolve differently than

*Bd*GPL itself, while *Bd*GPL spread around the globe fast and was more aggressive in infections, and thus necessitated no accentuation, as it already had the ideal infection characteristics.

4.3 Conclusion

The influence that invasive lineages could have on South African hosts, even if they are genetically similar to local lineages, is unquestionably negative from an amphibian conservation perspective. Direct exclusion of one lineage by the other (i.e. South African lineages experimentally) might not be the case in a natural environment, but the significantly quicker rate of mortality caused by the more virulent lineages is still possible. In a restricted corridor habitat the spread of any disease may be devastating, the presence of more than one lineage could exaggerate this effect due to inter-lineage competition and increased pathogen load. The possibility of multi-lineage infections also increases the need to limit potential introduction of invasive lineages as to prevent any unnecessary disease interactions that could increase the pathogen load above the tolerance of the host and allow for recombination and consequential hybrids to form with even more uncertain outcomes for local amphibian populations.

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