



Aspects of the ecology and behaviour of *Batrachochytrium dendrobatidis* on amphibians in South Africa

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Preface

Unpublished manuscripts contained in this thesis reflect the findings as analysed by Ruhan Verster, and changes may be made prior to publication with the input from co-authors. The database used in Chapter 4 for the prediction models will be published online together with the manuscript titled Environmental fitness of *Batrachochytrium dendrobatidis* lineages increases potential for recombination.

Published manuscripts contained within this thesis were included with the permission of all co-authors. In Chapter 6 variation will be seen from the original version as some references were incorrectly given in the published version and the correct references were included as part of the manuscript in this thesis. Contributions of myself and co-authors are explained in Chapter 1 under the heading Contribution of authors.

Abstract

Amphibians have been experiencing threats to their survival in terms of habitat destruction and disease. Some species are more sensitive than others, especially towards disease and the lineages of the disease that may impact on them. *Batrachochytrium dendrobatidis* (*Bd*) is a fungal organism causing chytridiomycosis in amphibians and has been responsible for declines and even extinctions of amphibian species around the globe. This fungus has been studied since 1998 and great progress has been made in terms of understanding the conservation implications and resolving its genome. Recent discovery of recombinant lineages, although rare, brought the possibility that new lineages may disrupt the equilibrium seen between amphibian populations and prevalence of this fungus therein. In the past lineage identification could only be done with whole genome sequencing, and this limited our investigation into the influence infection with *Bd* may have on the host. The development of lineage-specific primers for qPCR now enables us to study lineage level effects of *Bd* on the host as well as allowing for the study of co-infection with multiple lineages. Our experiments showed that although South Africa is host to both the hypervirulent *Bd*GPL and the hypovirulent *Bd*CAPE, the latter cannot be considered of less danger to amphibians, as we found that *Bd*CAPE had more transmission success to naïve individuals during our experiment, as compared to *Bd*GPL. Our experiments also showed that co-infection with multiple lineages on a single individual is possible, when individuals infected with different lineages of *Bd* inhabit the same habitat. This is a unidirectional situation, as only individuals already infected with *Bd*CAPE became infected with *Bd*GPL as well.

When studying the behaviour of *Bd* infected anuran larvae, it was seen that differences in behaviour could be seen based on the lineage of infection, hence not only is the virulence and transmission ability lineage-specific for the different lineages found in South Africa, but also the behavioural influences an infection has on the host. *Bd*CAPE was seen to increase certain movement parameters compared to the control animals, while *Bd*GPL decreased the same parameters when compared to the control animals. The influence of these altered behavioural patterns can potentially influence successful predation upon infected tadpoles, as well as their exploration and foraging efficiency in order to cope with their changing environments or physiological needs.

Since South Africa hosts more than one lineage of *Bd*, unidirectional co-infection was proven through experimentation and a recombinant lineage has already been isolated originating from genetic exchange between *Bd*GPL and *Bd*CAPE, it became necessary to model the lineage-specific predicted distribution for the different lineages within the South African landscape. Using a database consisting of *Bd* records from 26 African countries as well as

field data collected within South Africa, we were able to determine that large areas of South Africa are suitable for *Bd*GPL, including virtually all areas where *Bd*CAPE is currently found and predicted to occur. This is of conservation concern as *Bd*CAPE areas can potentially become infected with *Bd*GPL as well, increasing the risk for the amphibian host populations as well as increasing the risk of uncharacterised recombinant lineages forming. The modelling of the South African *Bd* landscape also showed that low to moderate likelihood areas were still *Bd* positive, with these sites often infected with both lineages. We also constructed a *Bd*-distribution prediction map for the African continent, that showed where previous models predicted that *Bd* can have a disjunct distribution between different countries, these areas are connected by low to moderate likelihood *Bd* prevalence areas, similar to areas often testing positive in South Africa.

In the past the identity of lineages infecting amphibian populations could only be done by whole genome sequencing and required significant financial input and skill. The development of lineage-specific qPCR primers reduced the need for whole genome sequencing and also the time needed to determine the lineage of infection during field surveys. This was tested by using these newly developed primers together with isolation attempts on a large scale transect survey of South Africa spanning from the Drakensberg, along the Orange River to the Namibian border. It showed that these genetic tools were highly successful in identifying the correct lineage of infection at the sample sites and also allowed for the faster recognition of sites that showed multiple lineage co-infection. It is also possible to use these new genotype identification tools in most laboratories previously capable of testing for *Bd* presence using ITS-qPCR. This, thus, increased the capacity to determine the lineage of infection during surveys, and thus increase our knowledge about *Bd* lineage distributions globally.

Based on recent genomic analysis of all *Bd* lineages available, it became apparent that *Bd* did not originate in Africa as previously thought. This necessitated the need to change the hypotheses that were accepted for approximately 15 years that *Bd* is an endemic pathogen to African amphibian species, to it being an introduced pathogen. The review that formed part of this showed that both *Bd*CAPE and *Bd*GPL can be found in disjunct reaches in Africa, and under the novel pathogen approach species vulnerability need to be tested and conservation programs need to increase their focus on the less virulent *Bd*CAPE as well.

Keywords:

Batrachochytrium dendrobatidis; qPCR; behavioural experiments; distribution modelling; disease transmission; pathogen behaviour influence; environmental envelope; chytrid; Africa

Chapter 1

General introduction

*A brief history of *Batrachochytrium dendrobatidis**

Batrachochytrium dendrobatidis, often referred to as *Bd* or amphibian chytrid fungus, was discovered after the cause was sought for a sudden mortality event and population declines in seemingly stable amphibian populations in Australia's Queensland region and in Panama (Berger *et al.*, 1998). The cause was found to be a fungus belonging to the Chytridiomycota phylum, but the formal description of *Bd* came in 1999 with the successful isolation and physical characterisation of the fungus causing the lethal skin infections seen in these amphibian populations experiencing dramatic population declines (Longcore *et al.*, 1999). By this time it was already apparent that amphibians can form an integral part in helping identify disturbances in ecosystems with amphibians showing sensitivity towards water quality associated with agriculture (Boyer & Grue, 1995) sediment levels (Welsh Jr & Ollivier, 1998), sensitivity towards water effluent from oil sand mining (Pollet & Bendell-Young, 2000) as well as indicators of dangers to human health by using them to identify toxic materials in natural environments ranging from ammonia to organic substances such as benzene and toluene (Van der Schalie *et al.*, 1999). This new threat towards amphibian survival thus quickly garnered attention, as the importance of the protection of this vertebrate also increased beyond the mere general protection of biodiversity premise.

Genetic analyses of previously recovered isolates from populations that suffered declines (James *et al.*, 2009), as well as seemingly healthy populations, especially in South Africa (Weldon *et al.*, 2004), showed in 2011 that there could be discerned between three distinct genotypes at that stage (Farrer *et al.*, 2011), with one lineage having a seemingly global spread, termed the Global Panzootic Lineage (*BdGPL*). Other lineages identified in parallel with this lineage were *BdCAPE* and *BdCH*, although the latter was only described in Switzerland, and the former localised at that stage to South Africa and a population infected through spill-over from frogs of South African origin in Mallorca, Spain (Walker *et al.*, 2008). Farrer *et al.* (2011) already postulated that *BdGPL* was hypervirulent when compared to *BdCAPE* and *BdCH* in terms of ability to cause mortality in infected individuals, by conducting experiments that resulted in survival curves showing that the animals infected with *BdGPL* had a shorter life expectancy than those infected with other lineages. However, in the years that followed, there has been a huge collective effort focussed on determining the distribution and genetic diversity within amphibian chytrid fungus, and collected isolates were studied to

great extent. This increased focus on the global distribution of lineages reached the point where it was known that more than one lineage of *Bd* could occur within the same geographical region, some of them already known to Farrer *et al.* (2011), but further ones being described in Rodriguez *et al.* (2014), including *BdBrazil*. Here this Brazilian lineage existed alongside the known *BdGPL*. Similarly, an endemic lineage of *Bd* has also been described in Korea (Bataille *et al.*, 2013) and a hybrid genotype was even described in the bullfrog trade by Shloegel *et al.* (2012). These endemic lineages were often seen to not be the lineage directly associated with sudden declines in populations of amphibians, at least as far as is known (Jenkinson *et al.*, 2016). This did however highlight that a comprehensive genetic analysis of as many lineages as possible, was needed, especially since evidence of recombination between lineages was starting to emerge and rare past hypotheses about the possibility of new lineages forming by different means of genetic reorganization started becoming more plausible (Morgan *et al.*, 2007; Shloegel *et al.*, 2012). This comprehensive genomic analysis of the amphibian chytrid fungus yielded evidence for six genetically different lineages of *Bd* with some of them, for the time being, termed as Hybrids (O'Hanlon *et al.*, 2018). These hybrids were localised to two different geographical locations, South Africa and Brazil. Given the premise that for hybrid genotypes to exist an exchange of genetic material between different lineages was required, it became important to determine if these hybrids are indeed associated with the convergence and interaction of two distinct genotypes in a single location (Samarasinghe *et al.*, 2020). The development of cost-effective qPCR techniques to identify different lineages of *Bd* especially for *BdGPL* and *BdCAPE* (Chapter 5 - Ghosh *et al.*, 2021), gave the opportunity to study these areas of contact in finer detail by being able to determine the lineage of infection without the additional time and economic burden of advanced culturing and whole genome sequencing. The robust and detailed review of the phylogeny of *Bd* by using virtually all available isolates showed that almost 80 % of these isolates came from the Global Panzootic lineage, with only three hybrids being found of the 234 isolates genotyped (Figure 1).

The fact that there is a globally present lineage responsible for most amphibian declines due to chytridiomycosis and being able to form hybrid genotypes, brought about the goal of not only determining the genetic diversity of *Bd* but identifying the zones where these hybrids are likely to occur due to overlapping distributions with other lineages, as well as determining the precise ability of different lineages to transmit to infected hosts and the influence lineages may have on the behaviour of the host. With these goals in mind, and the new tools available, the objectives for this PhD thesis were set.

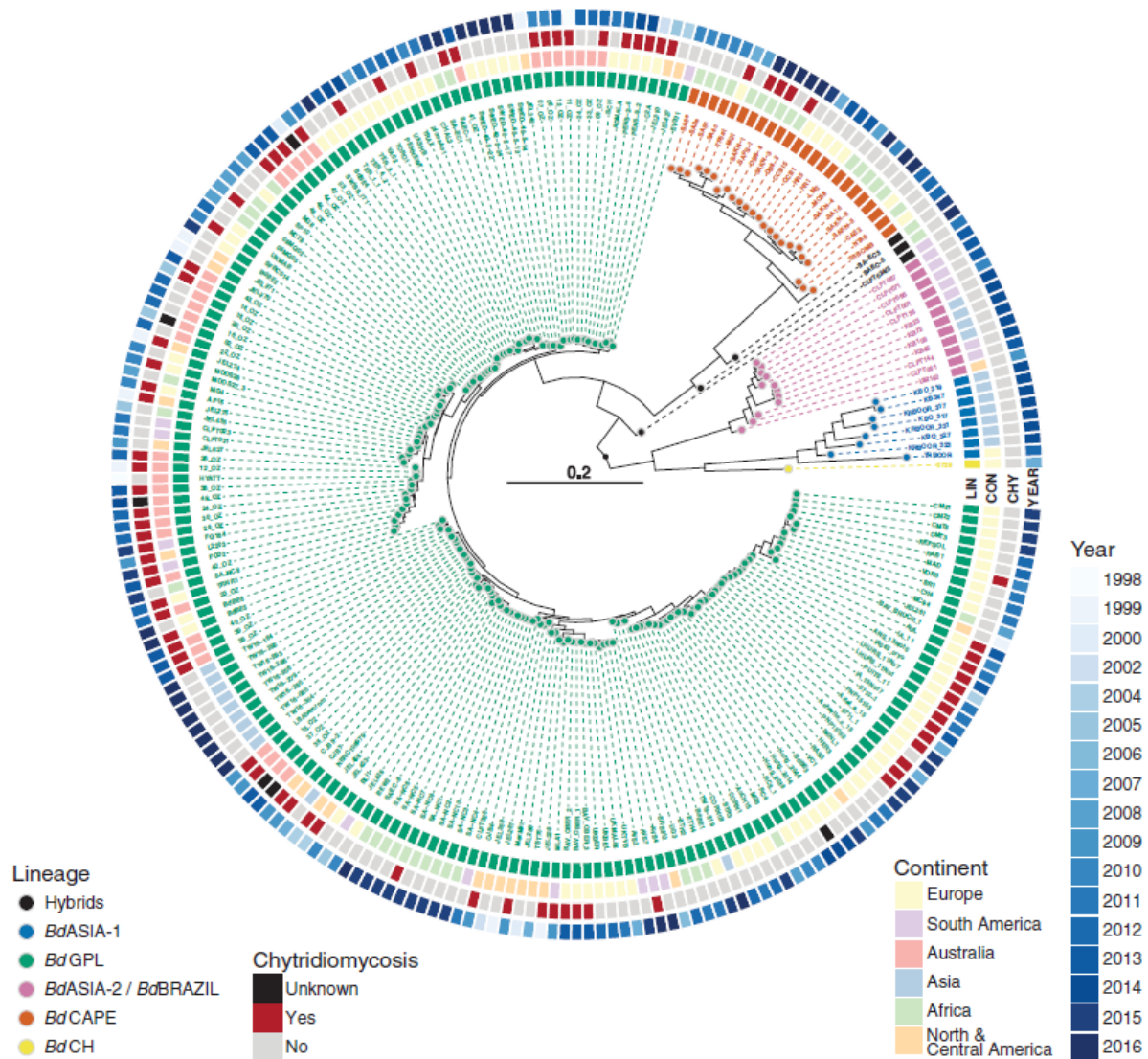


Figure 1: Radial phylogeny showing four divergent lineages (obtained from O'Hanlon et al. 2018)

Thesis layout

Through the development of the above goals, an experiment was designed that is described in further detail in Chapter 2 under the heading *In vivo* transmission of *Batrachochytrium dendrobatidis* lineages. The precise objective was to determine the probability that different lineages of *Bd* will directly interact in nature and potentially gain insight into the population dynamics of *Bd* when multiple lineages are present. This experiment was designed with the South African *Bd* context in mind, owing to the presence of more than one lineage of *Bd* in South Africa, as well as the widespread presence thereof in multiple amphibian species (Chapter 5 - Ghosh et al., 2021). Consequently, the proposed scenario that was experimentally created, was that two amphibian populations and their different genotypes of *Bd* may converge in a single location. Our experiment was set up to use a single host species, two lineages of *Bd*, and individuals with different infection statuses paired-up.

This experiment also allowed us to quantify the ability of the different lineages to infect individuals with no current *Bd* infection, thus allowing for the quantification of transmission capability to a naïve host when *Bd* is introduced into a population through an infected host. As Ghosh *et al.* (Chapter 5 published in 2021) also found a single individual animal testing positive for both *Bd*CAPE and *Bd*GPL, the general assumption was that the hypervirulent *Bd*GPL would displace or eradicate *Bd*CAPE infections. We wanted to know if animals can transmit a second lineage to already infected individuals to establish a multiple lineage infection on a single animal. Tissue samples from the animals used in this experiment were subjected to qPCR analyses that were developed as part of a collaboration that was published and included in this thesis as Chapter 5 (Ghosh *et al.*, 2021).

Although different species of amphibians exhibit different behaviour and seeing as tadpoles are even classified into different functional guilds as described by Wilson (1999), *Bd* displays no specificity towards host or developmental stage. Tadpoles don't always exhibit the extreme consequences of chytridiomycosis as what can be seen in adult individuals in terms of mortalities, but there has been evidence that they are morphologically altered by the infection, such as reduced body size (Hanlon *et al.*, 2015) and cardiac adaptations (Salla *et al.*, 2015). These studies focussed on the morphological differences between healthy and infected tadpoles, but these morphological differences, we hypothesized, must have a corresponding behavioural influence on the tadpole. Using modern behaviour tracking software from NoldusTM (Netherlands) we tracked the behaviour of not only healthy and infected tadpoles but also attempted to quantify the specific effect that the lineage of the *Bd* infection may have on tadpoles, as described in Chapter 3. This experiment thus holds the potential to identify behavioural characteristics that may serve as warning signs of infection, potentially making future detection of *Bd* less damaging to tadpole populations, as well as improving successful isolation attempts of *Bd* by using behaviour as a proxy. Consequently, this could reduce the possibility of overinvesting in detection or isolation efforts in healthy populations that could otherwise be identified in conjunctions with using behavioural cues. This is also to the extent of our knowledge, the first study on tadpoles to incorporate multiple lineages of *Bd* and this software analysis technique.

Chapter 4 of this thesis is the result of a collaborative effort to determine the environmental envelope for *Bd*CAPE and *Bd*GPL, and to investigate whether overlap exist between envelopes, potentially leading to areas where consequent recombination of lineages can occur. This was done by using data on lineage distributions on a transect of a large section of South Africa, identified employing new molecular techniques (Chapter 5 - Ghosh *et al.*, 2021), using sites ranging from the origin of the Orange River at Mt Aux Sources in the Drakensberg, to Vioolsdrif, approximately a 100km from the entry of the Orange River into the Atlantic Ocean

at Alexander Bay on the border with Namibia. This study was designed to determine the distribution of lineages along one of the largest watercourses, ranging over the widest range of climate envelopes in South Africa, to determine what climate can be termed as best befitting the envelope for *BdCAPE* and *BdGPL* including areas of overlap. The goal was to identify these areas for potential recombinants and develop a prediction model that could aid in identifying focal areas for future genomic studies on recombinant lineages. This has conservation importance since the virulence of South African recombinant lineages has not been studied to the extent that other lineages have and may still prove to be a threat to South African amphibian populations especially within these zones of overlap.

Thesis structure

This thesis has been written in manuscript format, with the intention of all unpublished manuscripts being submitted after thesis submission. Due to this fact, each chapter consists of all sections necessary for the submission of a manuscript (abstract, references, etc.). This thesis then ends with a general discussion attempting to summarize the results from all manuscripts contained here, discussing relevancy, as well as potential future research and focusses based on the results discussed in the various chapters.

Contribution of authors

Unpublished manuscripts

The manuscript titled *In vivo* transmission of *Batrachochytrium dendrobatidis* lineages (Chapter 2) was written by Ruhan Verster who was the principal investigator under the supervision of Ché Weldon. Ruhan designed and executed the experiment and was responsible for all animal husbandry. He analysed the samples with qPCR in the London based laboratories of Imperial College of London and the Zoological Society of London, funded by Mat Fisher (Imperial) and Trent Garner (ZSL). All statistical analyses, as well as preparation of the manuscript, were done by Ruhan Verster, with proofreading and editing by Ché Weldon and Trent Garner.

The manuscript titled Lineage dependent alteration in behaviour of *Cacosternum boettgeri* infected with *Batrachochytrium dendrobatidis* (Chapter 3) was written by Ruhan Verster. He was also responsible for the experimental design, execution, husbandry and statistical analyses. Statistical advice and effect size calculations were done by Erika Fourie from the statistical consultation services of the North-West University. Tarryn Botha was an advisor on

this experiment and allowed access to and training for the Noldus™ Ethovision® software that was used to track the movements of the tadpoles. Tarryn Botha also advised on the statistical analyses, and experimental design and edited the manuscript together with Ché Weldon.

The manuscript titled Environmental fitness of *Batrachochytrium dendrobatidis* increases potential for recombination (Chapter 4) was written by Ruhan Verster. Fieldwork was done in partnership between Ruhan Verster and Pria Ghosh, with the field sites based on the MSc dissertation of Ruhan Verster. Compilation of the *Bd* Africa database was also done by Ruhan Verster. Statistical analyses of rainfall and temperature data were done by Pria Ghosh, while Dirk Cilliers was responsible for the generation of the predicted distribution of *Bd* maps. All further manuscript preparation and editing were done by Ruhan Verster and Ché Weldon.

Published manuscripts

Chapter 5, titled Discriminating lineages of *Batrachochytrium dendrobatidis* using quantitative PCR published in the journal *Molecular Ecology Resources* 2021 (21:1452-1459; DOI: 10.1111/1755-0998.13299), was a collaborative effort between Imperial College London (UK), North-West University (ZA), Zoological Society of London (UK), The Royal Veterinary College (UK) and CIRAD (La Réunion, France). This collaboration resulted in the development by UK counterparts, and field testing (done by Ruhan Verster), of a novel lineage identification technique not necessitating the use of a tissue sample and culturing of isolates, on the Orange River system from Aliwal North to Vioolsdrif, also including sites not directly adjacent to the main watercourse, such as Springbok and Petrusville. This field testing was done with 461 samples being collected and processed by Ruhan Verster to identify the infecting lineage from skin swabs obtained from adult frog individuals belonging to nine different species. Ruhan Verster also did the qPCR analyses in the laboratories of collaborators at Imperial College London and the Zoological Society of London. To confirm the results obtained from this novel technique, simultaneous isolation attempts were made with the swabbing of the animals, and isolates obtained were whole-genome sequenced and the identification published as part of the global phylogenetic analysis as seen in O'Hanlon *et al.* (2018). The collection of field samples was done in the years 2016 and 2017 but only resulted in the co-authored manuscript in 2021.

Chapter 6 pertains to a review article titled Amphibian chytrid fungus in Africa – realigning hypotheses and the research paradigm published in *Animal Conservation* 2020 (DOI: 10.1111/acv.12538). This paper addressed the issue that the standing theory since the 2004 publication of Weldon *et al.* (2004) titled “Origin of the amphibian chytrid fungus” was that this pathogen and threat to amphibian biodiversity globally originated from Southern Africa, based on the oldest known positive samples found at the time. Thus, the working theory

was that at least Southern African amphibian populations were able to persist with stable infections due to the pathogen being endemic to the region. The oldest known positive individual at the time of publication for Weldon *et al.* (2004) was from 1938, from South Africa. However, in 2013 an earlier positive specimen was found, collected in 1928 California, USA (Huss *et al.*, 2013). Another earlier specimen that was originally collected in 1933 in China was also described as positive in 2014 (Zhu *et al.*, 2014). In 2015 two other studies appeared that also showed older specimens with indications of infection with *Bd*, one study from the USA with a specimen date of in the 1890's (Talley *et al.*, 2015) and a study from Korea indicating the oldest specimen they found to have signs of chytridiomycosis to date from 1911 (Fong *et al.*, 2015). By this time, it was apparent that *Bd* was not endemic to Africa, at least for the time being, and using museum specimens was not able to give us the origin point of this pathogen. Consequently, when the 2018 manuscript of O'Hanlon *et al.* was published in *Science*, it gave us a postulated origin point for *Bd*, seeking the ancestor responsible for most of the variation seen between lineages today. Thus, the thinking had to change for the African landscape, from *Bd* being an endemic pathogen and African frogs thus generally being adapted towards its presence and being able to persist despite infection, to the novel pathogen hypothesis with African amphibian populations being at risk of new variants, or even naïve populations being significantly at risk due to no previous co-evolved resistance. This article was conceptualized by Thomas Doherty-Bone, that took lead authorship, with fieldwork conducted by Ruhan Verster that served to highlight areas in South Africa, where no *Bd* had been detected for consecutive years, and which can be a haven for amphibians in the region but may also shelter amphibians most at risk if *Bd* is to be introduced. It thus became vitally important to identify the spread of lineages within corridors of suitable habitat in South Africa. Further contributions to this manuscript were testing and proving the suggestion made by the authors in this publication that the monitoring of species that are not currently vulnerable or threatened can provide valuable insight into the distribution dynamics of *Bd* lineages, especially in an environment as diverse as South Africa. Ruhan Verster also contributed in terms of improving the view of South African distribution of *Bd* and the isolation of the hybrid lineage that was found in South Africa, with this only being the second instance it has ever been done, with the only other locality with a hybrid lineage being Brazil (Jenkinson *et al.*, 2016). This publication thus laid the theoretical framework for the design of experiments determining the definitive influence different lineages have in terms of transmission, as well as on the behaviour of the host animals, to further enable future monitoring of African amphibian populations under this new *Bd* origin hypothesis.

References

- Bataille, A., Fong, J.J., Cha, M., Wogan, G.O., Baek, H.J., Lee, H., Min, M.S., ... Waldman, B. 2013. Genetic evidence for a high diversity and wide distribution of endemic strains of the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* in wild Asian amphibians. *Molecular Ecology*, 22(16): 4196-4209. DOI: 10.1111/mec.12385
- Berger, L., Speare, R., Daszak, P., Green, D.E., Cunningham, A.A., Goggin, C.L., Slocombe, R., ... Parkes, H. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences*, 95(15): 9031-9036. DOI: 10.1073/pnas.95.15.9031
- Boyer, R. & Grue, C.E. 1995. The need for water quality criteria for frogs. *Environmental Health Perspectives*, 103(4): 352-357. DOI: 10.1289/ehp.95103352
- Farrer, R.A., Weinert, L.A., Bielby, J., Garner, T.W.J., Balloux, F., Clare, F., Bosch, J., ... Fisher, M.C. 2011. Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proceedings of the National Academy of Sciences*, 108(46): 18732-18736. DOI:10.1073/pnas.1111915108
- Fong, J.J., Cheng, T.L., Bataille, A., Pessier, A.P., Waldman, B. & Vredenburg, V.T. 2015. Early 1900s detection of *Batrachochytrium dendrobatidis* in Korean amphibians. *PLoS ONE*, 10(3): e0115656. DOI: 10.1371/journal.pone.0115656
- Ghosh, P.N., Verster, R., Sewell, T.R., O'Hanlon, S.J., Brookes, L.M., Rieux, A., Garner, T.W.J., ... Fisher, M.C. 2021. Discriminating lineages of *Batrachochytrium dendrobatidis* using quantitative PCR. *Molecular Ecology Resources*, 21: 1452-1459. DOI: 10.1111/1755-0998.13299
- Hanlon, S.M., Lynch, K.J., Kerby, J. & Parris, M.J. 2015. *Batrachochytrium dendrobatidis* exposure effects on foraging efficiencies and body size in anuran tadpoles. *Diseases of Aquatic Organisms*, 112: 237-242. DOI: 10.3354/dao02810
- Huss, M., Huntley, L., Vredenburg, V., Johns, J. & Green, S. 2013. Prevalence of *Batrachochytrium dendrobatidis* in 120 Archived Specimens of *Lithobates catesbeianus* (American Bullfrog) Collected in California, 1924–2007. *EcoHealth*, 10(4): 339-343. DOI: 10.1007/s10393-013-0895-6
- James, T.Y., Litvintseva, A.P., Vilgalys, R., Morgan, J.A., Taylor, J.W., Fisher, M.C., Berger, L., ... Longcore, J.E. 2009. Rapid global expansion of the fungal disease chytridiomycosis into declining and healthy amphibian populations. *PLoS Pathogens*, 5(5): e1000458. DOI: 10.1371/journal.ppat.1000458

- Jenkinson, T.S., Betancourt Román, C.M., Lambertini, C., Valencia-Aguilar, A., Rodriguez, D., Nunes-de-Almeida, C.H.L., Ruggeri, J., ... James, T.Y. 2016. Amphibian-killing chytrid in Brazil comprises both locally endemic and globally expanding populations. *Molecular Ecology*, 25(13): 2978-2996. DOI:10.1111/mec.13599
- Longcore, J.E., Pessier, A.P. & Nichols, D.K. 1999. *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia*, 91(2): 219-227. DOI: 10.1080/00275514.1999.12061011
- Morgan, J.A.T., Vredenburg, V.T., Rachowicz, L.J., Knapp, R.A., Stice, M.J., Tunstall, T., Bingham, R.E., ... Taylor, J.W. 2007. Population genetics of the frog-killing fungus *Batrachochytrium dendrobatidis*. *Proceedings of the National Academy of Sciences*, 104(34): 13845-13850. DOI: 10.1073/pnas.0701838104
- O'Hanlon, S.J., Rieux, A., Farrer, R.A., Rosa, G.M., Waldman, B., Bataille, A., Kosch, T.A., ... Fisher, M.C. 2018. Recent Asian origin of chytrid fungi causing global amphibian declines. *Science*, 360: 621-627. DOI: 10.1126/science.aar1965
- Pollet, I. & Bendell-Young, L.I. 2000. Amphibians as indicators of wetland quality in wetlands formed from oil sands effluent. *Environmental Toxicology and Chemistry*, 19(10): 2589-2597. DOI: 10.1002/etc.5620191027
- Rodriguez, D., Becker, C.G., Pupin, N.C., Haddad, C.F.B. & Zamudio, K.R. 2014. Long-term endemism of two highly divergent lineages of the amphibian-killing fungus in the Atlantic Forest of Brazil. *Molecular Ecology*, 23(4): 774-787. DOI: 10.1111/mec.12615
- Salla, R.F., Gamero, F.U., Ribeiro, L.R., Rizzi, G.M., Medico, S.E.D., Rissoli, R.Z., Vieira, C.A., ... Costa, M.J. 2015. Cardiac adaptations of bullfrog tadpoles in response to chytrid infection. *Journal of Experimental Zoology*, 323A(7): 487-496. DOI: 10.1002/jez.1945
- Samarasinghe, H., You, M., Jenkinson, T.S., Xu, J. & James, T.Y. 2020. Hybridization Facilitates Adaptive Evolution in Two Major Fungal Pathogens. *Genes*, 11(1): 101. DOI: 10.3390/genes11010101
- Schloegel, L.M., Toledo, L.F., Longcore, J.E., Greenspan, S.E., Vieira, C.A., Lee, M., Zhao, S., ... Daszak, P.J., T. Y. 2012. Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. *Molecular Ecology*, 21(21): 5162-5177. DOI: 10.1111/j.1365-294X.2012.05710.x
- Talley, B.L., Muletz, C.R., Vredenburg, V.T., Fleischer, R.C. & Lips, K.R. 2015. A century of *Batrachochytrium dendrobatidis* in Illinois amphibians (1888–1989). *Biological Conservation*, 182: 254-261. DOI: 10.1016/j.biocon.2014.12.007

Van der Schalie, W.H., Gardner Jr, H.S., Bantle, J.A., De Rosa, C.T., Finch, R.A., Reif, J.S., Reuter, R.H., ... Folmar, L.C. 1999. Animals as sentinels of human health hazards of environmental chemicals. *Environmental Health Perspectives*, 107(4): 309. DOI: 10.1289/ehp.99107309

Walker, S.F., Bosch, J., James, T.Y., Litvintseva, A.P., Valls, J.A.O., Piña, S.I., García, G., ... Fisher, M.C. 2008. Invasive pathogens threaten species recovery programs. *Current Biology*, 18(18): R853-R854. DOI: 10.1016/j.cub.2008.07.033

Weldon, C., Du Preez, L.H., Hyatt, A.D., Muller, R. & Speare, R. 2004. Origin of the amphibian chytrid fungus. *Emerging Infectious Diseases*, 10(12): 2100-2105. DOI: 10.3201/eid1012.030804

Welsh Jr, H.H. & Ollivier, L.M. 1998. Stream amphibians as indicators of ecosystem stress: a case study from California's redwoods. *Ecological Applications*, 8(4): 1118-1132. DOI: 10.2307/2640966

Wilson, J.B. 1999. Guilds, functional types and ecological groups. *Oikos*: 507-522. DOI: <http://dx.doi.org/10.2307/3546655>

Zhu, W., Bai, C., Wang, S., Soto-Azat, C., Li, X., Liu, X. & Li, Y. 2014. Retrospective survey of museum specimens reveals historically widespread presence of *Batrachochytrium dendrobatidis* in China. *EcoHealth*, 11(2): 241-250. DOI: 10.1007/s10393-013-0894-7

Chapter 2

In vivo transmission capabilities of *Batrachochytrium dendrobatidis* lineages

Abstract

The amphibian chytrid fungus known as *Batrachochytrium dendrobatidis* (*Bd*) has garnered much focus as research subject and remains of paramount importance for conservation efforts. Since South Africa is host to multiple lineages of amphibian chytrid it became necessary to study multi-lineage co-infection in amphibian hosts. This led to the development of an experiment testing the virulence of multiple *Bd* lineages, not only in terms of mortality caused, but also in terms of transmission capabilities to naïve and already infected hosts. Using a model South African hosts species, *Sclerophrys gutturalis*, the transmission capabilities of both *Bd*CAPE and *Bd*GPL was studied, as well as the other quantifiers of virulence, infection intensity and the time till becoming symptomatic, as well as studying the possibility to induce multiple lineage co-infection. It was found that lineages previously described as hypovirulent, in this case *Bd*CAPE, was able to transmit to more lineage naïve individuals than the hypervirulent *Bd*GPL lineage. The competition trial showed that it is possible to induce multi-lineage infection with both *Bd*GPL and *Bd*CAPE on the same host by co-housing, but this possibility seemed to be one directional, with only a few *Bd*CAPE originally infected animals additionally becoming infected with *Bd*GPL. It was proposed that the increased transmissibility of the hypovirulent lineage could be a function of the time available to transmit as *Bd*GPL invariably caused individuals to become symptomatic, and consequently be removed from the experiment, before *Bd*CAPE. The confirmation that co-infection can be found highlighted the need for increased surveillance in areas where multiple lineages can be found, and the possibility that already *Bd* positive populations can still be at threat from novel lineages.

Keywords:

Batrachochytrium dendrobatidis; disease transmission; disease dynamics; experimental disease biology

Introduction

Batrachochytrium dendrobatidis (further referred to as *Bd*) is the lead causative agent of amphibian declines globally through the disease chytridiomycosis (Farrer *et al.*, 2011). Extensive and robust genomic analysis has shown that *Bd* comprises several genetic lineages with the most likely origin in Asia (O'Hanlon *et al.*, 2018). *Bd* can have severe detrimental effects for captive breeding programs, such as was seen at the Kihansi spray toad captive breeding facility in Tanzania in 2012 (Makange *et al.*, 2014). The knowledge that *Bd* can also infect captive anurans along with other disease-causing organisms (Miller *et al.*, 2008) such as ranavirus and *Aeromonas hydrophila*, resulted in the need to determine the transmission capability thereof, especially as South Africa is host to multiple lineages (O'Hanlon *et al.*, 2018; Fisher & Garner, 2020). The results from lineage-specific PCR's from a South African large scale *Bd* survey indicated that a single site, as well as a single individual, tested positive for both *Bd*CAPE and *Bd*GPL (the predominant lineages in Africa; Chapter 5 - Ghosh *et al.*, 2021). This further confirmed the need for a quantitative understanding of the transmission capabilities of these lineages.

One lineage in particular – *Bd*GPL – has been linked to enigmatic declines around the globe (Farrer *et al.*, 2011). It has also been found to form recombinant lineages in South Africa and Brazil, in areas where more than one lineage is present (Jenkinson *et al.*, 2016; O'Hanlon *et al.*, 2018). In Brazil these recombinants were found associated with the bullfrog trade (Schloegel *et al.*, 2012) while in South Africa they were found in completely natural settings. The possibility for recombination further exists in Southeast Asia and Northern Europe that also host more than one genetic lineage of *Bd* in close proximity distributions forming contact and possibly even overlapping distribution zones (Fisher & Garner, 2020). In South Africa *Bd*CAPE, *Bd*GPL and a recombinant lineage all occur in the same locality (O'Hanlon *et al.*, 2018; Ghosh *et al.*, 2021) necessitating a quantitative investigation into the transmission capabilities of these lineages in a host model. Host density increases the number of animals infected asymptotically in a controlled environment, but in a natural population a minimum threshold population size is needed for the disease to survive (Kilpatrick *et al.*, 2010), possibly restricting the recombinant lineage's distribution in natural settings. In animal experiments transmission of the disease will therefore depend on the ability of the disease-causing organism itself and the host to survive, and not on numbers exceeding a minimum threshold.

Variability of pathogen virulence can influence the outcome of experimental trials. *Bd*GPL is often described as hypervirulent when compared to other related lineages. Hypervirulence in *Bd*GPL has been described as being able to cause mortality in the host in a shorter time than other lineages (Farrer *et al.*, 2011), but virulence could be characterized by considering other parameters such as transmission capability, as mortality that is rapidly brought on invariably

prevents further transmission to susceptible hosts. In turn, transmission potential could be influenced by infection intensity and the frequency of contact between naïve and infected individuals. In this study we investigated transmission potential of two *Bd* lineages in a South African frog species. The multi-lineage presence of *Bd* in South Africa, and general lack of understanding of lineage-specific transmission dynamics highlights this study as a conservation research priority.

Material and methods

Three egg-strings of the Guttural toad, *Sclerophrys gutturalis*, were collected from natural ponds in the Potchefstroom area (permit: HQ 18/05/16-088 NW). *Bd* has not been detected in the area, and collection of animals as egg-strings negates the possibility of being infected upon collection as *Bd* grows using the keratin in tooth rows of tadpoles. Ethical approval for the experimental design and execution was obtained from the NWU Animal Research Ethics Committee (approval number: NWU-00015-16-S5). This species is abundantly available, can be raised in the laboratory with relative ease and is a suitable model due to the potential of being infected experimentally with *B. dendrobatidis* (De Jong *et al.*, 2018; Weldon, Unpublished data).

The eggs were hatched in aerated plastic trays containing a 1:1 ratio of aged borehole water and filtered water. After one week 160 tadpoles were randomly selected from the three egg strings and moved to an aerated aquarium (W = 30 cm; L = 50 cm; H = 30 cm) containing the same mixture of water. The remaining tadpoles were euthanized by submersion in a MS222 solution (250mg/L) and their carcasses disposed as biological waste. The experimental animals were fed Tetra Tabimin™ daily at a concentration of 100mg/100mL H₂O. Half of the water in the aquariums was replaced every second day, while concurrently removing debris from the tanks. This regime was maintained until the tadpoles successfully developed all four limbs, at which time they were transferred to slanted aquaria containing a shallow pool of water at the lower end and wetted tissue paper for substrate at the other end. The aquarium was fitted with a PVC halfpipe that acted as a shelter for the metamorphs. During this phase of development that lasted one to two days, the tails were resorbed and no additional food was provided and the substrate was replaced daily. Metamorphosed toadlets were transferred to individual 500 mL plastic tubs (W = 75 mm; L = 125 mm; H = 60 mm) with wet tissue paper for substrate. The feeding regime now changed to seven pinhead crickets per individual every second day, and tubs were cleaned every fourth day.

Experimental design

All 160 toadlets were weighed and randomly allocated to three groups (no significant difference in body mass between groups, $p = 0.2995$; $F=1.215$). The animals were photographed close-up using a macro lens mounted on a Canon EOS 600D, to distinguish dorsal colour patterns among paired animals. Photographs were taken before exposure to *Bd* and used to confirm individual identity upon completion of the experiment. Forty animals were exposed to *Bd*GPL (isolate 08MG04), 40 to *Bd*CAPE (isolate SA4c), and 80 animals to sterilised media that served as control groups or uninfected (naïve) animals. Isolate lineage was previously identified by whole genome sequencing and confirmed in O'Hanlon *et al.* (2018). Four exposures over five days were done in 20 mL water baths, and toadlets received 500 μ L media containing 110 000 - 145 000 zsp/mL of either lineage or sterile culture media. After dosing, a two-day acclimation period followed after which the animals were co-housed in pairs in sterile 500 mL tubs for a maximum of 14 days. There were 17 pairs for the *Bd*GPL vs Naïve treatment, 20 pairs for *Bd*CAPE vs Naïve treatment, 19 pairs for the *Bd*GPL vs *Bd*CAPE treatment and 19 pairs for the control treatment, with different number of pairs resulting from animals reaching humane endpoints and removed from the experiment during infection protocol completion prior to initiation of the transmission phase of the experiment. Animals were checked daily for signs of failing health associated with chytridiomycosis, such as lethargy, lack of ability to feed or reaction to external stimuli, such as opening of the enclosures or the experimenter gently touching the animal using a sterile pinset tweezers (Daszak *et al.*, 1999; Rosenblum *et al.*, 2009). If one of the paired animals reached the experimental end points, both animals were euthanised. All remaining animals were euthanized at the end of the experiment by MS222 (250mg/L) submersion. All carcasses were preserved in 70 % ethanol until tissue samples were subjected to lineage-specific real-time PCR analyses, as described in Chapter 5 (Ghosh *et al.*, 2021).

Statistical analyses

All statistical analyses were done in GraphPad Prism 8.0.2. An Ordinary one-way ANOVA was used for the description of the animal weights due to the normal distribution of the data. Kolmogorov-Smirnov tests were used to describe and compare the infection intensities for all datasets as most did not meet normal data distribution standards.

Results

The 19-pair control group which only housed animals that were exposed to enrichment media all tested negative for *Bd*. All *Bd*GPL infected animals from the *Bd*GPL infected vs naïve trial reached a humane endpoint (displayed clinical signs of moribund condition), requiring euthanasia before the full 14-day period transpired (Figure 2).

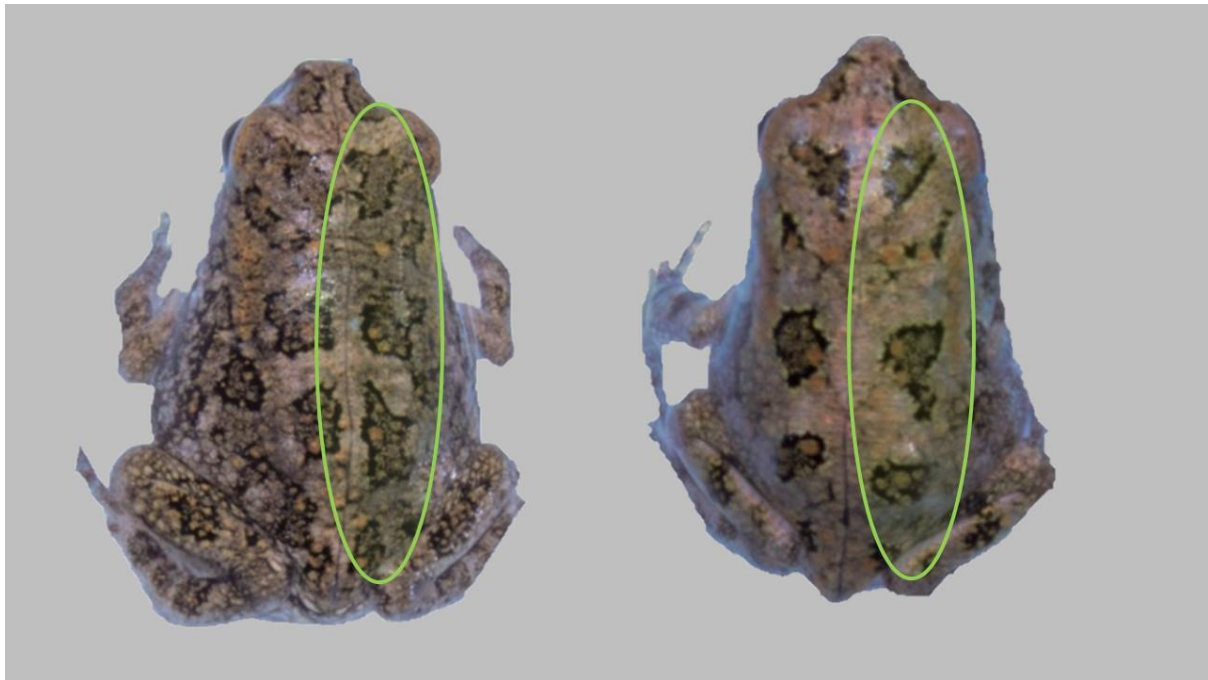


Figure 1: Variation in dorsal colour pattern allowed for identification of paired individuals from different treatment groups with green area showing focus area for identifying differences

Variation in dorsal colour pattern allowed distinction between treatment animals (Figure 1). The individuals from the naïve group with which these animals were paired were simultaneously euthanized, although they did not display clinical symptoms. Almost two thirds (64.7 %) of the naïve animals did test positive for *Bd*GPL, but mean infection intensity was significantly lower than in the source animals ($p < 0.0001$; K-S $D = 0.9412$; infected group = 52366 ± 12225 GE $\pm$ SE; naïve group = 865.7 ± 469.6 GE $\pm$ SE). Whereas mean infection intensity decreased slightly in the infected group during the latter half of the trial when they became moribund ($m = -0.011392$), infection intensity increased in the naïve group for the same period ($m = 0.1831424$; Figure 2).

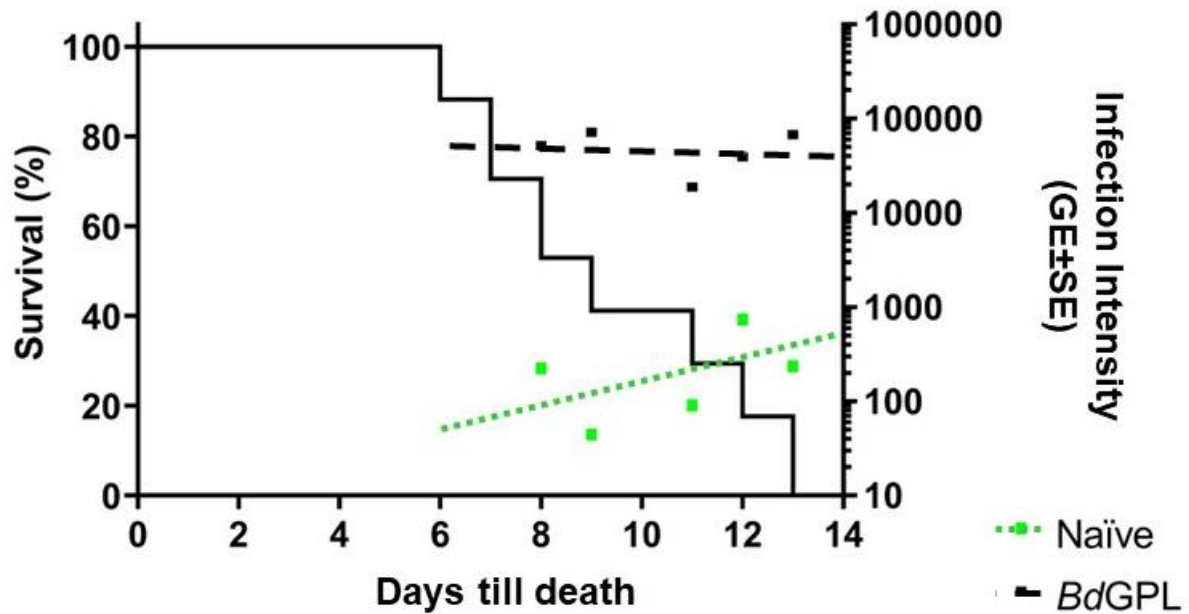


Figure 2: Kaplan-Meier survival curves for the *BdGPL* transmission trial with infection intensities determined for individuals euthanized

All animals from the *BdCAPE* transmission trial (*BdCAPE* infected vs naïve) survived the full duration of the experiment, with 85 % of the naïve animals acquiring a *BdCAPE* infection. Mean infection intensity of the originally infected animals at the time of experimental termination was significantly higher than that which had been established in the naïve animals at the same point in time ($p < 0.0001$; K-S D = 0.900; *BdCAPE* = 994.5 ± 251.7 GE $\pm$ SE; naïve = 60.56 ± 30.96 GE $\pm$ SE).

All except two of the *BdGPL* infected animals from the lineage competition trial (*BdGPL* infected vs *BdCAPE* infected) reached the experimental endpoint before the experiment was terminated (Figure 3). The *BdCAPE* group remained asymptomatic. Animals from both treatment groups retained their original infection, and three of the original *BdCAPE* infected animals tested positive for both lineages at conclusion of the experiment. Mean infection intensity differed significantly between the original *BdGPL* and *BdCAPE* infected individuals ($p < 0.0001$; K-S D = 1.000; *BdGPL* = 61375 ± 18873 GE $\pm$ SE; *BdCAPE* = 588.9 ± 83.81 GE $\pm$ SE), and remained almost constant over time for *BdGPL*, but increased slightly for *BdCAPE* ($m = -0.00545$ and 0.0342 respectively; Figure 3).

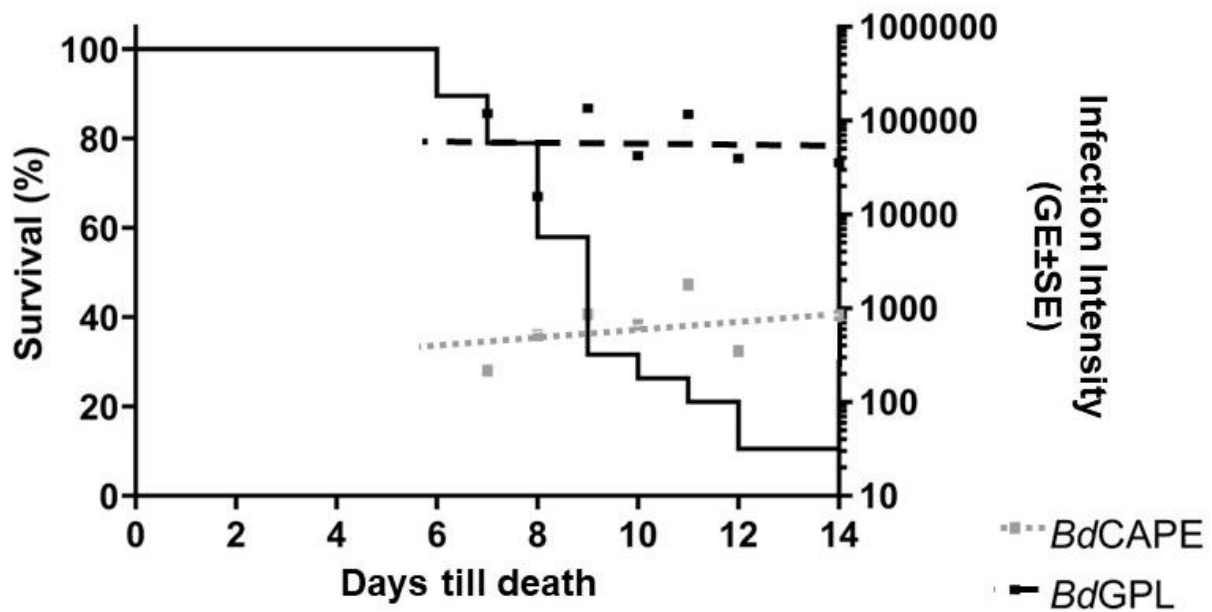


Figure 3: Kaplan-Meier survival curves for the *BdGPL* vs *BdCAPE* transmission trial with infection intensities determined for individuals euthanized.

The *BdGPL* infected toads in trials 1 (*BdGPL* vs naïve) and 3 (*BdGPL* vs *BdCAPE*) retained similar infection levels ($p=0.6877$; K-S $D=0.2384$), and so did the *BdCAPE* infected toads in trials 2 (*BdCAPE* vs naïve) and 3 ($p=0.3878$; K-S $D=0.2895$; Figure 4). The infection intensity of the two naïve groups did however differ significantly after exposure to *BdGPL* infected toads in trial 1 and *BdCAPE* infected toads in trial 2, with infection intensity in the group that contracted *BdGPL* being significantly higher ($p=0.0057$; K-S $D=0.5647$) compared to the naïve animals that became infected with *BdCAPE* during the transmission trial (trial 2). Overall, the mean infection intensity of the original *BdCAPE* infected toads was significantly lower ($p<0.0001$; K-S $D=1.000$) than that of the toads originally exposed to *BdGPL*.

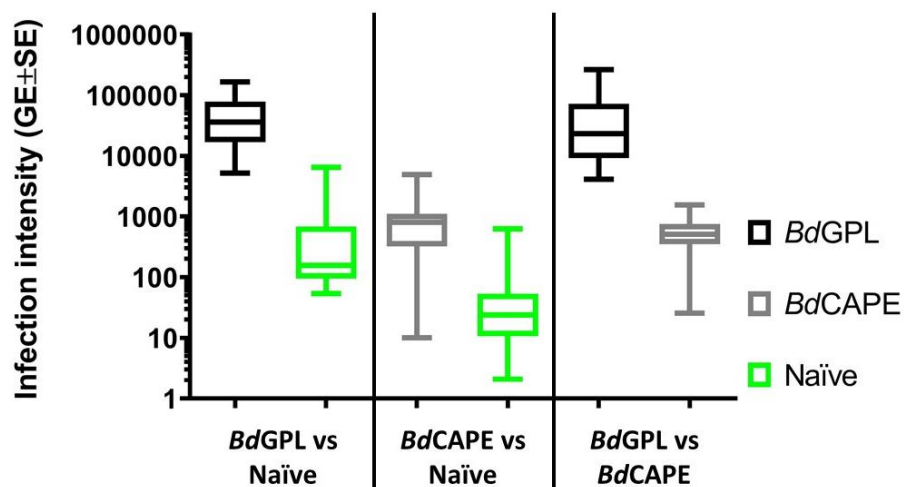


Figure 4: Box and whiskers diagram depicting the infection intensity average for every group of animals for each trial

Discussion

Previous experiments have shown that *Bd*GPL can be hypervirulent when using infection intensity and mortality of the host as parameters (Farrer *et al.*, 2011). One might assume that transmission potential is necessarily a reflection of virulence under the notion that it is a fundamental component of pathogen dynamics. Our experiments demonstrated that transmission took place at rates unrelated to the conventionally perceived lineage virulence. The hypovirulent *Bd*CAPE lineage, managed to infect more naïve individuals under the same controlled conditions than the hypervirulent *Bd*GPL. The premise that it can be beneficial for the disease to remain less virulent but more transmissible can be seen throughout pathogen history in other animal groups, from the Spanish flu originating from an avian ancestor, spreading to pigs and then to humans (Shortridge, 1999), the 2009 H1N1 pandemic (Girard *et al.*, 2010), to the more recent example of Coronavirus (Guo *et al.*, 2020; Umakanthan *et al.*, 2020). Our results indicate how transmission can be dictated by time exposed and that probability of transmission is increased over time, all of which can only happen if the disease and the host persists. When *Bd*GPL animals succumbed to chytridiomycosis, *Bd*CAPE animals persisted asymptotically and transmitted infection to naïve animals. Severely disease compromised individuals therefore lose the potential to infect new hosts when they die and are removed from the population, as witnessed in the *Bd*GPL trial (mean GE > 570000). However, the ability to transmit is also related to infection intensity and one has to assume that as long as animals with *Bd*GPL remain alive (with an infection intensity 50x higher than *Bd*CAPE), so too does transmission potential remain high based on the higher number of zoospore-producing sporangia being present and active on the host.

Where more than one lineage of *Bd* occur within a natural system surrounded by unfavourable habitat for both host and pathogen (Tarrant *et al.*, 2013; Ghosh *et al.*, 2021), their distributions are more likely to overlap provided that there are no geographical or other barriers preventing their dissemination. In fact, hybridization is known to occur in areas where their distribution overlap, such as in Brazil and South Africa (Jenkinson *et al.*, 2016; O'Hanlon *et al.*, 2018). It is highly probable that during these recombination events amphibian hosts become infected with more than one lineage at the same time. But, infection with multiple lineages of *Bd* has rarely been documented in wild amphibian populations. Through the use of lineage-specific qPCR, frogs infected with *Bd*CAPE and *Bd*GPL, as well as concurrent infections with both *Bd*CAPE and *Bd*GPL were detected in a region of South Africa previously identified as a corridor (Tarrant *et al.*, 2013) between the predominantly *Bd*GPL western reaches of the Orange River and the *Bd*CAPE dominated headwaters towards the eastern highlands (Chapter 5 - Ghosh *et al.*, 2021). The field data, however, did not explain the ecological principles that govern co-infection. When exposed to an experimental environment with the

potential of acquiring a second lineage, all of the animals in our study retained their original infection without exception. Of these, *BdGPL* transmitted to three *BdCAPE* animals, resulting in co-infection. The ecological interaction between *Bd* lineages once a lineage is established on a host (e.g. utilizing available resources), takes on the characteristics of competitive exclusion in preventing another lineage from developing and reproducing on the same resource, much the same as with examples from the *Salmonella* genus (Rabsch *et al.*, 2000) and probiotic control of bacterial infections in shrimp farming (Knipe *et al.*, 2021). We assume that cross-transmission is restricted in this competitive setting, because of the high transmission potential demonstrated in the infected vs naïve trials. The mechanism by which competitive exclusion takes place is not yet understood. The fact that amphibian skin is a finite resource and that carrying capacity can be reached by one lineage due to increased infection intensity before the other, can play a role by means of intraspecific competition (Begon *et al.*, 2006), and might very well explain this observation. This would explain why the more aggressive developing *BdGPL* maintained an almost stable high infection intensity in the group during the latter half of the experiment, while infection intensity increased with time in the animals infected with the slower developing *BdCAPE*. It is apparent though, that *BdGPL* has the competitive advantage in its ability to overcome the limitations of transmission when competitive exclusion predominates. Not only was transmission restricted but development and reproduction of both lineages were unimpeded in the presence of a competitive lineage. This is evident by the fact that similar infection intensities were retained among the same lineage groups from infected vs naïve trials and the competition trial, indicating that apart from being in competition for the initial colonisation of the habitat, no further competitive suppression of growth was observed.

Our results support the field observations in Chapter 5 (Ghosh *et al.*, 2021) that more than one lineage of *Bd* can infect amphibians simultaneously, and our experiment demonstrates that co-infection can be induced. The high transmission potential of *BdCAPE* may influence conservation approaches towards this lineage that is historically considered hypovirulent and of lesser concern in relation to the closely related global panzootic lineage (*BdGPL*). The possibility for co-infections that may arise due to the ability for invasion of *BdGPL* into naïve populations or populations from regions dominated by *BdCAPE* can lead to increased pressures on already threatened amphibian populations and lead to more fungal hybridization (Chapter 6 - Doherty-Bone *et al.*, 2020). Because inducing co-infection was proven to be rare, but possible, refining these experimental procedures will benefit future genetic studies aimed at understanding host-pathogen pressures that drive fungal recombination.

References

- Begon, M., Townsend, C.R. & Harper, J.L. 2006. *Ecology: From Individuals to Ecosystems*. 4th ed. Oxford, United Kingdom: Blackwell Publishing.
- Daszak, P., Berger, L., Cunningham, A.A., Hyatt, A.D., Green, D.E. & Speare, R. 1999. Emerging infectious diseases and amphibian population declines. *Emerging Infectious Diseases*, 5(6): 735-748. DOI: 10.3201/eid0506.990601
- De Jong, M.S., Van Dyk, R. & Weldon, C. 2018. Antifungal efficacy of F10SC veterinary disinfectant against *Batrachochytrium dendrobatidis*. *Medical Mycology*, 56(1): 60-68. DOI: 10.1093/mmy/myx023
- Doherty-Bone, T., Cunningham, A., Fisher, M., Garner, T., Ghosh, P., Gower, D., Verster, R., ... Weldon, C. 2020. Amphibian chytrid fungus in Africa—realigning hypotheses and the research paradigm. *Animal Conservation*, 23(3): 239-244. DOI: 10.1111/acv.12538
- Farrer, R.A., Weinert, L.A., Bielby, J., Garner, T.W.J., Balloux, F., Clare, F., Bosch, J., ... Fisher, M.C. 2011. Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proceedings of the National Academy of Sciences*, 108(46): 18732-18736. DOI:10.1073/pnas.1111915108
- Fisher, M.C. & Garner, T.W.J. 2020. Chytrid fungi and global amphibian declines. *Nature Reviews Microbiology*: 1-12. DOI: 10.1038/s41579-020-0335- x
- Ghosh, P.N., Verster, R., Sewell, T.R., O'Hanlon, S.J., Brookes, L.M., Rieux, A., Garner, T.W.J., ... Fisher, M.C. 2021. Discriminating lineages of *Batrachochytrium dendrobatidis* using quantitative PCR. *Molecular Ecology Resources*, 21: 1452-1459. DOI: 10.1111/1755-0998.13299
- Girard, M.P., Tam, J.S., Assossou, O.M. & Kieny, M.P. 2010. The 2009 A (H1N1) influenza virus pandemic: a review. *Vaccine*, 28(31): 4895-4902. DOI: 10.1016/j.vaccine.2010.05.031
- Guo, Y.-R., Cao, Q.-D., Hong, Z.-S., Tan, Y.-Y., Chen, S.-D., Jin, H.-J., Tan, K.-S., ... Yan, Y. 2020. The origin, transmission and clinical therapies on coronavirus disease 2019 (COVID-19) outbreak – an update on the status. *Military Medical Research*, 7(11): 1-10. DOI: 10.1186/s40779-020-00240-0
- Jenkinson, T.S., Betancourt Román, C.M., Lambertini, C., Valencia-Aguilar, A., Rodriguez, D., Nunes-de-Almeida, C.H.L., Ruggeri, J., ... James, T.Y. 2016. Amphibian-killing chytrid in Brazil comprises both locally endemic and globally expanding populations. *Molecular Ecology*, 25(13): 2978-2996. DOI:10.1111/mec.13599

- Kilpatrick, A.M., Briggs, C.J. & Daszak, P. 2010. The ecology and impact of chytridiomycosis: an emerging disease of amphibians. *Trends in Ecology and Evolution*, 25(2): 109-118. DOI: 10.1016/j.tree.2009.07.011
- Knipe, H., Temperton, B., Lange, A., Bass, D. & Tyler, C.R. 2021. Probiotics and competitive exclusion of pathogens in shrimp aquaculture. *Reviews in Aquaculture*, 13(1): 324-352. DOI: 10.1111/raq.12477
- Makange, M., Kulaya, N., Biseko, E., Kalenga, P., Mutagwaba, S. & Misinzo, G. 2014. *Batrachochytrium dendrobatidis* detected in Kihansi spray toads at a captive breeding facility (Kihansi, Tanzania). *Diseases of Aquatic Organisms*, 111(2): 159-164. DOI: 10.3354/dao02775
- Miller, D.L., Rajeev, S., Brookins, M., Cook, J., Whittington, L. & Baldwin, C.A. 2008. Concurrent infection with ranavirus, *Batrachochytrium dendrobatidis*, and *Aeromonas* in a captive anuran colony. *Journal of Zoo and Wildlife Medicine*, 39(3): 445-449. DOI: 10.1638/2008-0012.1
- O'Hanlon, S.J., Rieux, A., Farrer, R.A., Rosa, G.M., Waldman, B., Bataille, A., Kosch, T.A., ... Fisher, M.C. 2018. Recent Asian origin of chytrid fungi causing global amphibian declines. *Science*, 360: 621-627. DOI: 10.1126/science.aar1965
- Rabsch, W., Hargis, B.M., Tsois, R.M., Kingsley, R.A., Hinz, K.-H., Tschäpe, H. & Bäuml, A.J. 2000. Competitive exclusion of *Salmonella* Enteritidis by *Salmonella* Gallinarum in poultry. *Emerging Infectious Diseases*, 6(5): 443. DOI: 10.3201/eid0605.000501
- Rosenblum, E.B., Poorten, T.J., Settles, M., Murdoch, G.K., Robert, J., Maddox, N. & Eisen, M.B. 2009. Genome-wide transcriptional response of *Silurana* (*Xenopus*) *tropicalis* to infection with the deadly chytrid fungus. *PLoS ONE*, 4(8): e6494. DOI: 10.1371/journal.pone.0006494
- Schloegel, L.M., Toledo, L.F., Longcore, J.E., Greenspan, S.E., Vieira, C.A., Lee, M., Zhao, S., ... Daszak, P.J., T. Y. 2012. Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. *Molecular Ecology*, 21(21): 5162-5177. DOI: 10.1111/j.1365-294X.2012.05710.x
- Shortridge, K.F. 1999. The 1918 'Spanish' flu: pearls from swine? *Nature Medicine*, 5(4): 384-385. DOI: 10.1038/7383
- Tarrant, J., Cilliers, D., Du Preez, L.H. & Weldon, C. 2013. Spatial assessment of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) in South Africa confirms endemic and widespread infection. *PLoS ONE*, 8(7): e69591. DOI: 10.1371/journal.pone.0069591

Umakanthan, S., Sahu, P., Ranade, A.V., Bukelo, M.M., Rao, J.S.I., Abrahao-Machado, L.F., Dahal, S., ... Dhananjaya, K.V. 2020. Origin, transmission, diagnosis and management of coronavirus disease 2019 (COVID-19). *Postgraduate Medical Journal*, 96(1142): 753-758. DOI: 10.1136/postgradmedj-2020-138234

Weldon, C. Unpublished data. *Batrachochytrium dendrobatidis* experimental protocols and animal husbandry. Unpublished.

Chapter 3

Lineage dependent alteration in behaviour of *Cacosternum boettgeri* infected with *Batrachochytrium dendrobatidis*

Abstract

The amphibian chytrid fungus has been seen to have extensive impacts on population levels of amphibians. The various lineages that exist has been seen to exhibit different virulence levels and potential for causing mortality in host amphibians infected. The behavioural influences of *Batrachochytrium dendrobatidis* has yet to be extensively investigated on lineage level, while the dangers posed to amphibians by the different lineages have only become more prominent. Here we designed an experiment that made use of Noldus Ethovision XT14 software to track different behavioural parameters of tadpoles infected with two different lineages of amphibian chytrid fungus. It was found that not all behaviours are equally impacted by *Bd* infection, and often the impact is lineage specific. *Bd*CAPE was seen to increase measured parameters compared to control, while *Bd*GPL often decreased the movement parameters of the infected animals when compared with the control animals. These changes in movement parameters, whether it be an increase or decrease dependent on lineage, is considered as a change in the investment of resources in terms of time and energy by the host, also impacting its foraging ability. These changes can lead to increased predation risk by the host, ultimately leading to differing survival strategies within the same species of host based on the infecting lineage of *Bd*. It was also found that *Bd* can have a major impact, not just morphologically, on a life stage of amphibians generally not considered to be mortally influenced by infection.

Keywords:

Batrachochytrium dendrobatidis; tadpole behaviour experiment; tadpole lifestyle adaptation; behavioural fitness

Introduction

Behavioural fitness involves the ability of an individual animal to survive due to its behavioural traits and to successfully reproduce; and is fast becoming a monitoring tool for ecological effects (Greggor *et al.*, 2019). Owing to intrinsic and extrinsic differences associated with the metamorphic amphibian mode of life, the advantageous characteristics for survival are likely to differ in tadpoles and post-metamorphic individuals. When applied to amphibian larvae behavioural fitness is better defined by the behavioural traits that will allow them a greater chance of reaching metamorphosis and subsequently developing into reproducing adults. Survival fitness of the individual may have evolutionary benefits for the species by means of natural selection over extended time periods (Kardong, 2008). This evolution of fitness has been described as a continuous cycle of increase of advantageous characteristics due to natural selection and decrease of disadvantageous characteristics due to various forces of degradation (Fisher, 1958). Infectious disease, and the influence it has on individual behaviour, is an example of how survival fitness is decreased, even if the disease is non-lethal for the specific life stage in question, such as tadpoles (Garner *et al.*, 2009).

The amphibian chytrid fungus (*Batrachochytrium dendrobatidis* – hereon referred to as *Bd*) has been described as the single most significant cause of amphibian declines worldwide (Berger *et al.*, 1999; Rosenblum *et al.*, 2010; O'Hanlon *et al.*, 2018; Fisher & Garner, 2020). This fungus tends to show seasonal disease dynamics, especially in terms of prevalence, depending on the prevailing climate and habitat in which the amphibian hosts are found (Longo *et al.*, 2010; Petersen *et al.*, 2016). Most amphibian species, having to cope with the needs for both aquatic and terrestrial habitats, are challenged further by this fungus that displays an upwards flux in virulence and presence coinciding with season of host activity. Although disease progress and host extinction risk have been well documented (Scheele *et al.*, 2019; Weldon *et al.*, 2020; Fisher *et al.*, 2021), it has yet to be discerned how disease-altered host behaviour contributes to these extinction risks.

Although tadpoles generally harbour asymptomatic infections, deformities in mouthpart morphology can occur, which in turn reduces the amount of food ingested, often used as a measure of foraging efficiency (Venesky *et al.*, 2009; Hanlon *et al.*, 2015). Smaller body size at metamorphoses as a compromise for stable developmental time has been documented in *Bufo fowleri*, ultimately leading to compromised energetics (Parris & Cornelius, 2004). Infection in tadpoles can either benefit or disadvantage their ability to avoid predation and appears to be host species dependent. Parris *et al.* (2006) found that animals infected with *Bd* experience difficulty detecting predators, requiring both visual and chemical cues to elicit a defensive response. On the contrary, infected host survival chances can be increased in some

species due to them displaying lower activity levels or more actively seeking refuge when facing predator cues (Han *et al.*, 2011).

Although previous studies on *Bd*-altered host behaviour provided some insight into how behaviour was influenced, the changes have yet to be quantified. Nor has the role of *Bd*-genotype influence on host behaviour been defined and used to attempt clarifying the survival fitness of the host. Our study was designed to quantify behaviour using Noldus Ethovision software. This allowed for the measurement and comparison of a number of different parameters to test the behaviour of tadpoles in the presence of a chemical predator cue, but also how the different lineages of *Bd* have influenced the choice of habitat and movement when presented with a feed pellet. The specific species used has not been analysed, to our knowledge, in terms of behaviour while infected with *Bd*, and thus necessitated the baseline determination of behavioural parameters. The aim was thus to identify the major measurable differences in behaviour induced by two lineages of *Bd* in tadpoles of Boettger's dainty frog, *Cacosternum boettgeri*. The discovery that genetic recombination in *Bd* is still occurring and can give rise to novel lineages with variable virulence (Schloegel *et al.*, 2012; O'Hanlon *et al.*, 2018), has made the need apparent to discern potential lineage specific influences on host behaviour that different genotypes of the fungus may exhibit.

Material and methods

Egg clutches of Boettger's Caco, *Cacosternum boettgeri* were collected from breeding ponds in the vicinity of Koster (North West province), South Africa (-25.8610°, 26.9121°), with no previous history of *Bd* infection. During transport clutches were stored in water from the breeding pond, at 20°C in five-litre plastic containers stored within thermal insulated boxes. This species has known susceptibility to *Bd* (Tarrant *et al.*, 2013) and is an easy-to-rear species under laboratory condition (Weldon, Unpublished data). All animals were collected under the permit issued by the North West Department of Rural, Environmental and Agricultural Development (Permit number: NW 7650/02/2019). These tadpoles were then housed in the biosecure facility at the North-West University Potchefstroom campus. The biosecure room was climate controlled at 23°C with a 12h/12h day-night cycle. The room was also maintained under negative pressure, with disinfectant footbaths, double door access restriction, biohazard removal by approved NWU contractors and all consumables and tools were sterilised daily using Virkon® disinfectant. All animal husbandry and experimental procedures were approved by the NWU animal ethics committee, AnimCare (ethics number: NWU-00406-21-A5). Tadpoles were fed Tetra Tabimin™ daily at a concentration of 100 mg per tadpole. They were fed after any handling had taken place, or cleaning of the tubs at 4 pm

every day before experimentation. Once experimentation started, they were fed upon completion of the recording, with the only exception being the feeding trial where the animals were fed prior to the acclimation time starting in order to record their movements with food present. The animals were group reared to Gosner stage 36-39 at which time they were entered into the experiment.

In total 36 *C. boettgeri* tadpoles were used in this experiment. One group of 12 animals were infected with *BdCAPE*, another group of 12 with *BdGPL* and 12 animals were exposed to 1 mL sterile media on dose days. The infection protocol consisted of 10 doses over a period of 10 days with an average of 43 000 zoospores/mL, and 1 mL dose-volume per day. To limit the handling of the tadpoles as well as prevent any potential cross contamination, or injury due to netting of the tadpoles, the animals were dosed in their husbandry enclosures (W = 75 mm; L = 125 mm; H = 60 mm), whilst reducing the volume of water from 300 mL per enclosure, to 150 mL. The media containing zoospores, or sterile media in the case of control animals, was then added and the tadpoles were left in the reduced volume of water for 30 minutes. The enclosure water was then refilled with clean, aged borehole water. This protocol prevented the build-up of waste in the enclosures, while minimizing stress from handling or complete water changes. Twelve animals, four from each of the *BdGPL*, *BdCAPE* and control groups were euthanized (by submersion in MS-222 at 250mg/L) before behaviour recordings started, to confirm infection status, resulting in a total of 24 animals entering the recording phase of the experiment.

Non-invasive video recordings of behavioural traits commenced the day after dosing had finished. Recordings were taken between 8 am and 6 pm each day. Four trials were conducted on consecutive days to test behavioural differences. The trials consisted of a baseline trial, habitat selection, feeding ability, and behaviour in the presence of a predator cue. The first recording day consisted of determining the baseline behaviour for the tadpoles where behaviour without any manipulation apart from *Bd* were recorded, and the husbandry routine remained unaltered. The second recording day consisted of a habitat selection trial, in which the bottom of the transparent enclosures was externally altered to have half black and half white backgrounds, allowing for the estimation of time that the dark-bodied tadpoles spent potentially camouflaged against the darker background. This also allowed for determining tadpole exploratory behaviour by noting the number of times they transitioned between the zones. The third recording day consisted of a feeding trial. On this day, instead of tadpoles being fed after completion of the recording, a food pellet was placed in one corner of the enclosure before acclimation. During analysis the software was locked onto the feeding pellet's position, and the distance of the tadpole to the pellet was determined. The final recording day consisted of exposing tadpoles to a predator cue. Aquarium water of, the

African Sharptooth Catfish (*Clarias gariepinus*), a natural predator of amphibians, served as predator cue (obtained from the North-West University Aquarium). Thirty millilitres of seven-day *C. gariepinus*-exposed water were added to each tadpole enclosure at the start of the recording (10 % of enclosure water volume). All tadpoles were euthanized following this experiment by submersion in MS-222 solution (250mg/L).

Each trial consisted of tadpoles from three *Bd* infection status groups, namely *Bd*-negative (control group, n=8), *Bd*-positive with *Bd*GPL (n=8), and *Bd*-positive with *Bd*CAPE (n=8). Because of the physical limitations imposed by the camera's field of view and distance from the subject that would allow successful recording of tadpole movements, only six experimental enclosures could be recorded at a time. These enclosures were arranged in a 3x2 array and included two tadpoles from each group (Figure 1), with the enclosures externally modified for the habitat selection trial (Figure 2). Thus, each trial consisted of four recordings with the two animals from each of the above-mentioned groups being randomly assigned to replicates on the first day of recording, resulting in four recordings per trial with a different group of animals used for every recording. Enclosures had opaque sides in order to reduce external visual disturbances. A DSLR camera (Canon EOS 600D with a 18-55 mm lens) was mounted on a tripod and positioned directly above the enclosure array at a distance of 0.75 m. The focal point was set to the bottom of the enclosures and autofocus was turned off. Thirty-minute videos were recorded at a resolution of 1920 x 1080 at 25 frames/sec with a monochrome filter to improve detectability for the tracking program. The experimenter left the room after starting the recording and no further access was allowed during the recordings. The first five minutes of each recording was discarded as acclimation time. Ethovision XT 14 (from Noldus, Netherlands) was used to analyse the recordings and measure the different parameters used as indicators of behavioural traits.

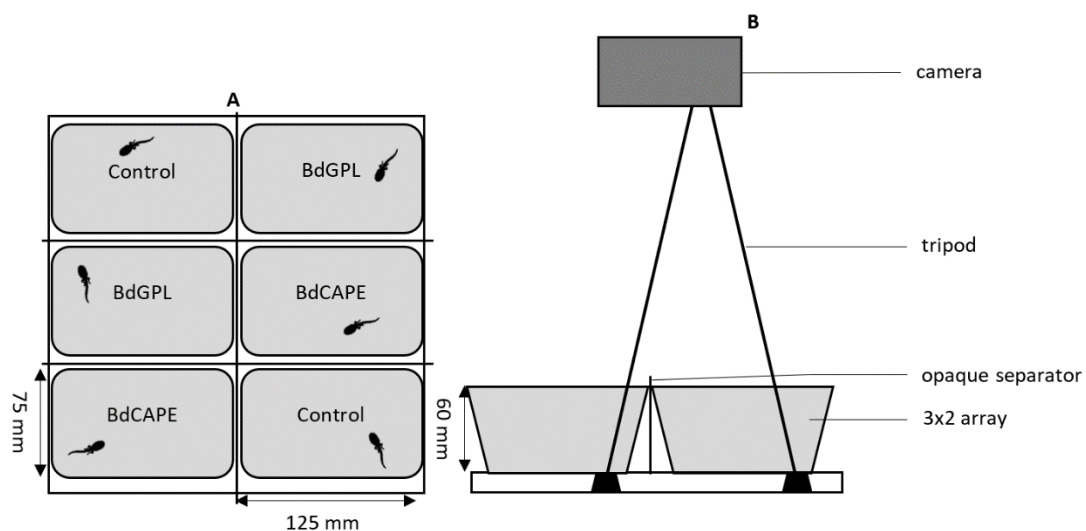


Figure 1: Experimental array setup for the various trials A) top view indicating the arrangement of the groups by infection status; B) side view indicating the position of the camera relative to the experimental animal enclosures.

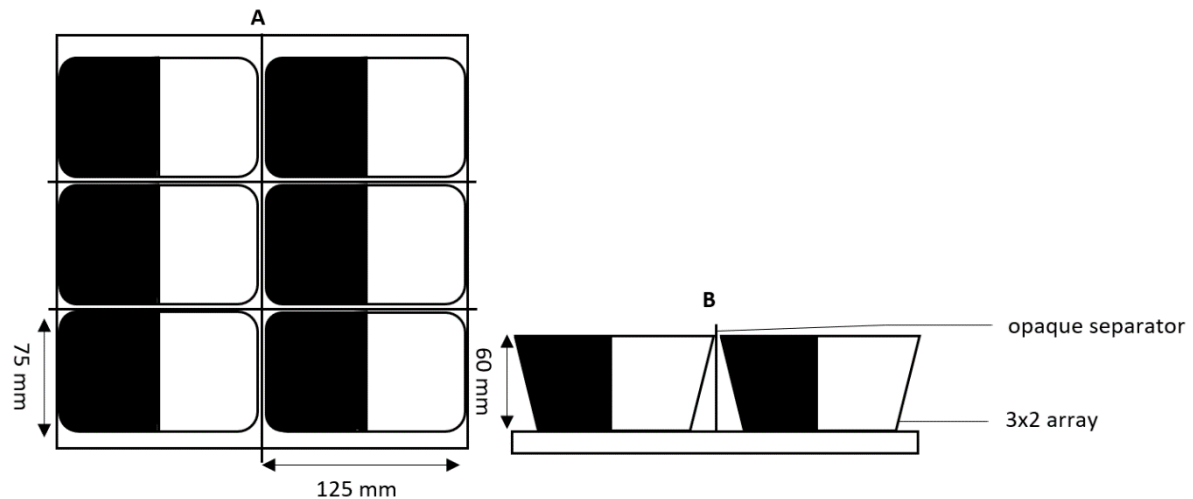


Figure 2: Illustration of enclosure modification for habitat selection trial

Ten different parameters were initially measured by the software. These included: *distance moved*, *velocity*, *mobility percentage* (determined in three different classes: highly mobile, mobile and immobile), *mobility time*, *movement time*, *no movement time*, *acceleration* and *surface breaching*. Mobility parameters measured the movement of the entire body of the animal subject, with mobility time measuring the time that any part of the whole body moved, while mobility percentage was calculated in terms of the proportion of the body that was detected to be moving, with above 80 % termed as highly mobile, less than 1 % immobile and remainder as mobile, with the entire body of the tadpole being used for detection by the programme. Time spent moving and not moving were determined as the speed at which the centre point of these animals moved, with any lack of movement as well as movement at a speed lower than 0.01 cm/s considered to be no movement. All movement at a speed greater than 0.01 cm/s was thus measured as Time Moved.

Mixed models effect analyses were used to determine the differences in the parameters measured, by taking the *Bd* infection status and trials as fixed effects. Means were calculated for each animal over the 25 minutes included in the recording, as well as for the same animal over the different trials. The interaction between the two was used to determine the effect size and significant difference between the estimated means. Effect sizes were ranged as follows: effect influences from 0.0 - 0.14 were termed too small to consider. Effect size 0.15 - 0.34 were termed as small and not visible, 0.35 - 0.44 as small to medium influence, 0.45 - 0.64 as medium, visible effect but not significant, 0.65 - 0.74 as medium to large effect visible, and effect sizes greater than 0.75 were termed as large practically visible and significant. Significance was calculated at 95 %. Canoco 4.5 was used to perform Canonical Correspondence Analysis (CCA) on the baseline trial to determine the subset of parameters measured that best described the data. When comparing the data within trials for the different

groups of animals, Ordinary one-way ANOVA were used, but when comparing different measurements for the same group within a trial Kolmogorov-Smirnov tests were used as not all the datasets had a normal distribution. Kruskal-Wallis tests were used to compare the pairs of data sets for the zone transition analyses.

Results

CCA was first performed on all ten parameters that were initially measured by the software, to determine the distribution of the different individuals' data. After removing the parameters that did not alter the distribution of the samples, four influential parameters remained (Figure 3-A vs B), namely Total Distance moved (*DistM*), Mean Time moving per event (*Move*), Mean Time not moving during stationary period (*No Move*), Absolute acceleration (*Abs_acc*), and Surface breaching (*Sur_Br*).

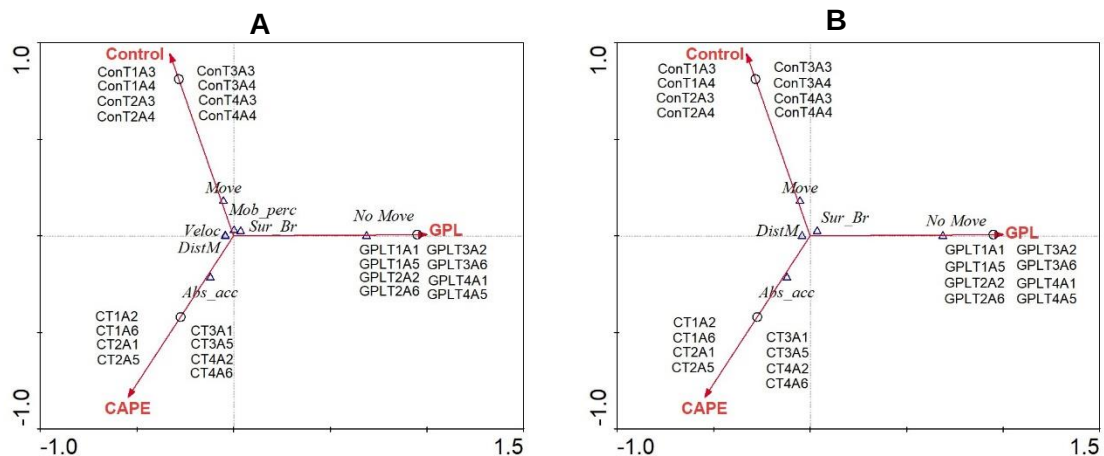


Figure 3: Canonical Correspondence Analysis graphs depicting the distribution of parameter data for the baseline trial for A) all parameters tested; B) the four most influential parameters

In general, it was seen that all the animals in the predator trial spent the longest time moving per moving event (effect size = 0.85), with the control animals in the feeding trial showing the shortest time moving per moving event compared to both the baseline and predator trials (effect size 0.89 and 0.85, respectively).

Baseline trial control animals had a mean time spent moving of 0.27 ± 0.11 s per moving event, feeding trial control animals had a mean of 0.16 ± 0.02 s per moving event and predator trial control animals had a mean of 0.27 ± 0.02 s per moving event, for the recording period. Large, visible and practically significant differences were seen when comparing the time spent per moving event for *BdGPL* groups of the predator trial (mean = 0.26 ± 0.07 s) with those of the

baseline (mean = 0.12 ± 0.02 s, effect size = 1.07) and feeding trials (mean = 0.13 ± 0.03 s, effect size = 1.01). *BdCAPE* animals showed a mean time spent moving of 0.25 ± 0.05 s per moving event for the baseline trial, 0.28 ± 0.06 s for the predator trial and 0.19 ± 0.06 s for the feeding trial. Although these values represented the time spent between motionless periods, it was seen (Figure 4-A vs Figure 5-A) that within both the baseline and predator trial the control and *BdCAPE* animals spent the same percentage of time moving (Baseline $p=0.66$, K-S $D=0.38$; Predator trial $p=0.98$, K-S $D=0.25$) at approximately 66 %. Within the baseline trial no significant difference could be seen when comparing the mean proportion of time spent moving versus not moving for any of the groups ($p=0.42$; $F=1.014$). Within the predator trial both the control and *BdCAPE* groups had significantly different proportions of time moving versus not moving ($p=0.002$, K-S $D=1.00$ and $p=0.019$, K-S $D=0.75$, respectively), with both groups having significantly higher times spent moving per event than being stationary between moving events (Figure 5-A).

When using effect sizes calculated on the mean time spent per moving or stationary event it was seen that a large and significant difference based on these effect sizes were seen when comparing control animals (mean = 8.69 ± 2.27 s) with *BdGPL* animals (effect size = 0.81; mean = 41.90 ± 15.35 s), while *BdCAPE* (mean = 12.76 ± 3.019 s) showed a medium to large effect size when compared to *BdGPL* (effect size = 0.72). All other pair wise comparisons for this parameter only showed small to medium effect sizes for *time not moving* (effect sizes ranged from 0.01 to 0.48).

Comparing the mean total distance moved by all animals within the baseline, predator and feeding trials, it was seen that in no instance a significant difference could be found between the different groups (Figure 4-B to Figure 6-B; $p=0.27$ to 0.99 , K-W=9.65). There was, however, large variation especially within the *BdCAPE* group. What was still apparent was that although the *BdCAPE* animals spent a larger proportion of their time moving when exposed to the predator cue, they travelled a shorter distance during that time (Figure 5-A vs B).

Absolute acceleration did not show any significant difference when comparing the animals across trial or *Bd* infection status (Figure 4-C to Figure 6-C; $p = 0.12$, $F=2.00$ to 0.25 , $F=1.42$). However, animals from the baseline trial achieved the highest mean *absolute acceleration*, but much variation within the data still occurred. This variation can be attributed to individual characteristics of the tadpoles, as it was seen that a single *BdCAPE* infected tadpole had a higher acceleration than the others, but this individual's data was still included in order to limit bias based on individual animal differences. In terms of effect sizes, *absolute acceleration* had a very small influence on behaviour when comparing the *BdGPL* groups

(mean = 0.05 ± 0.01 cm/s²) with *BdCAPE* groups (mean = 0.31 ± 0.21 cm/s²) across all four trials, since an effect size of 0.18 is too small to be practically visible.

During the feeding trial the control animals had a mean distance to the food pellet of 5.28 ± 0.96 cm; *BdCAPE* animals showed a mean of 5.19 ± 0.87 cm and *BdGPL* had a mean of 6.98 ± 0.97 cm. From an Ordinary One-Way ANOVA analysis (significance level 95 %) it was seen that these means did not differ significantly and significance varied between $p = 0.38$ to 0.997 ($F=1.17$). *BdGPL* did however have the largest mean distance to the feed pellet (Figure 6-E). It was noted during analysis of the video recordings that the animals' movement patterns were very similar when comparing the feeding trial to the predator trial. Although measured parameters may not have indicated statistically different measures, usage of the enclosure area seemed to have increased in the feeding and predator trials, as the movement was seen not to be restricted towards the sides of the enclosure. Animals were thus seen to cross the enclosure more often, while in the baseline trial they were seen to circle the enclosure in close proximity to the borders. It was also seen that the animals favoured the side at which the food pellet was placed.

Surface breaching did show small to medium effect sizes when comparing *BdGPL* (mean = 7.1 ± 1.55) with control animals (mean = 8.55 ± 1.92) and *BdCAPE* (mean = 9.15 ± 2.27) across the baseline, predator and feeding trials (Figure 4-D to Figure 6-D). These effect sizes ranged from 0.51 and 0.55 respectively for the baseline trial, and 0.15 and 0.27 respectively for the predator trial. The feeding trial showed a small effect size when comparing control with the *BdGPL* (0.34) and *BdCAPE* (0.29), indicating that the number of times these animals surfaced differed but was not practically visible.

The habitat selection trial showed no significant difference between the total time spent in the light zone for any of the three *Bd* infection status groups (Figure 7-A; $p=0.89$, $F=0.33$). A large, practically visible effect was however still seen when comparing the control group and *BdCAPE* group for the time they spent in the light zone (effect size = 0.80), with the time spent in the dark zone only showing a medium effect on behaviour (effect size = 0.47). When comparing the control group to *BdGPL*, a small to medium effect size was seen (0.42). The comparison between *BdCAPE* and *BdGPL* showed that the animals had a difference in behaviour to the effect size of 0.72, making it practically visible. It is important to note that the effect sizes were calculated using the mean amount of time every animal spent in either zone over 25 minutes. *BdCAPE* animals spent on average 45 % of their time in the light zone of the enclosure and 55 % in the dark zone (no significant difference; $p>0.99$, Figure 7-A). *BdGPL* spent 44 % of their time in the light zone of the enclosure and 56 % in the dark zone

(no significant difference; $p=0.97$). The control animals spent 55 % of their time in the light part of the enclosure and 45 % in the dark part of the enclosure (no significant difference; $p>0.99$).

There was also no significant difference in comparison of the time the three *Bd* infection status groups spent in the dark part of the enclosure ($p=0.36$, $F=1.07$). Considering this comparison between the different groups, small to medium effect sizes were obtained (Control vs *Bd*CAPE = 0.47; Control vs *Bd*GPL = 0.50; *Bd*CAPE vs *Bd*GPL = 0.23), which was not visible within our recording timeframe. Visual analysis of the recordings did however show that some animals from all three groups remained in the dark part of the enclosure and although there were animals that favoured the light part of the enclosure, they always moved into the dark part as well during the recording period. Two *Bd*CAPE infected animals, one control and one *Bd*GPL animal remained exclusively in the dark zone of the enclosure for the full duration of the habitat selection trial.

A Kruskal-Wallis test of the zone transitions per group showed that there was a significant difference between the number of times the control animals crossed the boundary compared to that of the *Bd*CAPE animals ($p=0.04$, $K-W=6.37$). The control group had the highest number of mean crossings at 12.13 ± 3.25 times (Figure 7-B). The *Bd*CAPE group crossed the boundary 4.5 ± 2.40 times and the *Bd*GPL group crossed 7.25 ± 2.13 times. The number of times the *Bd*GPL group crossed the boundary did not differ significantly from that of the control group ($p=0.82$, $K-W=6.37$) or the CAPE group ($p=0.47$, $K-W=6.37$).

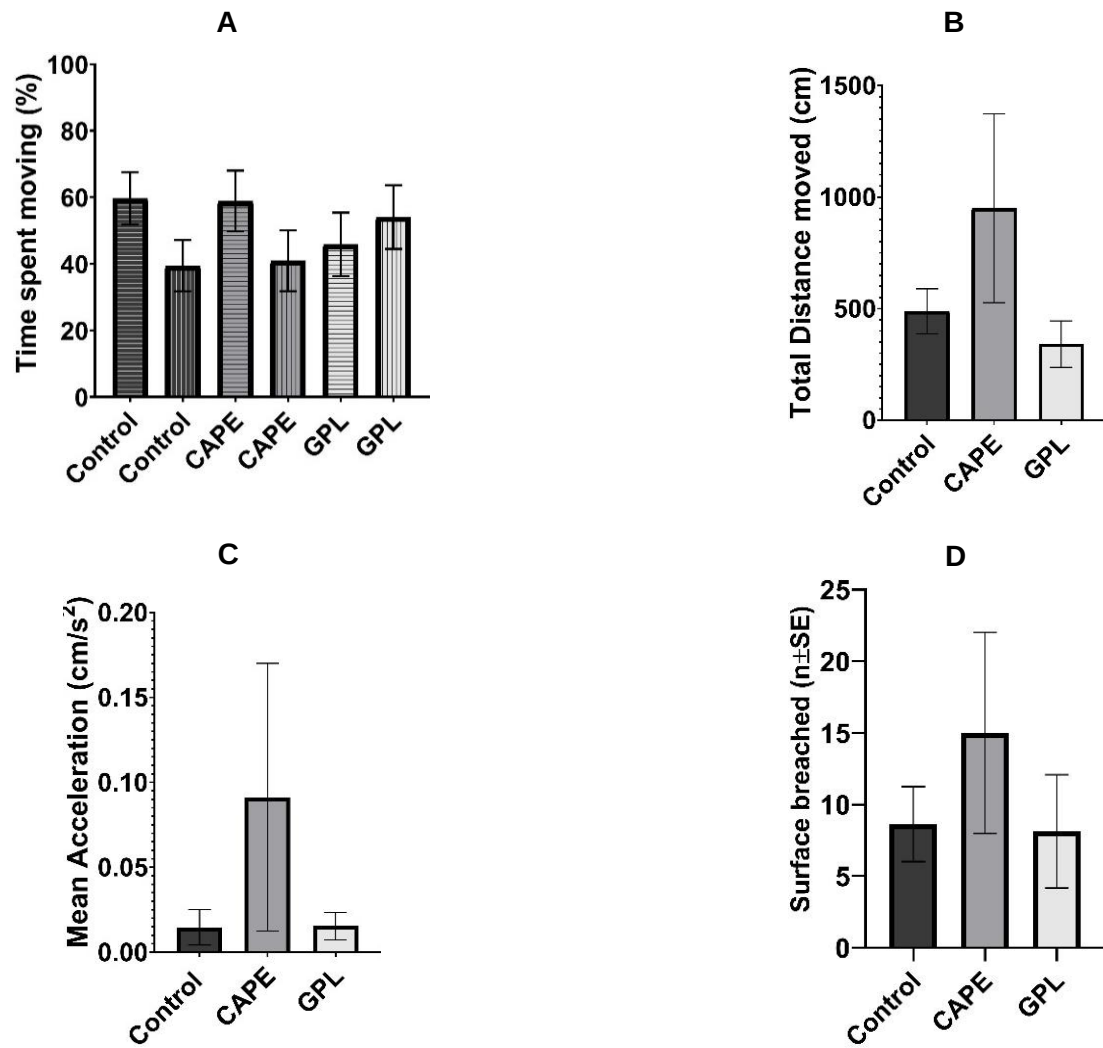


Figure 4: Baseline trial results: A) total times spent moving (horizontal lines) or not moving per event (vertical lines); B) total distance moved; C) mean acceleration throughout recording period; D) total number of times the surface was breached within the recording period (significance is shown at * where $p < 0.05$ and ** where $p < 0.01$)

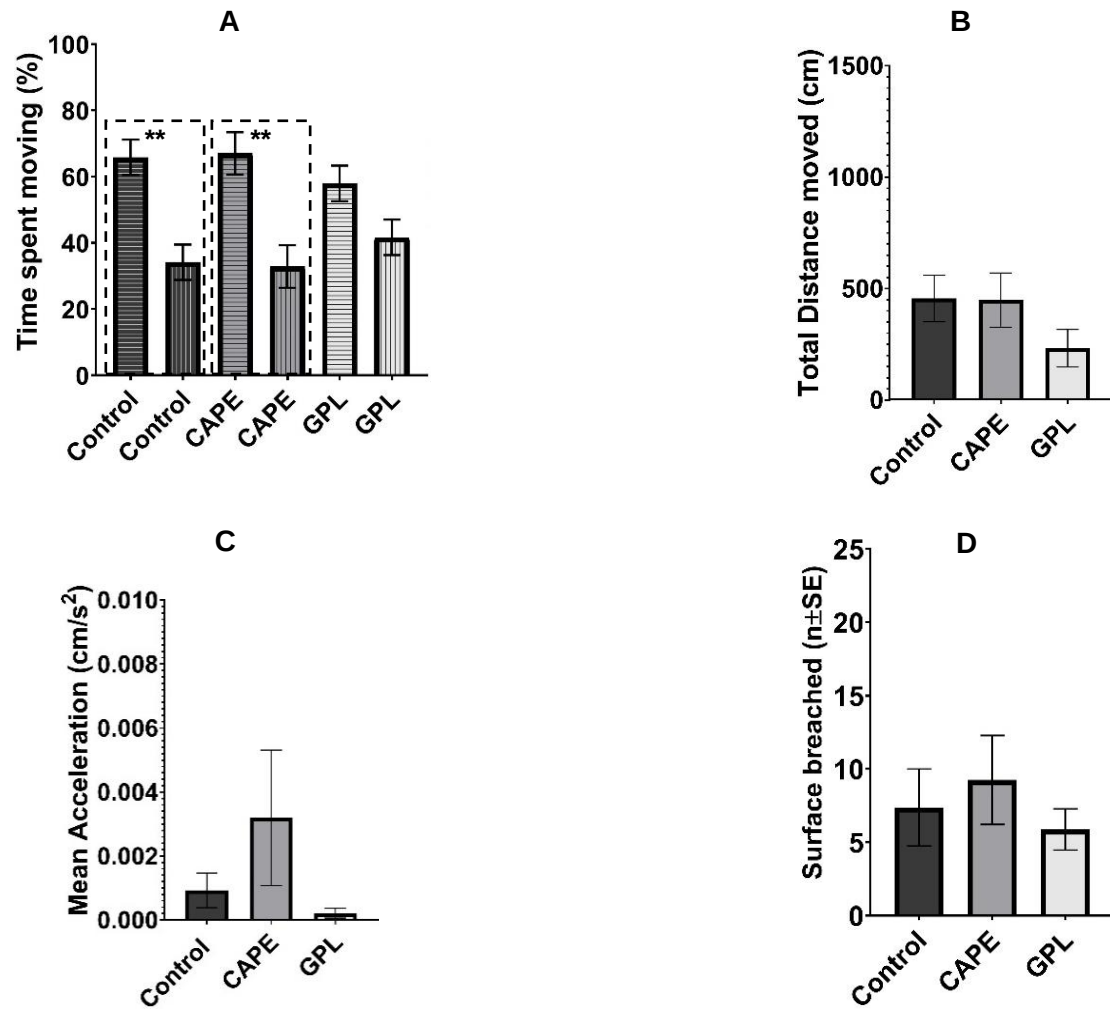


Figure 5: Predator trial results: A) total times spent moving (horizontal lines) or not moving per event (vertical lines); B) total distance moved; C) mean acceleration throughout recording period; D) total number of times the surface was breached within the recording period (significance is shown at * where $p < 0.05$ and ** where $p < 0.01$)

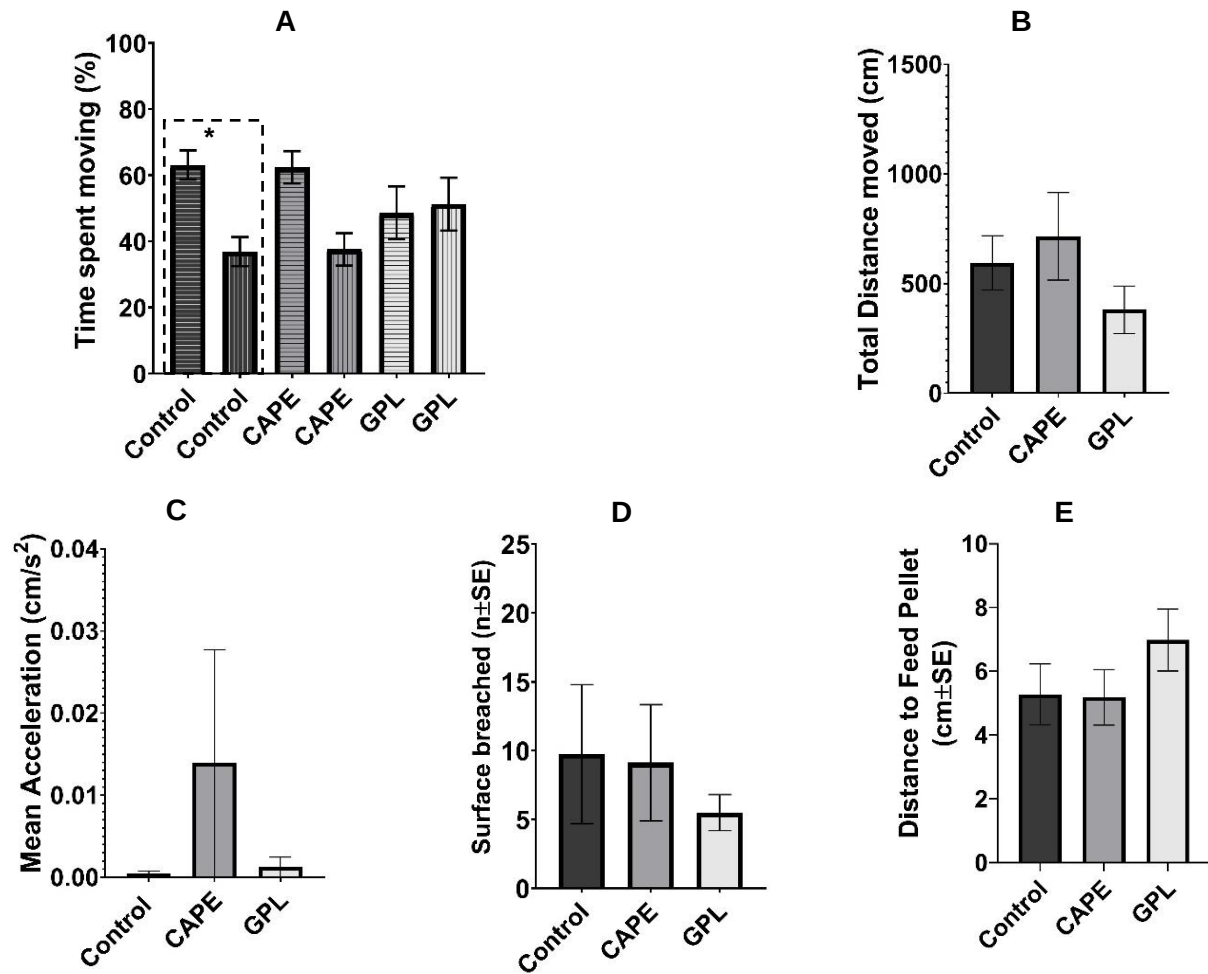


Figure 6: Feeding trial results: A) total times spent moving (horizontal lines) or not moving per event (vertical lines); B) total distance moved; C) mean acceleration throughout recording period; D) total number of times the surface was breached within the recording period; E) mean distance to feed pellet over the recording period (significance is shown at * where $p < 0.05$ and ** where $p < 0.01$)

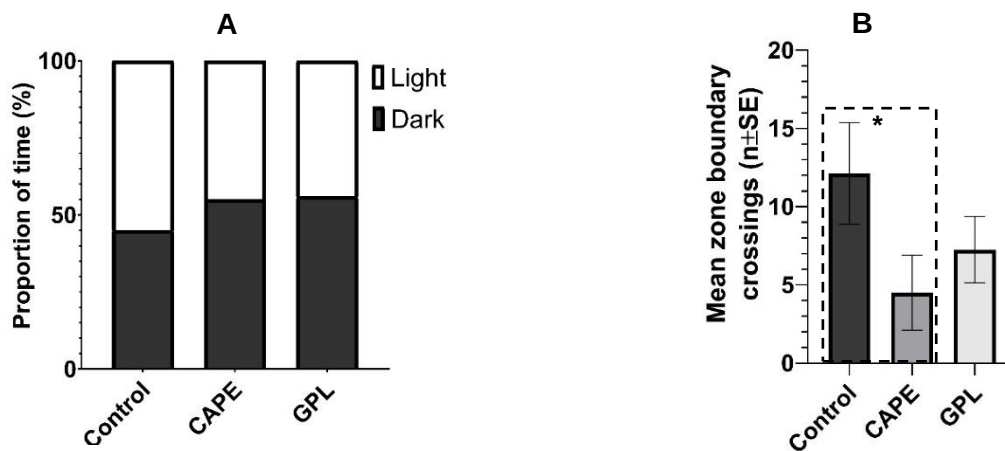


Figure 7: Habitat selection behaviour expressed as A) proportion of time in light or dark zones of the enclosures, B) total number of times the animals crossed the boundary between light and dark zones (significance is shown at * where $p < 0.05$ and ** where $p < 0.01$)

Discussion

Behavioural trials usually include distance moved and swimming speed as a standard, while this study showed that additional parameters need to be included to ensure no underreporting occurs. Tadpoles were observed to display different behaviours for the different trials, not only based on whether they were infected with *Bd* or not, but also based on the lineage of infection. Tadpoles were also observed to change their habitat usage, as they initially only moved around the borders of the enclosure, but during feeding trials and predator avoidance trials, started moving through the open sections of the habitat.

Baseline data showed that uninfected and *Bd*CAPE infected animals moved more often than not, while in *Bd*GPL infected animals the opposite trend occurred. The response to predation revealed all animals spent more time moving than they did for every stationary period, but the difference was most pronounced in the uninfected and *Bd*CAPE animals, while *Bd*GPL animals did not spend significantly different times in either state. The predator trial also saw *Bd*CAPE animals moving similar total distances to uninfected individuals, but in every other trial they had larger total distances moved than any other group. Only in the baseline trial did *Bd*GPL display similar total distance moved compared to the control group. In every other trial the *Bd*GPL animals had moved the shortest distances compared to the other groups.

Avoidance

The tadpoles used in this experiment was raised in the laboratory and consequently all behaviour that the tadpoles showed in reaction to the presence of a predator cue, is hereditary. Decreased mobility in the presence of a predator cue can be interpreted as a reliance on camouflage or remaining cryptic, tadpoles were observed staying towards the side of the container seeking shelter.

It was however seen from the predator trial that the *Bd*CAPE animals increased their time spent moving when a predator cue was present. It can be hypothesized that the increased time spent in a single zone could be an indirect threat response, avoiding potential detection by predators by avoiding the lighter zones of the habitat, a response only present when presented with a choice of habitats. By limiting their movements between areas, they mitigated the risk of being detected, as well as encountering potential predator threats. This could however mean that although *Bd*CAPE animals appeared to score on par with the control animals in the baseline trial, these animals may be less likely to move and occupy new areas due to the infection changing the way they manage the risk of predation, similar to ranavirus infection and perceived predation risk lowering the activity and feeding behaviour of tadpoles (Rae & Murray, 2019).

*Bd*GPL individuals also showed a preference (although not statistically significant) for the darker area of the arena. They did, however, transition between the regions more than *Bd*CAPE animals, but less than the control group. This may show that this group of animals ventured into the light part of the arena in an attempt to explore, thus forsaking the relative camouflage advantage that is preferred at the dark side of the arena. This may be due to the fact that they cannot afford it metabolically to forego foraging behaviour for safety. Past experiments by Farrer *et al.* (2011) showed that *Bd*GPL tend towards greater infection intensities on animals, thus increasing the physiological and metabolic load the fungus has on the animal, exacerbated by the fact that foraging (Venesky *et al.*, 2009) and developmental (Parris & Cornelius, 2004) factors of the individuals are impacted by infections of *Bd*.

Movement

The tadpoles can then use this conserved energy to spend more time foraging. Due to the short incubation time (usually two days) and rapid development of these animals during the tadpole stage (Du Preez *et al.*, 2009), these tadpoles are often concentrated at breeding ponds, since rapid development allows exploitation of temporary ponds for breeding. Conserving energy can be of great benefit during crowded conditions, and increased time spent not moving and decreased acceleration may help them become more cryptic to predators that rely on the movement of prey for detection. However, it is worthy to note that the tadpoles in the experiments were held individually in enclosures, thus there was no co-stimulation or crowd effect in reaction to predators, but removal of the co-stimulation source allowed greater discernment of the inherent predator avoidance behaviour of these tadpoles.

Habitat selection

Parris *et al.* (2006) suggested that two predatory cues, visual and chemical, are needed to elicit a response of choice for *Bd* infected animals in terms of location within their experimental habitat. In our habitat selection trial, the predator cue was absent, but the change in habitat, now effectively presented a choice for the animal within the enclosure, that can have both anti-predator and exploration behaviour implications, with exploration behaviour being negatively influenced if the animal chose to remain within a certain area of the habitat as a means of camouflage. *Bd*CAPE individuals opted to remain in the habitat they perceived as being safe, thus staying in a single area for longer. Han *et al.* (2011) found that animals tended to retreat to refugia faster when infected, although in the case of *Bd*CAPE the sought after refugium seemed to be camouflage and discreet habitat usage.

The choice of habitat for the animals differed based on their *Bd* infection status. Control animals spent more time in the light zone than in the dark and most often crossed the boundary

between the two zones, while the trend was the opposite for the *Bd* infected groups. These results can be viewed as potential exploratory behaviour, and something that the animals may invest in as they have the resources and relative health needed to explore and potentially reach more suitable habitats (Dugatkin, 2004). This could give uninfected animals an exploitation advantage as they will most probably reach the new habitats before the infected individuals (Begon *et al.*, 2006). However, all individuals displayed some degree of exploration through zone transitioning. It is therefore plausible that the uninfected individuals will only have a temporary advantage in exploiting the new habitat, followed by late arrival of infected individuals and potential transmission risk within the exploited habitat. The fact that these species often are concentrated in temporary ponds implies that uninfected individuals will probably not stay *Bd* negative for long, especially when viewing the results of the transmission experiment (Chapter 2) that showed that both lineages transmit readily to uninfected metamorphosed individuals housed in the same habitat, and the aquatic nature of tadpole habitats increasing the effective mobility area of zoospores. The temporary nature of breeding sites for the species in question does limit their potential to exploit new habitat, as these breeding sites are often isolated from extensive water systems, consequently they won't be able to expand their habitat presence during the tadpole phase. The greater investment of resources of the uninfected individuals in habitat exploration may have the additional advantage of obtaining more resources within the available habitat faster than infected individuals (Schmidt-Nielsen, 1997). This could also explain why tadpoles infected with *Bd* metamorphose at smaller body sizes (Parris & Cornelius, 2004) than uninfected individuals, because impaired exploration ability results in fewer encounters with resources.

The trials were designed to only introduce one environmental variable to impact the behaviour of the tadpoles, and in the case of the habitat selection trial, it was to see whether the animals preferred a dark or light backdrop in which to shelter. It is possible that the choice of habitat may be influenced differently if the additional stressor of a predator cue were to be introduced. The *Bd*CAPE group settled in an area of the habitat, and spent an extended amount of time there, although not significantly more within the recording timeframe, but also not changing zones and exploring the habitat. Although the *Bd*CAPE animals spent the same amount of time moving and thus potentially foraging in the baseline trial, which was confirmed with the *Bd*CAPE animals showing the same mean distance to feed pellet in the Feed trial, it is unexpected to see these animals not showing a significant preference towards a particular habitat and also spending a comparable amount of time in the dark zone with *Bd*GPL.

Feeding

When comparing infected tadpoles to uninfected tadpoles it was noted that the control animals and *Bd*CAPE animals both tended to use the centre part of the enclosures more than *Bd*GPL which tended to keep to the baseline observation of keeping to the sides of the enclosure. Direct comparison of the times the animals moved per event or remained stationary also revealed that *Bd*GPL tended to have longer periods of being stationary than any of the other groups, while *Bd*CAPE and uninfected animals had very similar results for the measured parameters.

Infection with *Bd* adds additional physiological stress on the tadpoles as there is a bigger demand for energy needed to cope with the symptoms of infection, often including sloughing of skin in post-metamorphic individuals (Ohmer *et al.*, 2015) and decrease in the keratinized mouthparts of the tadpoles (Marantelli *et al.*, 2004). These symptoms may lead to increased physiological load that could escalate to a lethal infection. Control animals in the Feeding trial spent less time foraging (although not significant) than the *Bd* infected animals. This reduced time spent moving potentially indicate that control animals were more efficient in finding food, especially when considering that distance to feed pellet for the control animals was the smallest of the three groups. *Bd* lineage did not significantly influence distance to feeding pellet, although the distance was larger in *Bd*GPL. We found evidence in our experiment that *Bd* affects the ability of tadpoles to detect food, but that differences are not further expressed by lineage. Previous testing has also shown that foraging efficacy of tadpoles infected with *Bd* does become compromised based on reduced amounts of food in the gut of infected tadpoles (Venesky *et al.*, 2009). It is probable that this could have been related to damaged jaws often associated with *Bd* infection (Fellers *et al.*, 2001). Further behavioural experimentation over longer periods could elucidate if lineage-specific differences become more pronounced over time, and could incorporate different feeding regimes (e.g., algae surface growth feeding vs feed pellet). Behavioural changes that lead to conserving of energy could compensate for this increased physiological load of the animal, especially when faced with environments that could potentially be metabolically demanding to the animal. The same trend was observed in the *Bd*GPL-infected animals, which spent the most time of all animals not moving and had the lowest acceleration readings for the Predator trial.

Physiological stress

Tadpoles of different stages can be dependent on different means of breathing. Earlier Gosner stage tadpoles are often unable to break the surface tension of water and are then dependent on bubble sucking (Schwenk & Phillips, 2020) in order to obtain the oxygen needed once the

lungs start to develop and to compensate for the increase in oxygen requirements during development (Phillips, 2019). Surface breaching is often linked to dissolved oxygen availability (Crowder *et al.*, 1998), and can be of great consequence for the survival of tadpoles depending on the environmental conditions. From an evolutionary and resource management perspective it makes sense to adapt the behaviour pre-emptively to fit the lifestyle of the morphotype to come, rather than expend the energy to evolve and grow bigger and more efficient gills only to lose them later. This was also important for our study, as we worked with late-stage tadpoles that were starting to show prominent hindlimbs and were thus well developed, potentially having lost some, if not all, of the functionality in their gills.

Bd should not have a direct influence on tadpole gills or their ability to absorb oxygen, because the absence of keratin prevents the fungus from establishing an infection and consequently damaging the gills directly. However, the cost of *Bd* infection could be an increased need of oxygen for the tadpole to fulfil its metabolic needs. The increased metabolic load on the tadpole due to decreased ability to forage and the nutrients lost to the infection itself may adversely affect the ability to return to the surface to breathe air. All animals in our experiment made use of surface breaching, but none of the groups showed significantly different values with regards to type of trial or *Bd* infection status. However, *Bd*CAPE infected animals did show the highest means from all the groups. *Bd*CAPE infected animals however still showed that they were able to actively differentiate between habitats, explore the habitat and score on par with control animals when presented with a food pellet during recording.

Conclusion

Behavioural trials present the possibility of exploring a multitude of interactions, and research questions relating to precise lineage influence of an infection with *Bd* on the behaviour of tadpoles. It would seem that *Bd*CAPE caused increases in the parameters tested, parameters expected to be decreased when compared to the control group due to the additional burden of *Bd* infection. These animals also showed increased acceleration and more time spent moving during feeding and predator detection trials, with the latter potentially impacting predation upon vulnerable tadpoles in a natural environment. *Bd*GPL reduces the relevant behavioural activity in *C. boettgeri*, potentially decreasing their survival fitness due to their reduced ability to forage, time they spend in camouflage and as well as decreased acceleration that could impede their ability to escape threats in a natural environment.

References

- Begon, M., Townsend, C.R. & Harper, J.L. 2006. *Ecology: From Individuals to Ecosystems*. 4th ed. Oxford, United Kingdom: Blackwell Publishing.
- Berger, L., Speare, R. & Hyatt, A. 1999. Chytrid fungi and amphibian declines: overview, implications and future directions. *Declines and disappearances of Australian frogs*: 23-33.
- Crowder, W.C., Nie, M. & Ultsch, G.R. 1998. Oxygen uptake in bullfrog tadpoles (*Rana catesbeiana*). *Journal of Experimental Zoology*, 280(2): 121-134. DOI: 10.1002/(SICI)1097-010X(19980201)280:2%3C121::AID-JEZ3%3E3.0.CO;2-Q
- Du Preez, L.H., Carruthers, V. & Burger, M. 2009. *A complete guide to the frogs of Southern Africa*. Cape Town, South Africa: Struik Nature.
- Dugatkin, L.A. 2004. *Principles of Animal Behavior*. New York, USA: WW Norton.
- Farrer, R.A., Weinert, L.A., Bielby, J., Garner, T.W.J., Balloux, F., Clare, F., Bosch, J., ... Fisher, M.C. 2011. Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proceedings of the National Academy of Sciences*, 108(46): 18732-18736. DOI:10.1073/pnas.1111915108
- Fellers, G.M., Green, D.E. & Longcore, J.E. 2001. Oral chytridiomycosis in the mountain yellow-legged frog (*Rana muscosa*). *Copeia*, 2001(4): 945-953. DOI: 10.1643/0045-8511(2001)001%5B0945:OCITMY%5D2.0.CO;2
- Fisher, M.C. & Garner, T.W.J. 2020. Chytrid fungi and global amphibian declines. *Nature Reviews Microbiology*: 1-12. DOI: 10.1038/s41579-020-0335-x
- Fisher, M.C., Pasmans, F. & Martel, A. 2021. Virulence and pathogenicity of chytrid fungi causing amphibian extinctions. *Annual Review of Microbiology*, 75: 673-693. DOI: 10.1146/annurev-micro-052621-124212
- Fisher, R.A. 1958. *The genetical theory of natural selection*. Second edition. New York: Dover.
- Garner, T.W.J., Walker, S., Bosch, J., Leech, S., Rowcliffe, J.M., Cunningham, A.A. & Fisher, M.C. 2009. Life history tradeoffs influence mortality associated with the amphibian pathogen *Batrachochytrium dendrobatidis*. *Oikos*, 118(5): 783-791. DOI: 10.1111/j.1600-0706.2008.17202.x,
- Greggor, A.L., Blumstein, D.T., Wong, B. & Berger-Tal, O. 2019. Using animal behavior in conservation management: a series of systematic reviews and maps. *Environmental Evidence*, 8(1): 1-3. DOI: 10.1186/s13750-019-0164-4

- Han, B.A., Searle, C.L. & Blaustein, A.R. 2011. Effects of an infectious fungus, *Batrachochytrium dendrobatidis*, on amphibian predator-prey interactions. *PLoS ONE*, 6(2): e16675. DOI: 10.1371/journal.pone.0016675
- Hanlon, S.M., Lynch, K.J., Kerby, J. & Parris, M.J. 2015. *Batrachochytrium dendrobatidis* exposure effects on foraging efficiencies and body size in anuran tadpoles. *Diseases of Aquatic Organisms*, 112: 237-242. DOI: 10.3354/dao02810
- Kardong, K.V. 2008. *An introduction to biological evolution*. Second edition. New York: McGraw-Hill.
- Longo, A.V., Burrowes, P.A. & Joglar, R.L. 2010. Seasonality of *Batrachochytrium dendrobatidis* infection in direct-developing frogs suggests a mechanism for persistence. *Diseases of Aquatic Organisms*, 92(2-3): 253-260. DOI: 10.3354/dao02054
- Marantelli, G., Berger, L., Speare, R. & Keegan, L. 2004. Distribution of the amphibian chytrid *Batrachochytrium dendrobatidis* and keratin during tadpole development. *Pacific Conservation Biology*, 10(3): 173-179. DOI: 10.1071/PC040173
- O'Hanlon, S.J., Rieux, A., Farrer, R.A., Rosa, G.M., Waldman, B., Bataille, A., Kosch, T.A., ... Fisher, M.C. 2018. Recent Asian origin of chytrid fungi causing global amphibian declines. *Science*, 360: 621-627. DOI: 10.1126/science.aar1965
- Ohmer, M.E.B., Cramp, R.L., White, C.R. & Franklin, C.E. 2015. Skin sloughing rate increases with chytrid fungus infection load in a susceptible amphibian. *Functional Ecology*, 29(5): 674-682. DOI: 10.1111/1365-2435.12370
- Parris, M.J. & Cornelius, T.O. 2004. Fungal pathogen causes competitive and developmental stress in larval amphibian communities. *Ecology*, 85(12): 3385-3395. DOI: 10.1890/04-0383
- Parris, M.J., Reese, E. & Storfer, A. 2006. Antipredator behavior of chytridiomycosis-infected northern leopard frog (*Rana pipiens*) tadpoles. *Canadian Journal of Zoology*, 84(1): 58-65. DOI: 10.1139/Z05-175
- Petersen, C.E., Lovich, R.E., Phillips, C.A., Dreslik, M.J. & Lannoo, M.J. 2016. Prevalence and seasonality of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* along widely separated longitudes across the United States. *EcoHealth*: 1-15. DOI: 10.1007/s10393-016-1101-4
- Phillips, J. 2019. *The Biomechanics and Evolution of Air-Breathing in Anuran Tadpoles*. University of Connecticut. (Master's Theses).
https://opencommons.uconn.edu/qs_theses/1378.

- Rae, J. & Murray, D. 2019. Pathogen vs. predator: ranavirus exposure dampens tadpole responses to perceived predation risk. *Oecologia*, 10.1007/s00442-019-04501-1
- Rosenblum, E.B., Voyles, J., Poorten, T.J. & Stajich, J.E. 2010. The deadly chytrid fungus: a story of an emerging pathogen. *PLoS Pathogens*, 6(1): e1000550. DOI: 10.1371/journal.ppat.1000550
- Scheele, B.C., Pasmans, F., Skerratt, L.F., Berger, L., Martel, A., Beukema, W., Acevedo, A.A., ... Catenazzi, A. 2019. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science*, 363(6434): 1459-1463.
- Schloegel, L.M., Toledo, L.F., Longcore, J.E., Greenspan, S.E., Vieira, C.A., Lee, M., Zhao, S., ... Daszak, P.J., T. Y. 2012. Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. *Molecular Ecology*, 21(21): 5162-5177. DOI: 10.1111/j.1365-294X.2012.05710.x
- Schmidt-Nielsen, K. 1997. *Animal Physiology Adaptation and Environment*. Cambridge University Press.
- Schwenk, K. & Phillips, J.R. 2020. Circumventing surface tension: tadpoles suck bubbles to breathe air. *Proceedings of the Royal Society B*, 287(1921): 20192704. DOI: 10.1098/rspb.2019.2704
- Tarrant, J., Cilliers, D., Du Preez, L.H. & Weldon, C. 2013. Spatial assessment of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) in South Africa confirms endemic and widespread infection. *PLoS ONE*, 8(7): e69591. DOI: 10.1371/journal.pone.0069591
- Venesky, M.D., Parris, M.J. & Storfer, A. 2009. Impacts of *Batrachochytrium dendrobatidis* infection on tadpole foraging performance. *EcoHealth*, 6(4): 565-575. DOI: 10.1007/s10393-009-0272-7
- Weldon, C. Unpublished data. Husbandry and amphibian experimentation. Unpublished.
- Weldon, C., Channing, A., Misinzo, G. & Cunningham, A.A. 2020. Disease driven extinction in the wild of the Kihansi spray toad, *Nectophrynoides asperginis*. *African Journal of Herpetology*, 69(2): 151-164. DOI: 10.1080/21564574.2020.1752313

Chapter 4

Environmental fitness of *Batrachochytrium dendrobatidis* lineages increases potential for recombination

Abstract

Amphibian chytrid fungus is widespread throughout Africa and several prediction models have attempted to discern its potential presence in areas not frequently sampled. Recent increases in the number of surveys, as well as the more cost-effective means of determining the lineage from field swab samples, allowed for a new more comprehensive model with greater resolution to be constructed, as well as a lineage specific distribution prediction model focussed on South Africa. Combining data from publications and field samples obtained in our own surveys led to a comprehensive database of 26 African countries that has been sampled for *Bd*. The predicted distribution of *Bd* for Africa, compared to other published models showed that vast unstudied areas are predicted to have a low to moderate likelihood of *Bd* to be present. The South African focussed *Bd* lineage models showed that these lower to moderate likelihood areas are important in the surveillance for *Bd*. The latter also highlighted that these areas of South Africa show a probability for both lineages to be present, resulting in large areas that need to be under constant surveillance in order to identify the potential ingress of *Bd*GPL and potential recombinant lineages due to probable contact with *Bd*CAPE.

Keywords:

Batrachochytrium dendrobatidis; environmental envelope; distribution modelling; Africa; recombinant zone

Introduction

Batrachochytrium dendrobatidis, (hereafter referred to as *Bd*), and *B. salamandrivorans* are two pathogenic chytrid fungi that infect amphibians around the globe (Martel *et al.*, 2013). *Bd* has been studied to the extent that it is known to consist of multiple lineages, with differing infection abilities, and virulence toward the host (Farrer *et al.*, 2011; O'Hanlon *et al.*, 2018; Ghosh *et al.*, 2021). Although this evidence initially came from whole genome sequences of isolates from around the globe, it became increasingly important to distinguish lineage in field samples. A rapid and cost-effective solution came in the form of lineage-specific primers for *Bd*GPL and *Bd*CAPE (Ghosh *et al.*, 2021; Chapter 5). The recent focus on deciphering the genome of *Bd* has led to the discovery that *Bd* originated in Asia based on the genotypic heterogeneity contained in isolates from Asia and them being ancestral to other isolates from around the globe (O'Hanlon *et al.*, 2018). This reiterated the need for testing and understanding the inherent complexities in the natural environment and the effects thereof on the management of pathogens (Barrett *et al.*, 2009).

Bd has been studied in terms of the seasonality of infection intensity (Longo *et al.*, 2010), as well as environmental niche modelling within South Africa that identified suitable habitats within otherwise highly unsuitable areas for the survival of this fungus (Tarrant *et al.*, 2013). There have been attempts to link climate change and chytrid-related declines of amphibians, but Rohr *et al.* (2008) commented that a single hypothesis cannot explain chytrid related amphibian declines in their entirety, and Garner *et al.* (2011) showed that although dynamics of infection may change with different temperature-seasons (warmer winters vs. colder winters) survival of the host was not dependent on it. *In vitro* experiments and field observations in temperate regions of Australia and Europe demonstrated that *Bd* can alter its growth patterns to survive in a wide range of temperatures (Woodhams *et al.*, 2008; Rödder *et al.*, 2010). Lower temperatures induced an increase in the number of zoospores (the infective motile stage of *Bd*) released from sporangia, which would increase its infectivity in cold environments (Woodhams *et al.*, 2008). Conversely, temperature and precipitation, especially the temperature in the warmest months and the rainfall in the driest months, increased the prevalence of *Bd* in Jamaica (Holmes *et al.*, 2014). It therefore appears that *Bd* is capable of changing its growth patterns to survive in a wider range of temperatures, in spite of predictions based on the IPCC predicted climate changes in 2010 that the geographic range of *Bd* may become restricted in future (Rödder *et al.*, 2010). In 2016 a machine learning model was used together with a global database of *Bd* for an updated model of *Bd* distribution, concurring with the Woodhams *et al.* (2008) and Rödder *et al.* (2010) findings that the distribution of *Bd* will most likely shift towards temperate areas in future (Xie *et al.*, 2016). This also highlighted the fact that temperature is the main driver of this shift. This further highlighted

the need to further study the distribution of different lineages of *Bd* to have a better understanding of the climatic variables pertinent to the distribution of the fungus. There has been cases where amphibian populations remained stable despite of *Bd* infection, for example, *Bombina variegata* in the Netherlands (Spitzen-van der Sluijs *et al.*, 2017). There have also been cases where there were fluctuating infections and uncorrelated population growth in the host, *Alytes obstetricans*, which showed that host vulnerability seems to be context dependent and species-specific at least in Northern Europe (Doddington *et al.*, 2013; Spitzen-Van Der Sluijs *et al.*, 2014). This has also been substantiated by the *thermal mismatch hypothesis* that describes amphibians as being increasingly vulnerable to infection with *Bd* if the climate shifts away from the optimum for the host, although this is dependent on the host species and the direction of the shift (Cohen *et al.*, 2017). Importantly, the ecological interactions between genetically distinct batrachochytrids have been largely unstudied.

The Orange River system in South Africa was predicted to be suitable for *Bd* in highly restricted zones associated with the river (Tarrant *et al.*, 2013), and consequently provides an ideal environmental transect to study both the environmental niches of different *Bd* lineages as well as the potential for transition/recombinant zones. Disjunct regions associated with the Orange River in the Karoo and the Drakensberg have previously been surveyed for *Bd* disease dynamics and association with host genotype and microbial community (Weldon & Du Preez, 2006; Smith & Weldon, 2007; Griffiths *et al.*, 2018). A wide environmental gradient across altitude, temperature and rainfall makes this single water course an ideal system to study the lineage climate interactions on the epidemiology of *Bd*.

With the development of new tools to easily identify lineage, and the varied landscape in South Africa offering the possibility for different lineage niches, a widescale transect survey of South Africa was timely. Our objective was to increase the resolution of predictive models for South Africa, apply it per lineage and identify overlapping zones of distribution to identify potential recombinant zones. We also intend to expand the predictive *Bd* distribution model to the whole of Africa, including a comprehensive dataset regarding the prevalence of *Bd* in Africa.

Material and methods

Field sampling and processing

Field sampling formed part of two related studies. The first was the field-testing phase to determine the success of specifically designed lineage-specific primers, firstly as published in Ghosh *et al.* (2021), Chapter 5, and secondly the lineage distribution modelling for South Africa

in this thesis. The Orange River was targeted for both these studies as it stretches for over 2 300 km from the headwaters that originate in the highlands of Lesotho to where it reaches the Atlantic Ocean on the South African West Coast, with a catchment area spanning four different countries (Lange *et al.*, 2007). Climates along the watercourse vary widely, from low-temperature winters with high rainfall in the Drakensberg to the arid warmer climate of western South Africa (Cambray *et al.*, 1986; Conley & van Niekerk, 2000).

We sampled 32 sites that comprised a 1 500km section between Vioolsdrift (furthest west site, 155 m alt.) and Lotheni camp in the Ukhahlamba Drakensberg National Park (furthest east site, 1 600 m alt.). All sample sites were associated with the Orange River, even if the site itself was not within the main watercourse. Primary criteria for the sites included the presence of a waterbody with either larval or adult amphibians. Sites were selected to be no more than 100 km apart, while ponds or streams situated within a 10 km radius of each other were treated as single sites. Sterile cotton swabs were used to collect DNA from the ventral skin surface of adult frogs. When tadpoles were sampled, they were euthanized (submersion in 250mg/L MS222) and excised tissue preserved in 70 % ethanol, before qPCR analyses.

The fieldwork trips culminated in 1 477 samples and 40 new isolates, of which 22 were subjected to whole genome sequencing (see O’Hanlon *et al.*, 2018).

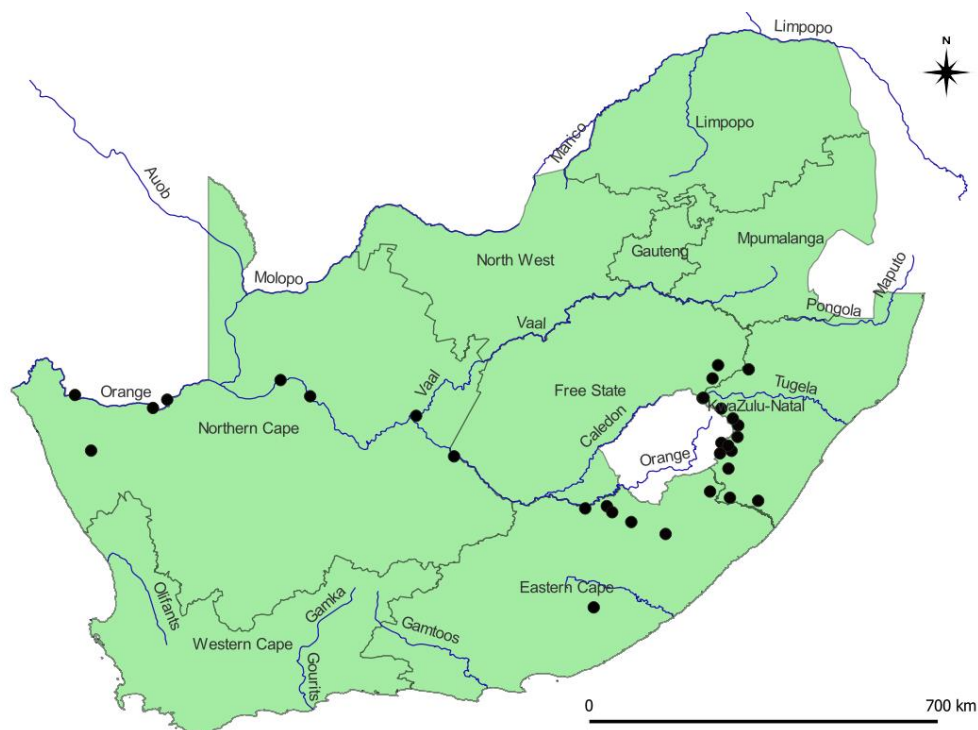


Figure 1: Location of *Bd* sampling sites for South Africa

Before lineage-specific qPCRs were run on field swab samples, normal pan-lineage ITS-PCR was run to determine if samples were positive (Boyle *et al.*, 2004). Samples that showed amplification in duplicate with a GE > 0.1 were then subjected to lineage-specific qPCR testing using methods developed by Ghosh *et al.* (2021; Chapter 5).

All *Bd* positive sites within South Africa were characterised in terms of mean temperature (average daily surface temperature between 1981 and 2018), mean daily precipitation (per day averaged from 1981 to 2018) and mean annual dew/frost point at 2 m above ground level (daily average between 1981 and 2018; Figure 4). Mean temperature, mean daily precipitation and mean annual dew point was selected based on work done by Puschendorf *et al.* (2009), Tarrant *et al.* (2013) and Murray *et al.* (2011), and were downloaded from the NASA POWER website (<https://power.larc.nasa.gov>).

Kruskal-Wallis tests were used to determine if significant differences could be found between mean temperature, mean precipitation, mean annual dew point and elevation, as well as prevalence, for the sites that tested positive for different lineages of *Bd*. Elevation was predicted to correlate with other variables used, and a Pairwise Wilcox test was carried out on significantly different variables, to identify groups that differed from each other, using Holm as the p-value adjustment method.

Modelling

A Maxent (version 3.3.3k) modelling approach (Phillips *et al.*, 2006) was used to map different lineage distributions of *Bd* with algorithms described by Elith *et al.* (2011) to correlate environmental parameters with the presence data of *Bd*. A comprehensive database for known African *Bd* records was created with the following information: Country, locality and coordinates of sample origin, sample size, the prevalence of *Bd*, lineage found (if available), date of sampling, and host genus and species name. This database contained 12 222 samples, originating from 26 countries, 4 552 of which was tested to be positive. 1 172 of the positive samples were shown to be *Bd*GPL, 725 samples tested positive for *Bd*CAPE, 306 samples originated from sites that tested positive for both *Bd*CAPE and *Bd*GPL. Lineage had not been assigned for the remaining 2 349 positive samples. These data were compiled from 40 published sources and three unpublished sources (n=135 localities). Eleven different environmental variables were used (Table 1), with the selection based on the predictive model created by Tarrant *et al.* (2013). The original model in 2013 only incorporated the presence of *Bd* and was used as a basis for our models, while our database also included *Bd*-lineage, mostly for South Africa, allowing for lineage-specific prediction models for South Africa. Three different models were created: firstly, a model for the predicted *Bd* distribution throughout the

entire African continent and Madagascar regardless of lineage (Figure 2) and secondly lineage-specific distribution models for *BdCAPE* (Figure 3-A) and *BdGPL* (Figure 3-B). Maxent used 80 % of the positive records for training of the model and the remaining 20 % to test for accuracy of predictions. Model replications were set to 100 using subsample, with the regularisation multiplier set to 1. Random seed was set to true with all other settings left at default.

Model performances were evaluated using the area under curve (AUC) of the receiving operating characteristic (ROC) curve and by using jack-knife tests. The jack-knife tests examined the importance of each variable, firstly by removing one variable at a time and secondly by testing each variable in isolation (Supplemental information). AUC values of 1 indicates perfect models, while values of 0.5 indicates that the model has no predictive ability. The lineage-specific prediction models (Figure 3) had a “10 percentile training presence logistic threshold” using the average results from the 100 model replications in order to convert the results to a binary output for display purposes.

Table 1: Environmental parameter contribution to model				
Key	Variable	Contribution to model		
		Fig. 2	Fig. 3(A)	Fig. 3(B)
BIO2	Mean monthly diurnal range (Max temp – Min temp)	3.6%	0.6%	13.1%
BIO9	Mean temperature of the driest quarter (°C)	1.6%	50.8%	3.7%
BIO10	Mean temperature of the warmest quarter (°C)	18.2%	0%	11%
BIO11	Mean temperature of the coldest quarter (°C)	8.1%	2.2%	4.3%
BIO15	Precipitation seasonality (coefficient of variation)	5.2%	0.9%	4.8%
BIO16	Precipitation of the wettest quarter (mm)	3%	2.7%	2.1%
BIO19	Precipitation of the coldest quarter (mm)	10.1%	6.4%	10.7%
Alt	Altitude above sea level	2.3%	2.2%	4.5%
Riv	Presence of rivers	39.3%	23.2%	26.4%
Slope	Slope	8.3%	8.8%	18.6%
Aspect	Aspect	0.4%	2%	0.7%

Results

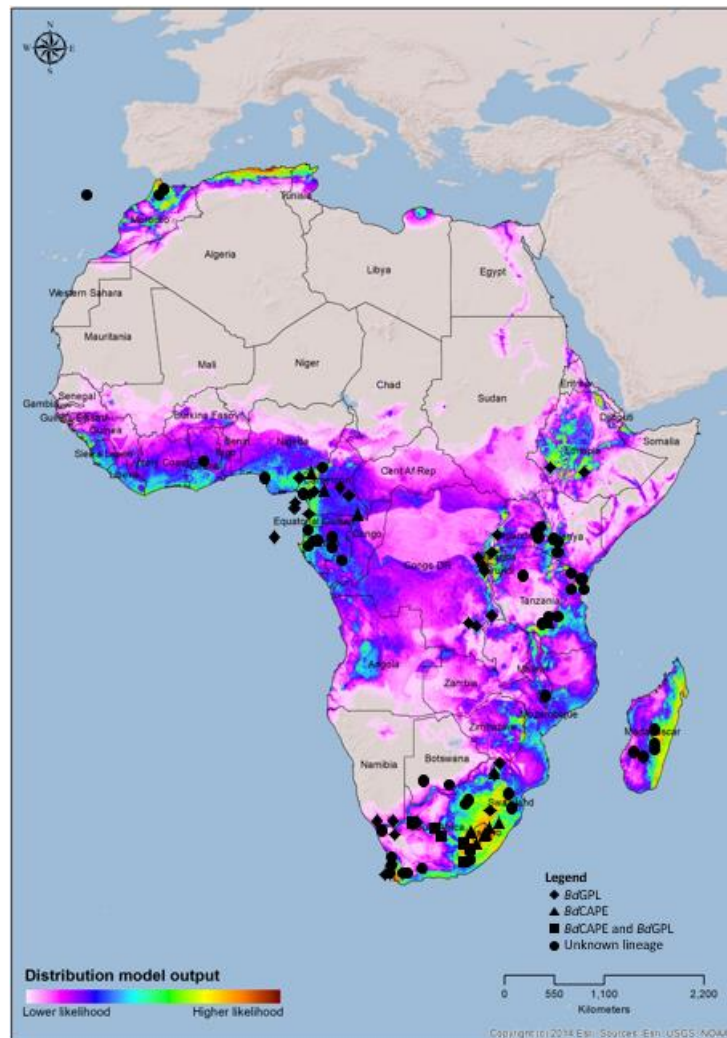


Figure 2: Predicted distribution of *Bd* for Africa and Madagascar

The African *Bd* model had an AUC score of 0.917, indicating that it has a high efficacy in predicted *Bd* distribution. The distribution model of *Bd* for Africa showed that the Savanna and Tropical Lowland Forest vegetation regions have a likelihood for *Bd* to occur according to White's vegetation map of Africa (1983). Predicted distribution is mostly absent from the Desert and Semi-desert vegetation regions, while high likelihood areas are predicted for coastal West and North Africa, the Great Rift Valley, and eastern South Africa and Madagascar. This model also showed that the presence of rivers was the highest contributing variable, accounting for 39.3 % contribution to the model.

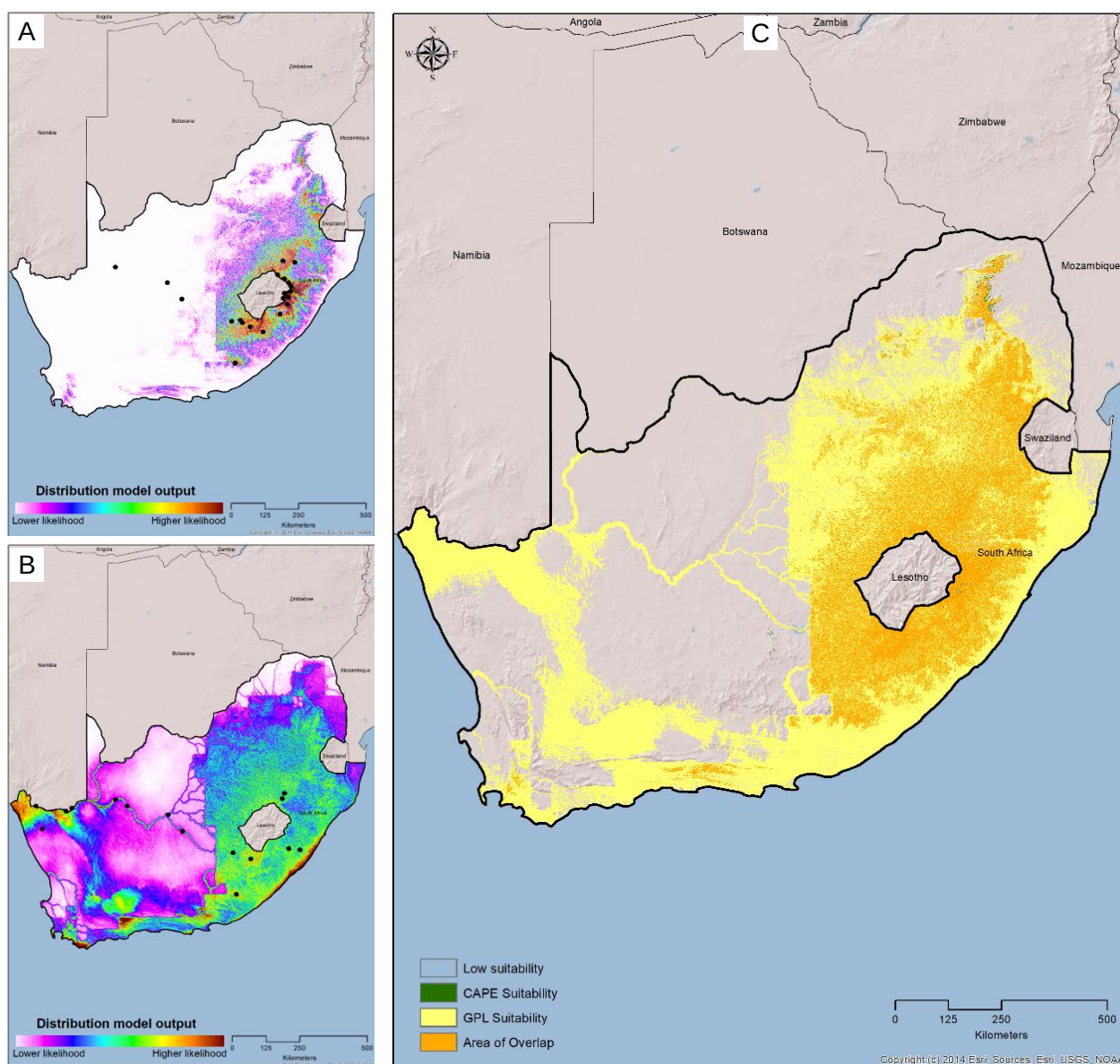


Figure 3: Prediction map of *Bd* distribution in South Africa for *Bd*CAPE (A), *Bd*GPL (B), and both lineages combined indicating predicted areas of overlap (C)

The *Bd*CAPE model (Figure 3-A) had an AUC value of 0.989 and the *Bd*GPL (Figure 3-B) model had an AUC value of 0.946, indicating that both these models also have high predictability. The lineage-specific predicted distribution maps also show that *Bd*CAPE has a restricted distribution, largely in eastern and southern coastal South Africa, often occupying higher elevation regions (Figure 3-A). *Bd*GPL has an almost complete predicted distribution in South Africa including all areas where *Bd*CAPE is predicted to occur (Figure 3-B, C). However, in the predicted hotspot for *Bd*CAPE, the eastern Great Escarpment, *Bd*GPL is predicted to have a moderate likelihood of occurrence. Analysis of the contribution of variables to the model showed that *Bd*GPL and *Bd*CAPE are not equal in variable contribution. For the *Bd*CAPE model mean temperature of the driest quarter contributed 50.8 % to the model, while for *Bd*GPL the presence of rivers was the highest contributing variable at 26.4 %.

Analysis of environmental parameters associated with lineage distribution showed a correlation between drier, warmer sites and *BdGPL* presence. But it also showed that the range of environmental parameters where *BdGPL* occurred was larger than the range of environmental parameters for *BdCAPE* (Figure 4). Correlations were seen where *BdCAPE* occurred and high precipitation, and low-temperature environments, while the opposite seemed to be the case for *BdGPL*. Although there was one exception where *BdGPL* was isolated from a typical *BdCAPE* area. When considering the lineage-specific qPCR results from the fieldwork (Ghosh et al. 2021; Chapter 5), it is especially apparent that the two basic lineages in South Africa have different distributions due to mean annual temperature and mean annual precipitation.

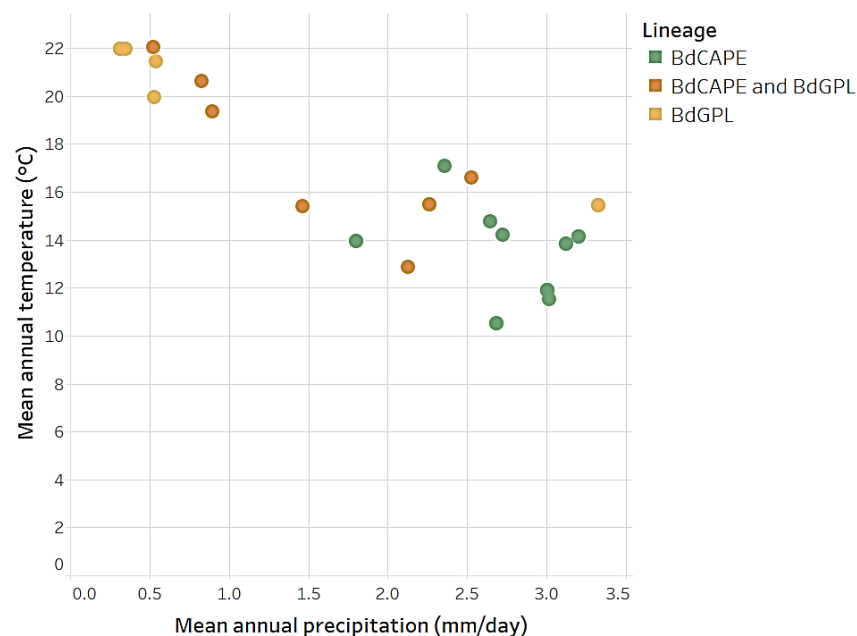


Figure 4: Scatterplot showing qPCR lineage-typed sites only according to lineage

A significant difference was seen for mean annual temperature, mean daily precipitation and elevation when comparing the different groups of lineage-specific infections, using Kruskal-Wallis tests ($p < 0.05$). Wilcoxon Rank Sum tests showed a significant difference between the mean annual temperature and mean annual precipitation for *BdCAPE* positive sites, and those that were positive for *BdGPL* or both *BdGPL* and *BdCAPE*, but the same significant difference was not seen when comparing the mixed lineage sites to the *BdGPL* positive sites. It is interesting to note that although mixed lineage sites were significantly different from *BdCAPE* sites in terms of mean annual temperature and mean daily precipitation, they were not significantly different from *BdCAPE* positive sites in terms of elevation.

Discussion

Predicted *Bd* distribution in Africa

Our model for predicted *Bd* prevalence in Africa was based on a dataset containing sample data from 26 countries compiled from publications and field surveys, and represents the most comprehensive model for the continent to date. Surveillance of Africa is far from complete because no data was available for the remaining 28 African countries. Targeted surveillance may yield more records, improving the model further, since most of the countries that have not been surveyed are predicted to have suitable regions for *Bd* albeit with lower likelihood. These areas should not be ignored since some of the areas with lower predicted distribution do have confirmed records of *Bd*. Noticeable exceptions of unsurveyed countries with higher likelihood are the coastal regions of North Africa. The higher likelihood regions of our model correlated well with the continental prediction map of Zimkus *et al.* (2020), but differed in that areas not predicted to be suitable to *Bd* by Zimkus *et al.* were shown to have a low to moderate likelihood for *Bd* distribution in our model. Zimkus *et al.* did, however, only use 490 unique records in their model, due to the exclusion of localities where precise coordinate data was unavailable, or sites that fell within a 1 km range from each other. Our model still considered the latter data points, even if it fell within the 1 km range from each other. When considering lineage identity, it is apparent that considerable effort needs to be made to resolve the distribution of *Bd* lineages in Africa. Lineage has only been identified for 59 % of records. Based on this restricted evidence, *Bd*CAPE is present in the highlands of Cameroon, Tanzania (Sewell *et al.*, 2021), Lesotho and South Africa, whereas evidence for *Bd*GPL indicates that it is present in Cameroon, Congo, Equatorial Guinea, São Tomé and Príncipe, Ethiopia, South Africa and Uganda.

Predicted *Bd* lineage distribution in South Africa

The localities used as data for our South African *Bd* models highlighted the need to also extend surveys in areas identified as having a lower likelihood of occurrence. In South Africa the central region generally has a lower amphibian diversity, referred to as the transitional fauna (Poynton, 1961), and yet *Bd*GPL was frequently detected. When investigating the distribution of known lineages along the Orange River, it became apparent that the two lineages in South Africa are associated with different climate environments. The majority of *Bd*GPL records were found at predominantly western sites, while *Bd*CAPE records dominated towards the east. Plotting the mean annual temperature for the different lineage sites in South Africa against the mean annual precipitation showed that both *Bd*GPL and *Bd*CAPE have grouped within different environmental envelopes. At these hotspots, the corresponding lineage was highly

prevalent, with *BdCAPE* associated with the cooler, higher precipitation and montane regions of the Orange River's watercourse, and *BdGPL* associated with the warmer, drier landscape associated with the western part of South Africa.

The areas determined to be hotspots were mutually exclusive for lineage, thus where one lineage was highly prevalent the other lineage was not present. This could be due to founder-controlled distributions of the lineages, essentially pertaining to occupying resources before the other lineage can reach sustainable population levels (Begon *et al.*, 2006). However, our prediction models showed that *BdCAPE* is more restricted in potential range than *BdGPL*, leading to the threat that *BdCAPE* may be displaced, and eventually replaced due to virtually all its habitat being suitable for *BdGPL* as well. But since *Bd* has been in South Africa for some time, it would be expected that if one lineage (*BdCAPE*) was to be easily displaced by the other (*BdGPL*) then the latter should be seen throughout the range of the former, which is not the case. This is supported by the models not showing equal likelihood for presence of both lineages in the same vicinity, specifically when it comes to areas where the likelihood for *BdCAPE* is higher, showing at least a degree of niche separation. *BdCAPE* may also be more competitive when compared to *BdGPL* within areas showing a high likelihood for *BdCAPE* as this lineage can transmit to naïve individuals faster than *BdGPL* (Chapter 2). Further support for this type of community may be the single time that *BdGPL* was isolated from an area typical for the environmental envelope of *BdCAPE*. This single event happened in 2015 and no other *BdGPL* records were subsequently found in the *BdCAPE* envelope, despite extensive sampling in the area post-2015 and the inclusion of lineage-specific qPCR testing, as well as isolation tactics.

When considering only the samples that were lineage-typed using the lineage-specific primers, it is confirmed that the lineages in South Africa are separated by environmental niche, with a zone of overlapping niches between the two clusters. If it becomes apparent in future surveys that lineage niche constriction or expansion is taking place, then it may be better explained by succession of lineages. A similar event occurred in Switzerland where *BdCH* was endemic (Farrer *et al.*, 2011), but more recent isolation attempts only managed to find *BdGPL*, leading to the assumption that *BdGPL* displaced *BdCH* (Garner, 2016). Displacement of *BdCAPE* by *BdGPL*, especially considering the large area that is suitable for both lineages may ultimately lead to the extirpation of *BdCAPE* from South Africa, regardless of its ability to transmit to more hosts in shorter time than *BdGPL* (Chapter 2), but that remains to be determined in line with possible shifts in niches due to climate change. The second possibility is that this contact between the lineages lead to recombination events, leading to a third genotype in areas where there has been contact. This recombinant could be more lethal to

the host than the original lineages, or it could become established and displace the original lineages from the area. There is also the possibility for both these scenarios to occur upon contact. Recombinant lineages might be found as well as displacement of *BdCAPE* by *BdGPL*/recombinant cohort leading to only *BdGPL* and the recombinant lineage being present in future.

Both lineages showed an area of relatively low prevalence in the approximate middle of the Orange River. If this is due to the lineage-specific environmental envelope, then it would be expected that an area where both occur in low prevalence may be at the edge of both lineage's optimal environmental conditions (Wilson, 1999). This low likelihood of presence areas will consequently be a constant area of interaction between the two lineages, and thus the area where recombinant lineages can be found – termed here as a potential recombinant zone. If it is the case that one lineage is displacing the other, then this recombinant zone will be expected to shift over time, resulting in potential recombinant lineages being found over a wider area. This does assume that the recombinants have high survivability, which is yet to be determined. Ultimately, due to the relative cost of recombination for fungi (Lee *et al.*, 2010), and the fact that most fungi are clonal in nature (Drenth *et al.*, 2019), recombination is rare. But there have been cases where recombination within *Phytophthora infestans* led to a new lineage with major destructive capacity (Fry *et al.*, 1992), but this remains to be seen for *Bd*. The discovery of *Bd* recombinant lineages (Schloegel *et al.*, 2012; O'Hanlon *et al.*, 2018) together with the vast areas of South Africa showing a possibility for overlapping distributions and consequently large areas available for possible contact to lead to recombinants requires the consideration of all possible scenarios.

Conclusion

South Africa is host to several *Bd* lineages over a wide range of environmental parameters. This provides us with the opportunity to see not only how a single lineage can differ in prevalence determined by environmental parameters, but also how different lineages can interact on a gradient of environmental change within a single system. The large overlap zone for the two known lineages of *Bd* within South Africa constitutes a worrying prediction for the potential of recombinants to be formed and can be a threat to South African amphibian populations. Especially as our experiments (Chapter 2) showed that *BdCAPE*-infected individuals can become infected with *BdGPL* as well. The experiments showed that *BdCAPE* can transmit to the entire uninfected population faster than *BdGPL*, but *BdGPL* can infect individuals already infected with *BdCAPE*, consequently direct interaction between the two lineages will be inevitable. This inevitability can pose a threat to South African amphibian populations, or cause a new threat in terms of recombinant lineages, of which the virulence

and precise impact on the amphibian populace are not yet known. The identification of recombination zones, as was done with this study, remains of paramount importance in being prepared for future declines possibly caused by recombinant lineages.

References

- Barrett, L.G., Kniskern, J.M., Bodenhausen, N., Zhang, W. & Bergelson, J. 2009. Continua of specificity and virulence in plant host–pathogen interactions: causes and consequences. *New Phytologist*, 183(3): 513-529. DOI: 10.1111/j.1469-8137.2009.02927.x
- Begon, M., Townsend, C.R. & Harper, J.L. 2006. *Ecology: From Individuals to Ecosystems*. 4th ed. Oxford, United Kingdom: Blackwell Publishing.
- Boyle, D.G., Boyle, D.B., Olsen, V., Morgan, J.A.T. & Hyatt, A.D. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms*, 60: 141-148. DOI: 10.3390/s130708551
- Cambray, J.A., Davies, A.J. & Ashton, P.J. 1986. The Orange-Vaal River system. In: Davies, B.R. & Walker, K.F., eds. *The Ecology of River Systems*. Dordrecht, Netherlands: Springer. pp. 89-161.
- Cohen, J.M., Venesky, M.D., Sauer, E.L., Civitello, D.J., McMahon, T.A., Roznik, E.A. & Rohr, J.R. 2017. The thermal mismatch hypothesis explains host susceptibility to an emerging infectious disease. *Ecology Letters*, 20(2): 184-193. DOI: 10.1111/ele.12720
- Conley, A.H. & van Niekerk, P.H. 2000. Sustainable management of international waters: The Orange River case. *Water Policy*, 2(1-2): 131-149.
- Doddington, B.J., Bosch, J., Oliver, J.A., Grassly, N.C., Garcia, G., Schmidt, B.R., Garner, T.W.J., ... Fisher, M.C. 2013. Context-dependent amphibian host population response to an invading pathogen. *Ecology*, 94(8): 1795-1804. DOI: 10.1890/12-1270.1
- Drenth, A., McTaggart, A.R. & Wingfield, B.D. 2019. Fungal clones win the battle, but recombination wins the war. *IMA fungus*, 10(1): 1-6. DOI: 10.1186/s43008-019-0020-8
- Elith, J., Phillips, S.J., Hastie, T., Dudík, M., Chee, Y.E. & Yates, C.J. 2011. A statistical explanation of MaxEnt for ecologists. *Diversity and Distributions*, 17: 43-57. DOI: 10.1111/j.1472-4642.2010.00725.x
- Farrer, R.A., Weinert, L.A., Bielby, J., Garner, T.W.J., Balloux, F., Clare, F., Bosch, J., ... Fisher, M.C. 2011. Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proceedings of the National Academy of Sciences*, 108(46): 18732-18736. DOI:10.1073/pnas.1111915108
- Fry, W.E., Goodwin, S.B., Matuszak, J.M., Spielman, L.J. & Milgroom, M.G. 1992. Population genetics and intercontinental migrations of *Phytophthora infestans*. *Annual Review of Phytopathology*, 30: 107-130. DOI: 10.1146/annurev.py.30.090192.000543

- Garner, T. 2016. Switzerland: *BdGPL* vs. *BdCH* [Correspondence]. 4 February, Potchefstroom, South Africa.
- Garner, T.W.J., Rowcliffe, J.M. & Fisher, M.C. 2011. Climate change, chytridiomycosis or condition: an experimental test of amphibian survival. *Global Change Biology*, 17(2): 667-675. DOI: 10.1111/j.1365-2486.2010.02272.x
- Ghosh, P.N., Verster, R., Sewell, T.R., O'Hanlon, S.J., Brookes, L.M., Rieux, A., Garner, T.W.J., ... Fisher, M.C. 2021. Discriminating lineages of *Batrachochytrium dendrobatidis* using quantitative PCR. *Molecular Ecology Resources*, 21: 1452-1459. DOI: 10.1111/1755-0998.13299
- Griffiths, S.M., Harrison, X.A., Weldon, C., Wood, M.D., Pretorius, A., Hopkins, K., Fox, G., ... Antwis, R.E. 2018. Genetic variability and ontogeny predict microbiome structure in a disease-challenged montane amphibian. *The ISME Journal*, 12(10): 2506-2517. DOI: 10.1038/s41396-018-0167-0
- Holmes, I., McLaren, K. & Wilson, B. 2014. Precipitation constrains amphibian chytrid fungus infection rates in a terrestrial frog assemblage in Jamaica, West Indies. *Biotropica*, 46(2): 219-228. DOI: 10.1111/btp.12093
- Lange, G.-M., Mungatana, E. & Hassan, R. 2007. Water accounting for the Orange River Basin: an economic perspective on managing a transboundary resource. *Ecological Economics*, 61(4): 660-670. DOI: 10.1016/j.ecolecon.2006.07.032
- Lee, S.C., Ni, M., Li, W., Shertz, C. & Heitman, J. 2010. The evolution of sex: a perspective from the fungal kingdom. *Microbiology and Molecular Biology Reviews*, 74(2): 298-340. DOI: 10.1128/MMBR.00005-10
- Longo, A.V., Burrowes, P.A. & Joglar, R.L. 2010. Seasonality of *Batrachochytrium dendrobatidis* infection in direct-developing frogs suggests a mechanism for persistence. *Diseases of Aquatic Organisms*, 92(2-3): 253-260. DOI: 10.3354/dao02054
- Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M.C., Woeltjes, A., ... Pasmans, F. 2013. *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *Proceedings of the National Academy of Sciences*, 110(38): 15325-15329. DOI: 10.1073/pnas.1307356110
- Murray, K.A., Retallick, R.W.R., Puschendorf, R., Skerratt, L.F., Rosauer, D., McCallum, H.I., Berger, L., ... VanDerWal, J. 2011. Assessing spatial patterns of disease risk to biodiversity: implications for the management of the amphibian pathogen, *Batrachochytrium dendrobatidis*. *Journal of Applied Ecology*, 48(1): 163-173. DOI: 10.1111/j.1365-2664.2010.01890.x

- O'Hanlon, S.J., Rieux, A., Farrer, R.A., Rosa, G.M., Waldman, B., Bataille, A., Kosch, T.A., ... Fisher, M.C. 2018. Recent Asian origin of chytrid fungi causing global amphibian declines. *Science*, 360: 621-627. DOI: 10.1126/science.aar1965
- Phillips, S.J., Anderson, R.P. & Schapire, R.E. 2006. Maximum entropy modeling of species geographic distributions. *Ecological Modelling*, 190(3-4): 231-259. DOI: 10.1016/j.ecolmodel.2005.03.026
- Poynton, J.C. 1961. Zoogeography of eastern Africa: an outline based on anuran distribution. *Nature*, 189(4767): 1217-1219. DOI: 10.1038/1941217a0
- Puschendorf, R., Carnaval, A.C., VanDerWal, J., Zumbado-Ulate, H., Chaves, G., Bolaños, F. & Alford, R.A. 2009. Distribution models for the amphibian chytrid *Batrachochytrium dendrobatidis* in Costa Rica: proposing climatic refuges as a conservation tool. *Diversity and Distributions*, 15(3): 401-408. DOI: 10.1111/j.1472-4642.2008.00548.x
- Rödger, D., Kielgast, J. & Lötters, S. 2010. Future potential distribution of the emerging amphibian chytrid fungus under anthropogenic climate change. *Diseases of Aquatic Organisms*, 92(2): 201-207. DOI: 10.3354/dao02197
- Rohr, J.R., Raffel, T.R., Romansic, J.M., McCallum, H. & Hudson, P.J. 2008. Evaluating the links between climate, disease spread, and amphibian declines. *Proceedings of the National Academy of Sciences*, 105(45): 17436-17441. DOI: 10.1073/pnas.0806368105
- Schloegel, L.M., Toledo, L.F., Longcore, J.E., Greenspan, S.E., Vieira, C.A., Lee, M., Zhao, S., ... Daszak, P.J., T. Y. 2012. Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. *Molecular Ecology*, 21(21): 5162-5177. DOI: 10.1111/j.1365-294X.2012.05710.x
- Sewell, T.R., Van Dorp, L., Ghosh, P.N., Wierzbicki, C., Caroe, C., Lyakurwa, J.V., Tonelli, E., ... Fisher, M.C. 2021. A series of terribly unfortunate events: how environment and infection synergized to cause the Kihansi spray toad extinction. *bioRxiv*, DOI: 10.1101/2021.11.10.468118
- Smith, K.G. & Weldon, C. 2007. A conceptual framework for detecting oral chytridiomycosis in tadpoles. *Copeia*, 2007(4): 1024-1028. DOI: 10.1643/0045-8511(2007)7%5B1024:ACFFDO%5D2.0.CO;2
- Spitzen-van der Sluijs, A., Canessa, S., Martel, A. & Pasmans, F. 2017. Fragile coexistence of a global chytrid pathogen with amphibian populations is mediated by environment and demography. *Proceedings of the Royal Society B*, 284(1864): 20171444. DOI: 10.1098/rspb.2017.1444

- Spitzen-Van Der Sluijs, A., Martel, A., Hallmann, C.A., Bosman, W., Garner, T.W.J., Van Rooij, P., Jooris, R., ... Pasmans, F. 2014. Environmental determinants of recent endemism of *Batrachochytrium dendrobatidis* infections in amphibian assemblages in the absence of disease outbreaks. *Conservation Biology*, 28(5): 1302-1311. DOI: 10.1111/cobi.12281
- Tarrant, J., Cilliers, D., Du Preez, L.H. & Weldon, C. 2013. Spatial assessment of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) in South Africa confirms endemic and widespread infection. *PLoS ONE*, 8(7): e69591. DOI: 10.1371/journal.pone.0069591
- Weldon, C. & Du Preez, L.H. 2006. Quantitative measurement of *Batrachochytrium dendrobatidis* in amphibian skin. *Diseases of Aquatic Organisms*, 72(2): 153-161. DOI: 10.3354/dao072153
- White, F. 1983. *Vegetation of Africa - a descriptive memoir to accompany the UNESCO/AETFAT/UNSO vegetation map of Africa*. Paris, France: U. N. Educational , Scientific and Cultural Organization
- Wilson, J.B. 1999. Guilds, functional types and ecological groups. *Oikos*: 507-522. DOI: 10.2307/3546655
- Woodhams, D.C., Alford, R.A., Briggs, C.J., Johnson, M. & Rollins-Smith, L.A. 2008. Life-history trade-offs influence disease in changing climates: strategies of an amphibian pathogen. *Ecology*, 89(6): 1627-1639. DOI: 10.1890/06-1842.1
- Xie, G.Y., Olson, D.H. & Blaustein, A.R. 2016. Projecting the global distribution of the emerging amphibian fungal pathogen, *Batrachochytrium dendrobatidis*, based on IPCC climate futures. *PLoS ONE*, 11(8): e0160746. DOI: 10.1371/journal.pone.0160746
- Zimkus, B.M., Baláž, V., Belasen, A.M., Bell, R.C., Channing, A., Doumbia, J., Fokam, E.B., ... Penner, J. 2020. Chytrid pathogen (*Batrachochytrium dendrobatidis*) in African amphibians: a continental analysis of occurrences and modeling of its potential distribution. *Herpetologica*, 76(2): 201-215. DOI: 10.1655/0018-0831-76.2.201

Chapter 5

Discriminating lineages of *Batrachochytrium dendrobatidis* using quantitative PCR

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Abstract

The ability to detect and monitor infectious disease in a phylogenetically informative manner is critical for their management. Phylogenetically informative diagnostic tests enable patterns of pathogen introduction or changes in the distribution of genotypes to be measured, enabling research into the ecology of the pathogen. *Batrachochytrium dendrobatidis* (*Bd*), a causative agent of chytridiomycosis in amphibian populations, emerged worldwide in the 21st century and is composed of six lineages which display varying levels of virulence in their hosts. Research into the distribution, ecology and pathogenicity of these lineages has been hampered by an inability to type lineage efficiently. Here, we describe a lineage-specific TaqMan qPCR assay that differentiates the two lineages of *Bd* most commonly associated with chytridiomycosis: *Bd*GPL and *Bd*CAPE. We demonstrate how this assay can be used for the surveillance of wild populations of amphibians in Southern Africa using skin swabs, tissue samples and cultured isolates.

Keywords:

Batrachochytrium dendrobatidis, chytrid, diagnostic, lineage, qPCR

Introduction

Bd, a fungal pathogen of amphibians, causes the potentially lethal disease chytridiomycosis. This fungus is a member of the Chytridiomycota, and one of only two species within the phylum known to infect vertebrates (Longcore *et al.*, 1999; Martel *et al.*, 2013). *Bd* has been detected infecting almost 700 species of amphibians, with chytridiomycosis implicated as the cause of population declines in over 500 species (Olson & Ronnenberg, 2014; Scheele *et al.*, 2019). This has led to the extinction of an estimated 90 species of amphibians globally (Scheele *et al.*, 2019).

Within *Bd* there are at least six phylogenetically distinct lineages – *BdCAPE*, *BdGPL*, *BdASIA-1*, *BdASIA-2/BRAZIL*, *BdASIA-3* and *BdCH*. However, *BdCAPE* and *BdGPL* are primarily associated with mass mortality events and species declines or extinctions (Farrer *et al.*, 2011; Doddington *et al.*, 2013; Jenkinson *et al.*, 2016; O'Hanlon *et al.*, 2018; Byrne *et al.*, 2019). While only *BdGPL* has undergone a rapid, recent and global range expansion, recent research indicates that the other five lineages, which had previously been considered largely spatially restricted, have achieved intercontinental distributions. This raises the possibility of lineage contact and interactions occurring. One such interaction is interlineage genetic recombination (Jenkinson *et al.*, 2016; O'Hanlon *et al.*, 2018; Byrne *et al.*, 2019) which may lead to increased virulence (Greenspan *et al.*, 2018). Given the infrequency with which recombinations are isolated, molecular tools that enable the identification and monitoring of areas where they might occur are needed in order to focus research effort effectively. To date, this has been hampered by the lack of a suitable lineage-specific diagnostic test for *Bd*.

Currently, a pan-lineage qPCR assay targeting the ITS region of the genome is the most widely-used method for detecting *Bd* (Boyle *et al.*, 2004). This method has high specificity, is sensitive enough to detect subclinical infections, and works with swabs taken from adult amphibian skin or tadpole mouthparts, so is minimally invasive (Boyle *et al.*, 2004; Kriger *et al.*, 2006; Hyatt *et al.*, 2007; Garland *et al.*, 2011; Skerratt *et al.*, 2011). A further advantage of this assay working with swab samples is that these are light, easy to carry in difficult field settings and are easy to store (Hyatt *et al.*, 2007; Van Sluys *et al.*, 2008). The primary drawback to this pan-lineage qPCR assay is its inability to type the lineage of *Bd* present in a sample. Although there have been attempts to design lineage-specific diagnostics based on the targeted genomic region, the ITS is unable to resolve interspecific phylogenies within *Bd* owing to a breakdown in concerted evolution across this multiallelic array (Schoch *et al.*, 2012; O'Hanlon *et al.*, 2018).

The most accurate method for determining lineage is whole genome sequencing (WGS) from a pure culture of *Bd*. Although comparative genomic analyses have been extremely valuable

in the field of chytrid research, the expense and requirement for a pure isolate culture make this approach impractical on the large scale required to answer fundamental questions about *Bd* lineage distribution and ecology. The most reliable alternative approach to WGS, multilocus sequence typing (MLST) is suitable for use with swabs but requires access to a specialised technology, the Fluidigm Access Array platform, and a reasonably high infection burden (>150 genomic equivalents [GE]). It is, therefore, not applicable for all *Bd* positive cases, especially in hosts with a subclinical infection burden (Byrne *et al.*, 2017).

Recent research has shown that phylogenetic analyses based on *Bd* mitochondrial DNA (mtDNA) recovers the same lineages as those recovered by WGS (O’Hanlon *et al.*, 2018). The *Bd* mitochondrial genome has recently been fully sequenced, has a low rate of recombination relative to the nuclear genome, and is present at high copy number so is likely detectable at low infection burdens. There is also a precedent for using mtDNA for phylogenetic studies across a wide range of taxa (Barr *et al.*, 2011; Li *et al.*, 2016; Schreeg *et al.*, 2016; Song *et al.*, 2016; Penry *et al.*, 2018). Here, we report a lineage-specific qPCR assay for *Bd* based on mtDNA which detects the two lineages of *Bd* most commonly associated with chytridiomycosis in wild populations – *Bd*GPL and *Bd*CAPE – with high specificity and sensitivity.

Material and methods

Assay design and optimisation

A 175,295 kb long mtDNA alignment for 150 chytrid isolates, with 145 isolates of *Bd* representing *Bd*PGL, *Bd*CAPE, *Bd*ASIA-1, *Bd*ASIA-2/BRAZIL and *Bd*CH, was manually screened for lineage-specific single nucleotide polymorphisms (SNPs) (O’Hanlon *et al.*, 2018). Candidate primers and probes were designed using Primer Express Software 3.0 (ThermoFisher Scientific).

We optimised primer and probe concentrations separately using a checkboard system in singleplex with DNA quantitation standards for the DNA template. We selected final primer and probe concentrations which maximised reaction efficiency compared to oligonucleotide concentration, while ensuring that the mean C_t values for a given quantity of DNA remained as similar as possible between the *Bd*CAPE and *Bd*GPL reactions. Consistency between the reactions made it possible to multiplex the two qPCR reactions into a single assay diagnosing both *Bd*GPL and *Bd*CAPE. All optimisation and validation reactions were carried out on both

a 7300 Real-Time PCR system and a QuantStudio 7 Flex Real-Time PCR system (Applied Biosystems).

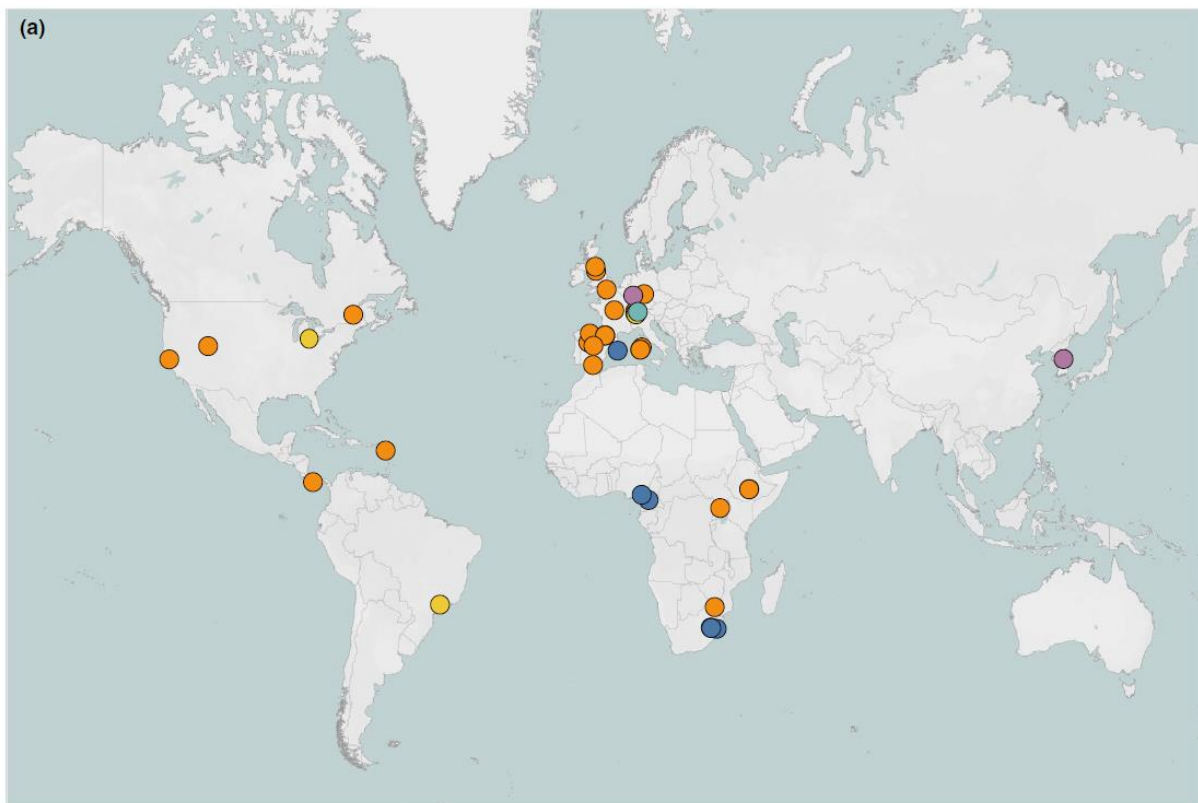
The cycling conditions were identical to the pan-lineage *Bd* diagnostic of Boyle *et al.* (2004), except that we reduced the number of cycles from 50 to 40 and increased the annealing temperature from 60 to 62°C in order to increase the specificity. We set the C_t threshold for all reactions at $\Delta R_n = 0.1$, and the total reaction volume in all cases were 25 µl in total (20 µl master mix and 5 µl DNA template). The optimised multiplex reaction mix and full cycling conditions are provided in the Supporting Information. When the reactions were run in singleplex the same quantities of all reactions were used, except for an increase in the volume of filtered water commensurate with the quantity of primer and probe removed. We quantified the starting DNA present in a reaction using standard curve analysis calculated from DNA quantitation standards made from isolate IA042 (*Bd*GPL) and isolate TF5a1 (*Bd*CAPE) (Table S1). Both these isolates were previously typed to their respective lineages using WGS (O’Hanlon *et al.*, 2018). The final primer and TaqMan MGB probe sequences are shown in Figure 1. We carried out pan-lineage *Bd* detection using the protocol of Boyle *et al.* (2004).



Figure 1: *Bd* mtDNA sequence sections showing the (a) *Bd*CAPE-specific and (b) *Bd*GPL-specific primer and TaqMan MGB probe sequences

Assessing lineage-specific qPCR specificity, sensitivity and reliability

A panel of 52 isolates (38 *Bd*GPL, six *Bd*CAPE, four *Bd*ASIA-1, three *Bd*ASIA-2/BRAZIL and one *Bd*CH) collected from 17 countries as well as from traded amphibians was used to test the specificity of the lineage-specific qPCR (Figure 2-A). All isolates had been previously assigned a lineage by WGS (O’Hanlon *et al.*, 2018) (Figure 2-B). We extracted DNA from the live cultures following RACE protocols (Supporting Information) for 50 isolates (Table S2). DNA extracts for isolates UM142 and CLFT-065 were provided by University of Michigan. We tested all 52 isolates with both the lineage-specific qPCR reactions in singleplex to check for cross-reactivity. All other reactions were carried out in multiplex. To test for sensitivity, we ran the lineage-specific qPCR assay with a 10-fold dilution series of *Bd*GPL and *Bd*CAPE DNA quantitation standards in duplicate. Once a suitable range of DNA concentrations for standard curve analysis had been identified, we tested the repeatability of the assay within and between plates by comparing reaction efficiency, and R^2 and C_t values.



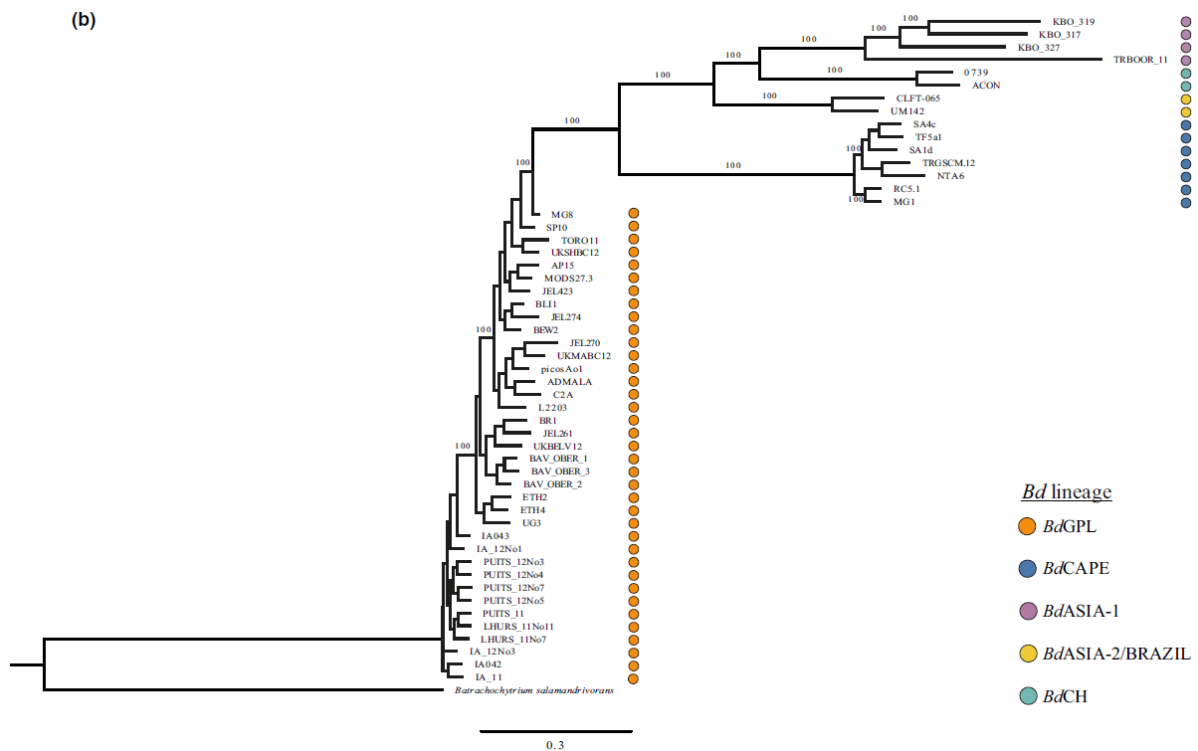


Figure 2: (a) Map showing locations of *Bd* isolates used for assay specificity testing; (b) phylogeny of isolates used for assay specificity testing, generated from WGS analysis. Lineage is indicated by dot colour. Map generated in Tableau and formatted in Adobe Illustrator

Field sampling and processing

WGS analysis has revealed that *Bd*GPL and *Bd*CAPE are both present in wild populations in South Africa in the Orange River Basin (Figure 3-A). This region was therefore selected as a study system to see if the lineage-specific qPCR assay diagnosed the same lineages from field sites as WGS. Sites were surveyed along the Orange River at approximately 100 km apart from the source in the Drakensberg Mountains to near the estuary on the border with Namibia. At each site, we attempted to culture and isolate *Bd* using non-lethal tissue sampling from adult amphibians and lethal mouthpart sampling from tadpoles (Fisher *et al.*, 2018). Any resulting chytrids were lineage-typed by WGS using the protocol described by O’Hanlon *et al.* (2018). All adult amphibians were also swabbed following “Risk Assessment of Chytridiomycosis to European Amphibian Biodiversity (RACE)” protocols (Biodiversa, 2013; Fisher *et al.*, 2018). DNA extraction also followed the RACE protocol (Supporting Information), following which swab samples were tested for *Bd* using the pan-lineage qPCR diagnostic. Any samples which were positive in duplicate with an infection load of more than one genomic equivalents (GE) were tested with the lineage-specific qPCR diagnostic in duplicate with DNA quantitation standards and two no-template controls.

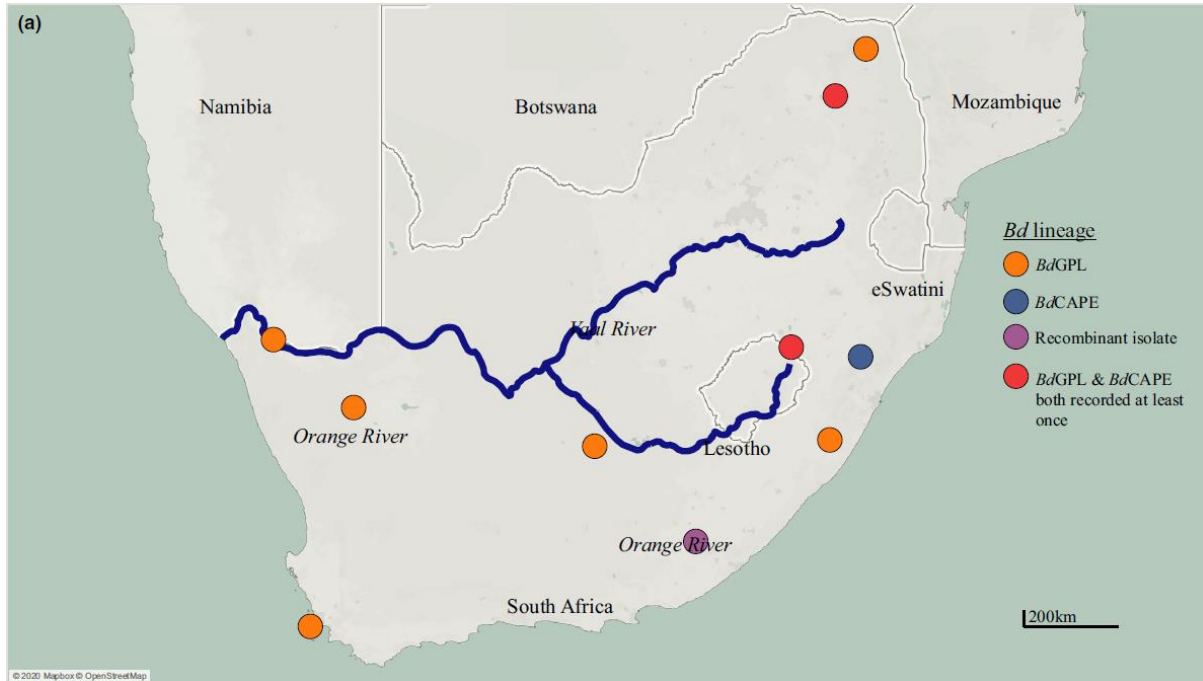
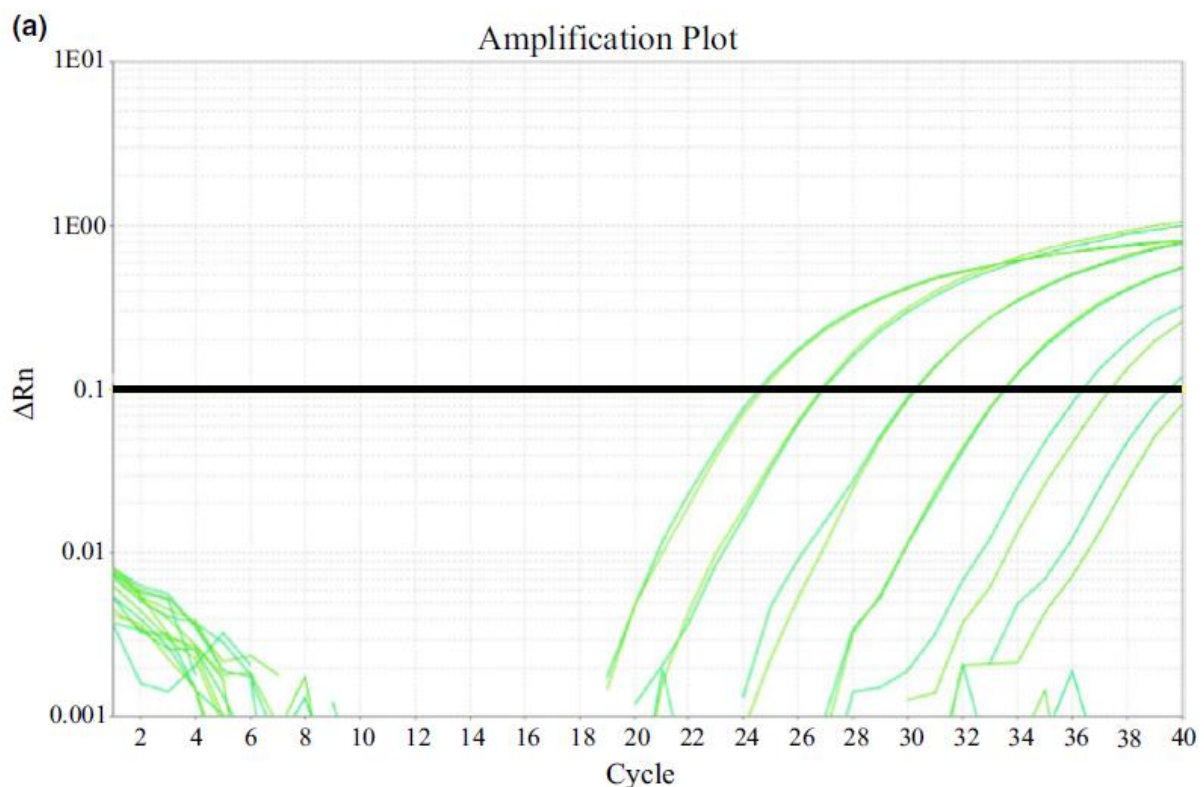


Figure 3: Maps showing (a) *Bd* lineage locations in South Africa identified by isolation and WGS analysis and (b) comparison of *Bd* lineage typing of sites in South Africa using WGS analysis and lineage-specific qPCR. Agreement of diagnostics is indicated by shape of the mark; lineages identified at the site are identified by the colour. Map generated in Tableau and formatted in Adobe Illustrator; additional data downloaded from naturalearthdata.com

Results

Lineage-specific qPCR performance under laboratory conditions

The *Bd* lineage-specific assay was able to detect all isolates assigned to the relevant lineage by WGS. The *Bd*GPL assay also detected DNA from one isolate from the *Bd*ASIA-2/BRAZIL lineage (UM142) but at an unrealistic concentration. This reaction also did not cross the reaction threshold during the exponential phase, as would be expected in a true positive result (Figure S1). Both lineage-specific assays had a limit of detection of 1GE (Figure 4). The *Bd*CAPE-specific recorded a plateauing of fluorescence at 10,000 GE, resulting in an underestimation of *Bd* load when comparing with the other quantitation standard fluorescence levels (Figure 4-A). This plateauing owes to too much target DNA present in a sample quenching the PCR reaction due to the depletion of reagents. Based on these results, we recommend a DNA quantitation standard range for both lineage-specific qPCRs of 1000 GE, 100 GE, 10 GE and 1 GE for standard curve analysis.



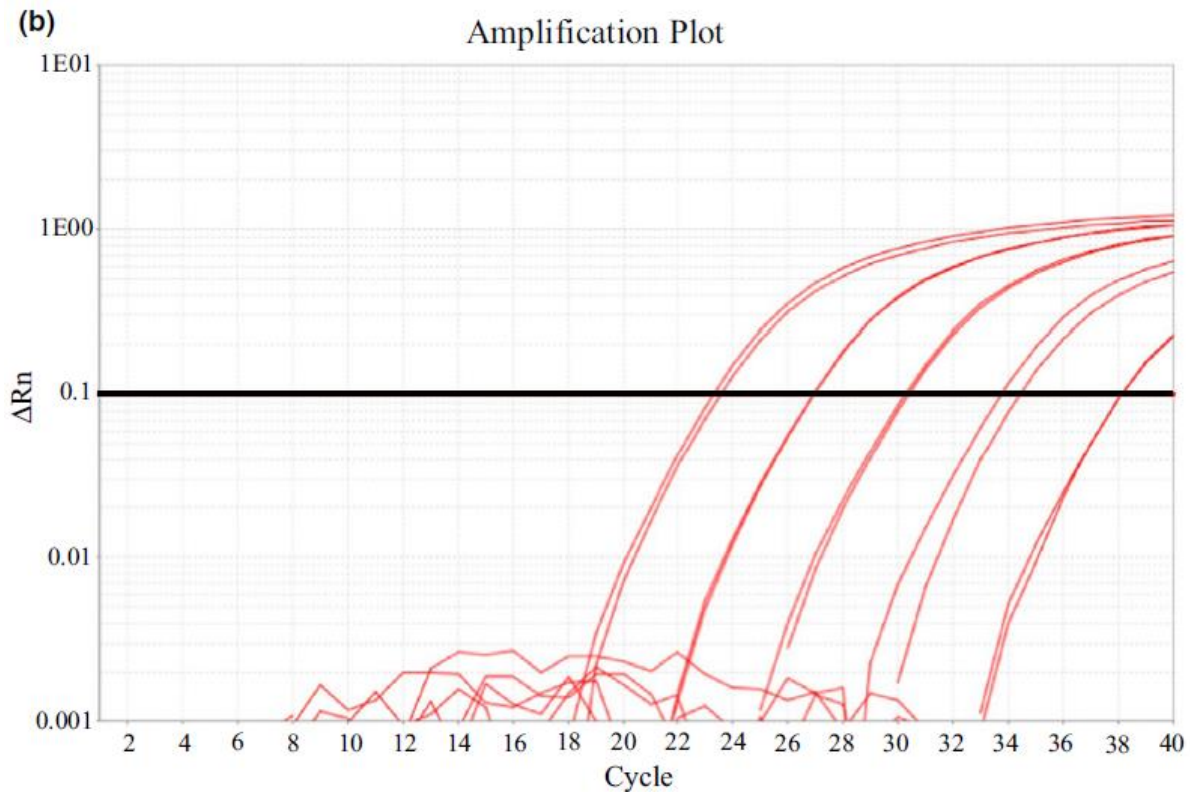


Figure 4: qPCR fluorescence amplification plots showing amplification of 10,000 GE, 1000 GE, 100 GE, 10 GE and 1 GE DNA quantitation standards with (a) *BdCAPE*-specific primers and TaqMan MGB probe and (b) *BdGPL*-specific primers and TaqMan MGB probe

Both assays performed well over three separate qPCR plates. The overall mean percentage efficiency for the *BdCAPE*-specific qPCR was 108.87 % (ranging from 99.35 % to 104.21 %). All R^2 values, whether averaged across and within plates were >0.95 (ranging from 0.98 to 0.99) and the C_t values of DNA quantitation standards were also consistent, all crossing the C_t threshold within one cycle of each other in all cases.

Lineage-specific qPCR performance under field conditions

We obtained at least one pure culture of *Bd* and at least one *Bd*-positive skin swab that met the threshold for qPCR lineage-testing from 10 sampling sites. At all sites except at Aliwal North, WGS analysis and the lineage-specific qPCR agreed in their lineage typing (Figure 3-B). At Aliwal North, the lineage-specific qPCR was positive for *BdCAPE*, but WGS analysis revealed that the isolate recovered from the site was a CAPE/GPL recombinant. At Douglas, a single swab returned a positive for both *BdGPL* and *BdCAPE*. Both lineages were also recovered from the same pond at Douglas by isolation and WGS.

Discussion

We have designed a *Bd*CAPE- and *Bd*GPL-specific qPCR diagnostic that will facilitate research into the ecology and distribution of these epidemiologically important *Bd* lineages. The diagnostic gap that has persisted until now resulted in an inability to gather baseline data on *Bd* distribution at a phylogenetically and spatially useful scale. This lack has led to difficulties investigating the ecology and epidemiological significance of lineage interactions. Our lineage-specific qPCR complements existing diagnostics for *Bd* while meeting the need to decipher lineage in large numbers of samples and in animals with low infection burdens.

The qPCR assay was able to correctly detect whether *Bd*GPL or *Bd*CAPE was present in 51 out of 52 isolates. The most likely explanation for the low-level cross-reactivity of the *Bd*GPL-specific qPCR assay with a *Bd*ASIA-2/BRAZIL isolate is that the DNA extract was contaminated with DNA from a *Bd*GPL isolate at some point after WGS had been carried out. Sequencing reads from this isolate's mtDNA were screened for the presence of the *Bd*GPL-specific probe, which was not found. This result highlights the importance of manually examining amplification plots to assess whether they appear typical, and to continue to utilise WGS methods where possible to corroborate results from the lineage-specific qPCR.

The assay was sufficiently sensitive for use in cases of low infection burden, with a sensitivity of one genomic equivalent. We note, however, that mtDNA copy number has not been quantified for any *Bd* isolate and that ITS copy number, which is used to quantify infection burden in the majority of *Bd* surveillance studies, is known to be highly variable (Longo *et al.*, 2013; Rebollar *et al.*, 2017). We recommend therefore that in order to remain consistent with the current literature, that infection intensity be reported based on the pan-lineage *Bd* diagnostic, but that this quantification should be viewed as semi-quantitative rather than absolute. Under experimental conditions, this problem should be resolved by quantifying the copy number of the targeted region for specific isolates before carrying out any experimental work.

Novel diagnostics are challenging to test in a field setting, due to the lack of a priori knowledge of the location of the target pathogen. However, agreement of the lineage-specific qPCR diagnostic with WGS analysis for the sites in South Africa strongly supports the use of the qPCR assay under field conditions. Crucially, the results from Douglas indicate that the assay is able to identify where mixed-lineage infections are occurring at both the population and individual host level. This indicates that the assay is appropriate for use in putative lineage contact zones where lineage interactions may occur. The lineage-specific qPCR identified the only recombinant lineage collected as *Bd*CAPE. This suggests uniparental inheritance of mitochondria in *Bd*, a new insight into the pathogen's biology.

In this study, we focussed on designing diagnostics for *Bd*CAPE and *Bd*GPL not only as these are the lineages most commonly associated with chytridiomycosis, but also due to knowledge barriers making similar work for the remaining lineages challenging until very recently. The *Bd* isolates that have been fully sequenced with published mitochondrial genomes are biased towards *Bd*GPL and, to a lesser extent, *Bd*CAPE. As a result, these two lineages have until recently had much more clearly resolved internal phylogenies than the others, allowing greater confidence when identifying lineage-specific SNPs. The comparative lack of confidence with which SNPs for the remaining lineages could be assigned made investing time and financial costs of designing lineage-specific assays unreasonable. However, recently the global *Bd* phylogeny has been more clearly resolved, and we therefore recommend that similar lineage-specific mtDNA based diagnostics for *Bd*ASIA-1, *Bd*ASIA-2, *Bd*ASIA-3 and *Bd*CH should be designed as a research priority. This would allow the rapid and widespread generation of baseline data from field samples, allowing much finer delineation of *Bd* lineage distributions globally and further insight into *Bd* ecology.

The consistency of any diagnostic assay across multiple laboratories is essential to its utility and ease of use. The wide application of the pan-lineage *Bd* qPCR diagnostic to date means that any laboratory working on *Bd* is likely to have access to all the reagents and facilities required to perform the lineage-specific assay described here, at a similar cost per sample. Lineage-specific diagnostics also enable *in vivo* and *in vitro* experimental manipulation of *Bd* lineages, thus providing further insight into *Bd* population dynamics and structure.

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Author contributions

The research was planned by P.N.G., T.W.J.G., and M.C.F. mtDNA sequences were compiled and aligned by A.R. Primer and TaqMan MGB probe design was done by P.N.G. Assay validation was carried out by P.N.G., and L.M.B., P.N.G., R.V., T.W.J.G. and C.W. carried out fieldwork in South Africa. S.J.O.H and T.R.S carried out whole genome sequences analyses. P.N.G. wrote the article with input from all authors.

Data availability

The mtDNA alignment is deposited in DRYAD Data Repository <https://datadryad.org/stash/dataset/doi:10.5061/dryad.r2280gbbb>.

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References

- Barr, A., Premasuthan, A., Satkoski, J., Smith, D.G., George, D. & Kanthaswamy, S. 2011. A rapid quantitative real-time PCR-based DNA quantification assay coupled with species – assignment capabilities for two hybridizing *Macaca* species. *Folia Primatologica*, 82(2): 71-80. DOI: 10.1159/000328124
- Biodiversa. 2013. Wildlife diseases on the increase: a serious threat for Europe's Biodiversity. The example of the *Bd* fungus disease.
- Boyle, D.G., Boyle, D.B., Olsen, V., Morgan, J.A.T. & Hyatt, A.D. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms*, 60: 141-148. DOI: 10.3390/s130708551
- Byrne, A.Q., Rothstein, A.P., Poorten, T.J., Erens, J., Settles, M.L. & Rosenblum, E.B. 2017. Unlocking the story in the swab: a new genotyping assay for the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. *Molecular Ecology Resources*, 17(6): 1283-1292. DOI: 10.1111/1755-0998.12675
- Byrne, A.Q., Vredenburg, V.T., Martel, A., Pasmans, F., Bell, R.C., Blackburn, D.C., Bletz, M.C., ... Rosenblum, E.B. 2019. Cryptic diversity of a widespread global pathogen reveals expanded threats to amphibian conservation. *Proceedings of the National Academy of Sciences*, 116(41): 20382-20387. DOI: 10.1073/pnas.1908289116
- Doddington, B.J., Bosch, J., Oliver, J.A., Grassly, N.C., Garcia, G., Schmidt, B.R., Garner, T.W.J., ... Fisher, M.C. 2013. Context-dependent amphibian host population response to an invading pathogen. *Ecology*, 94(8): 1795-1804. DOI: 10.1890/12-1270.1
- Farrer, R.A., Weinert, L.A., Bielby, J., Garner, T.W.J., Balloux, F., Clare, F., Bosch, J., ... Fisher, M.C. 2011. Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proceedings of the National Academy of Sciences*, 108(46): 18732-18736. DOI:10.1073/pnas.1111915108
- Fisher, M.C., Ghosh, P., Shelton, J.M.G., Bates, K., Brookes, L., Wierzbicki, C., Rosa, C.M., ... Garner, T.W.J. 2018. Development and worldwide use of non-lethal, and minimal population-level impact, protocols for the isolation of amphibian chytrid fungi. *Scientific Reports*, 8(1): 7772. DOI: 10.1038/s41598-018-24472-2
- Garland, S., Wood, J. & Skerratt, L.F. 2011. Comparison of sensitivity between real-time detection of a TaqMan assay for *Batrachochytrium dendrobatidis* and conventional detection. *Diseases of Aquatic Organisms*, 94(2): 101-105. DOI: 10.3354/dao02327

- Greenspan, S.E., Lambertini, C., Carvalho, T., James, T.Y., Toledo, L.F., Haddad, C.F.B. & Becker, C.G. 2018. Hybrids of amphibian chytrid show high virulence in native hosts. *Scientific Reports*, 8(1): 9600. DOI: 10.1038/s41598-018-27828-w
- Hyatt, A.D., Boyle, D.G., Olsen, V., Boyle, D.B., Berger, L., Obendorf, D., Dalton, A., ... Colling, A. 2007. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms*, 73: 175-192. DOI: 10.1002/adhm.201300318
- Jenkinson, T.S., Betancourt Román, C.M., Lambertini, C., Valencia-Aguilar, A., Rodriguez, D., Nunes-de-Almeida, C.H.L., Ruggeri, J., ... James, T.Y. 2016. Amphibian-killing chytrid in Brazil comprises both locally endemic and globally expanding populations. *Molecular Ecology*, 25(13): 2978-2996. DOI:10.1111/mec.13599
- Kruger, K.M., Hines, H.B., Hyatt, A.D., Boyle, D.G. & Hero, J. 2006. Techniques for detecting chytridiomycosis in wild frogs: comparing histology with real-time Taqman PCR. *Diseases of Aquatic Organisms*, 71(2): 141-148. DOI: 10.3354/dao071141
- Li, Q., Wei, S., Tang, P., Wu, Q., Shi, M., Sharkey, M.J. & Chen, X. 2016. Multiple lines of evidence from mitochondrial genomes resolve phylogenetic relationships of parasitic wasps in Braconidae. *Genome Biology and Evolution*, 8(9): 2651-2662. DOI: 10.1093/gbe/evw184
- Longcore, J.E., Pessier, A.P. & Nichols, D.K. 1999. *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia*, 91(2): 219-227. DOI: 10.1080/00275514.1999.12061011
- Longo, A.V., Ossiboff, R.J., Zamudio, K.R. & Burrowes, P.A. 2013. Lability in host defenses: terrestrial frogs die from chytridiomycosis under enzootic conditions. *Journal of Wildlife Diseases*, 49(1): 197-199. DOI: 10.7589/2012-05-129
- Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M.C., Woeltjes, A., ... Pasmans, F. 2013. *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *Proceedings of the National Academy of Sciences*, 110(38): 15325-15329. DOI: 10.1073/pnas.1307356110
- O'Hanlon, S.J., Rieux, A., Farrer, R.A., Rosa, G.M., Waldman, B., Bataille, A., Kosch, T.A., ... Fisher, M.C. 2018. Recent Asian origin of chytrid fungi causing global amphibian declines. *Science*, 360: 621-627. DOI: 10.1126/science.aar1965
- Olson, D. & Ronnenberg, K. 2014. Global Bd mapping project: 2014 update. *FrogLog*, 22: 17-21.

- Penry, G.S., Hammond, P.S., Cockcroft, V.G., Best, P.B., Thornton, M. & Graves, J.A. 2018. Phylogenetic relationships in southern African Bryde's whales inferred from mitochondrial DNA: further support for subspecies delineation between the two allopatric populations. *Conservation Genetics*, 19(6): 1349-1365. DOI: 10.1007/s10592-018-1105-4
- Rebollar, E.A., Woodhams, D.C., LaBumbard, B., Kielgast, J. & Harris, R.N. 2017. Prevalence and pathogen load estimates for the fungus *Batrachochytrium dendrobatidis* are impacted by ITS DNA copy number variation. *Diseases of Aquatic Organisms*, 123(3): 213-226. DOI: 10.3354/dao03097
- Scheele, B.C., Pasmans, F., Skerratt, L.F., Berger, L., Martel, A., Beukema, W., Acevedo, A.A., ... Canessa, S. 2019. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science*, 363(6434): 1459-1463. DOI: 10.1126/science.aav0379
- Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., ... Consortium, F.B. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for *Fungi*. *Proceedings of the National Academy of Sciences*, 109(16): 6241-6246. DOI: 10.1073/pnas.1117018109
- Schreeg, M.E., Marr, H.S., Tarigo, J.L., Cohn, L.A., Bird, D.M., Scholl, E.H., Levy, M.G., ... Birkenheuer, A.J. 2016. Mitochondrial genome sequences and structures aid in the resolution of *Piroplasmida* phylogeny. *PLoS ONE*, 11(11): e0165702. DOI: 10.1371/journal.pone.0165702
- Skerratt, L.F., Mendez, D., McDonald, K.R., Garland, S., Livingstone, J., Berger, L. & Speare, R. 2011. Validation of diagnostic tests in wildlife: the case of chytridiomycosis in wild amphibians. *Journal of Herpetology*, 45(4): 444-450. DOI: 10.1670/10-193.1
- Song, S., Tang, P., Wei, S. & Chen, X. 2016. Comparative and phylogenetic analysis of the mitochondrial genomes in basal hymenopterans. *Scientific Reports*, 6(1): 1-11. DOI: 10.1038/srep20972
- Van Sluys, M., Kriger, K.M., Phillott, A.D., Campbell, R., Skerratt, L.F. & Hero, J.M. 2008. Storage of samples at high temperatures reduces the amount of amphibian chytrid fungus *Batrachochytrium dendrobatidis* DNA detectable by PCR assay. *Diseases of Aquatic Organisms*, 81: 93-97. DOI: 10.3354/dao01953

Chapter 6

Amphibian chytrid fungus in Africa – Realigning hypotheses and the research paradigm

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Abstract

The amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), responsible for numerous amphibian declines and extinctions, was previously thought to originate from the African continent. This was based on infected museum specimens from early 20th century South Africa, Cameroon and Uganda. Further research on archived specimens from other continents eventually revealed early 20th century records also in Brazil and Japan. Recent robust analysis of genomic diversity and phylogeny of *Bd* has shown origin from Asia to be more plausible. This raises the issue that the threat of *Bd* for African amphibians as a novel pathogen has been underestimated. There are now cases where dramatic amphibian declines in disparate mountains on the continent could be attributed to *Bd*, and this necessitates an urgent realigning of hypotheses and the research agenda for amphibian conservation on the continent. Notably, hotspots of amphibian host naivety include West Africa where this pathogen has so far not been detected. We discuss research gaps that amphibian conservationists might focus on, notably more genomic sequencing of the *Bd*CAPE (the less virulent) lineage to determine its date of emergence, and assessing the susceptibility of different amphibian species to infection, disease and decline to better prioritize conservation actions.

Keywords:

Emerging infectious disease; conservation; chytridiomycosis; *Batrachochytrium*; Anura; caecilian; Africa; amphibian declines

Review

Based on its phylogeny and genomic diversity, the amphibian chytrid fungal pathogen (*Batrachochytrium dendrobatidis*; hereafter *Bd*), a causative agent of the lethal disease, amphibian chytridiomycosis, appears to have recently originated from Asia (O'Hanlon *et al.*, 2018). This discovery challenges some of the working hypotheses about *Bd* and its likely conservation impacts (Weldon *et al.*, 2004; Rachowicz *et al.*, 2005; Lips *et al.*, 2008). One new perspective, for example, is that this widespread and destructive pathogenic chytrid fungus has been recently introduced to the African continent. Prior to the latest study by O'Hanlon *et al.* (2018), *Bd* was hypothesized to originate from Africa, based on: records of infected frogs in the early 20th century in disjunct countries (Weldon *et al.*, 2004; Soto-Azat *et al.*, 2010; Vredenburg *et al.*, 2013; Seimon *et al.*, 2015); widespread occurrence in contemporary amphibian communities on the continent either side of the Sahara in the absence of invasive alien amphibian species (Kielgast *et al.*, 2010; El Mouden *et al.*, 2011; Reeder *et al.*, 2011; Tarrant *et al.*, 2013); scant support for obvious amphibian declines in the presence of *Bd* (e.g. Smith *et al.*, 2007); and the presence of multiple lineages of *Bd* in South Africa suggesting broad genetic diversity of a source pathogen population (Farrer *et al.*, 2011). Additionally, substantial international export of clawed frogs (*Xenopus* spp.) from South Africa was initiated in the early 20th century (Weldon *et al.*, 2007), which is contemporaneous with the posited time of emergence and initial global spread of *Bd* (Farrer *et al.*, 2011; O'Hanlon *et al.*, 2018). In addition to the O'Hanlon *et al.* (2018) study, the 'chytrid out of Africa' hypothesis had been challenged by an absence of *Bd* in surveys in Africa west of the Dahomey Gap (the dry savanna separating moist forests of Ghana and western Nigeria, forming a separate West African biogeographic hotspot Penner *et al.*, 2013). Furthermore, *Bd* has been detected in archived amphibian specimens collected in the early 20th century from South America (Carvalho *et al.*, 2017), North America (Talley *et al.*, 2015) and Asia (Japan) (Goka *et al.*, 2009). Results from the examination of museum specimens, however, should be treated with caution because, within such collections, there often are numerous contemporaneous and historical (many of which are unknowable) opportunities for cross-contamination between specimens (of even different species) from various points in time and space, and these are rarely mitigated (i.e., by rinsing specimens with ethanol before sampling) by studies of historical *Bd* 'infection'.

If, as now appears to be the case, *Bd* is a novel pathogen to the African continent, it could be a substantially underestimated threat to African amphibian biodiversity. Research on amphibian population trends in Africa is rare, and there have been few reports of so-called 'enigmatic declines' where previously abundant amphibians vanish in relatively unchanged habitat. A notable exception is the Kihansi Spray toad *Nectophrynoides asperginis* that was

driven to extinction in the wild in Tanzania during a rapid *Bd* epidemic wave (Weldon *et al.*, 2020). The susceptibility of this species to lethal *Bd* infection has repeatedly been demonstrated in captive populations (McAloose *et al.*, 2008; Makange *et al.*, 2014). Although mass mortalities of the Lake Clawed Oku frog *Xenopus longipes* in Cameroon have been observed, *Bd* was ruled out as a causative factor (Doherty-Bone *et al.*, 2013). More recently, however, the presence of *Bd* has been found to correlate with amphibian declines elsewhere in Cameroon, with puddle frogs (*Phrynobatrachus* spp.), long-fingered frogs (*Cardioglossa* spp.) and small-tongue toads (*Werneria* spp.) being particularly affected (Hirschfeld *et al.*, 2016; Scheele *et al.*, 2019; Tchassem *et al.*, In press). These previously common species have declined dramatically in both forest and agricultural habitats in the space of 4 years, with no evidence of recovery (Doherty-Bone & Gvoždík, 2017). The *Bd* infection loads in these Cameroon species were lower than in other frog populations succumbing to chytridiomycosis (*sensu* Briggs *et al.*, 2010), but this might be explained by low lethality thresholds of infection loads, as might occur in *Rhinoderma darwinii* in Chile (Valenzuela-Sánchez *et al.*, 2017). In South Africa, where *Bd* is known to have been present over a long period of time and where amphibians are relatively well studied, sporadic *Bd*-associated mortality events appear to be exacerbated by environmental conditions in Poynton's River Frog *Amietia poyntoni* from Namaqualand and the Eastern Cape Province (Hopkins & Channing, 2003; Lane *et al.*, 2003), and in a high-altitude species in the Drakensberg Mountain range, the Phofung River Frog *Amietia hymenopus* (Griffiths *et al.*, 2018). Conversely, the cause(s) of enigmatic declines of the Western Cape endemic Rose's Mountain Toad *Capensibufo rosei*, which has disappeared from all but two historical sites, remain(s) unknown (Cressey *et al.*, 2015), but *Bd* is known to cause amphibian population declines and extinctions in the absence of notable mortality events (Valenzuela-Sánchez *et al.*, 2017).

Given the recent discovery that *Bd* is an introduced pathogen to Africa (O'Hanlon *et al.*, 2018), the population monitoring of a wide range of African amphibian species in concert with contemporaneous *Bd* surveillance is urgently required. Although species already of conservation concern should be monitored (restricted range species, mountain specialists), the monitoring of less obviously threatened species should also be considered due to the uncertainty of which species might be susceptible to *Bd*-driven declines. The species putatively impacted by *Bd* in Cameroon include frogs (*Phrynobatrachus* sp.) that were previously abundant, assessed to be Vulnerable by the IUCN, and broadly distributed across mountains (Hirschfeld *et al.*, 2016; Doherty-Bone & Gvoždík, 2017). Collection of data on environmental parameters that can influence host-pathogen dynamics, such as temperature, rainfall and hydroperiod, also should be considered in these further studies (Doddington *et al.*, 2013; Bosch *et al.*, 2018; Hudson *et al.*, 2019). Knowledge of environmental parameters is

useful for differentiating causes of dramatic declines that have occurred both in the presence of *Bd* and extensive habitat loss, such as in the highlands of Ethiopia (Gower *et al.*, 2012; Gower, Abera, *et al.*, 2013). This knowledge can be used to mitigate declines should severity of *Bd* infection interact with habitat change, such as habitat management that influences microhabitat quality or density of amphibian hosts. Understanding environmental parameters is also vital to explain population declines that occur in what might be considered an apparently stable host-pathogen system, such as those influenced by season and latency of infection severity (Longo & Burrowes, 2010; Longo *et al.*, 2013; Valenzuela-Sánchez *et al.*, 2017).

Genotyping *Bd* has become a valuable tool in understanding virulence and the potential outcome of infection. Several lineages of *Bd* have been found in Africa (Figure 1). The hypervirulent global panzootic lineage (*Bd*GPL) is known to occur in South Africa, Uganda and Ethiopia, while the hypovirulent *Bd*CAPE lineage was found initially in South Africa (Farrer *et al.*, 2011), and more recently in frogs and caecilians within, and originating from, Cameroon (Fisher *et al.*, 2018; O'Hanlon *et al.*, 2018). In South Africa, there is evidence of hybridization between these two lineages. Although the virulence of this hybrid is unknown, the only other recorded hybrid lineage (from Brazil), can be more virulent than either of the parent strains (Greenspan *et al.*, 2018). Assessing the distribution of these (and possible additional) lineages across the rest of Africa should be a priority for better understanding the evolutionary history of *Bd*CAPE and other strains, and to determine possible routes of spread.

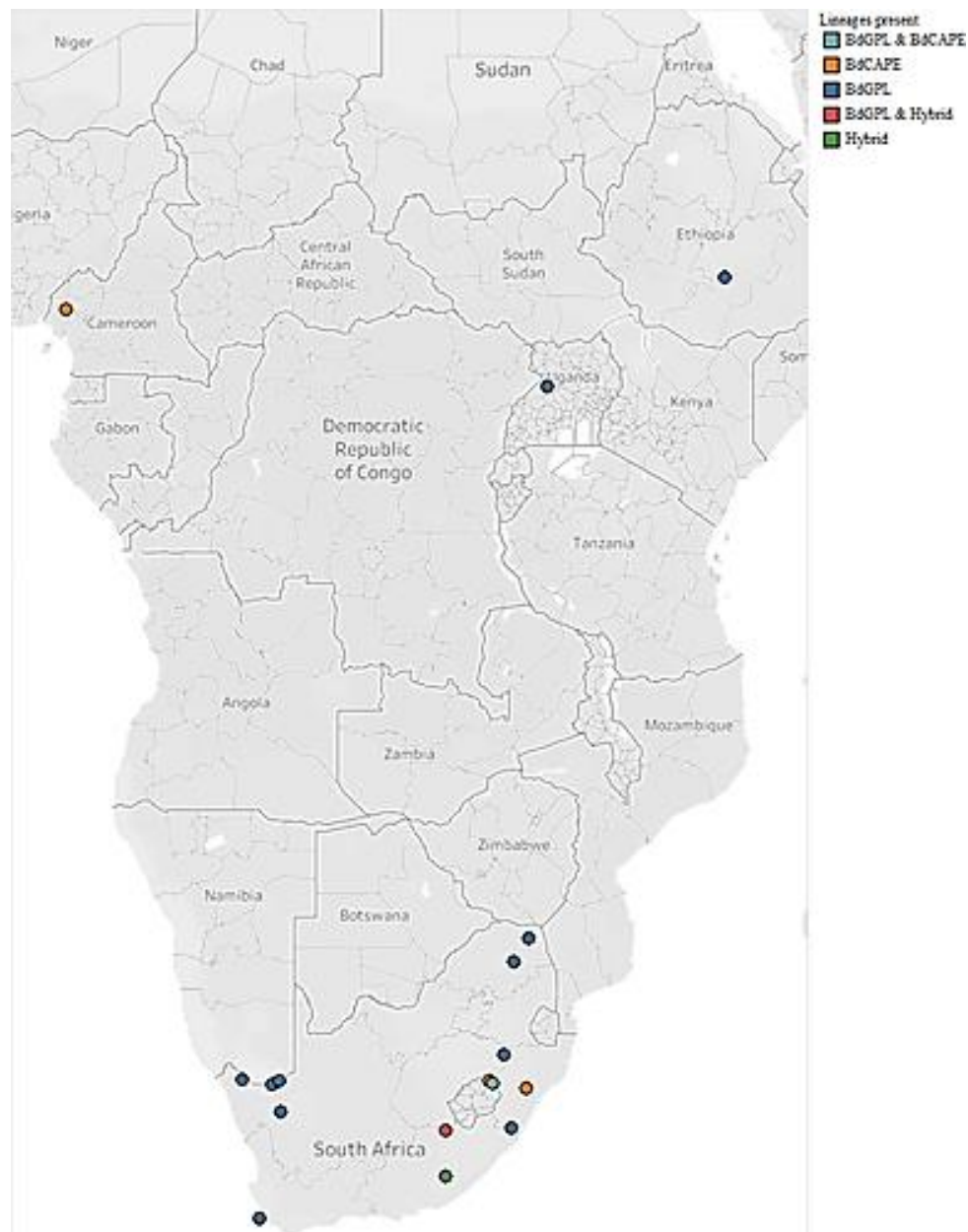


Figure 1: Distribution of strains of *Batrachochytrium dendrobatidis* on the African continent

Within parts of Africa (e.g., the Albertine Rift, Cameroon, Gabon and the Gulf of Guinea islands), the presence of *Bd*GPL has been identified via sequencing of the ITS region (Seimon *et al.*, 2015; Hydeman *et al.*, 2017; Miller *et al.*, 2018). Use of the ITS region to identify *Bd* lineages, however, has been found to be inaccurate compared to genomic sampling (O'Hanlon *et al.*, 2018), demonstrating the need for more genomic sampling and/or an alternative assay to identify *Bd* lineages (Bletz *et al.*, 2015). O'Hanlon (2018) were unable to estimate the date of emergence of *Bd*CAPE due to a paucity of isolates, however the emergence of *Bd*GPL was confidently dated to the late 19th/early 20th century, thus this lineage has only recently arrived in Africa as elsewhere. Studies of archived amphibians have identified the presence of *Bd* in Cameroon since at least 1933, Kenya 1934, Uganda 1935, in South Africa since 1938, the eastern Democratic Republic of Congo 1950 and Bioko Island 1966 (Weldon *et al.*, 2004;

Soto-Azat *et al.*, 2010; Vredenburg *et al.*, 2013; Seimon *et al.*, 2015; Hydemann *et al.*, 2017), but in each case the PCR techniques used could not identify the lineage of *Bd* present. Assuming these results are a true reflection of historical *Bd* presence in Africa, and are not a result of specimen contamination, establishing a technique to establish the strain from preserved specimens would facilitate our understanding of the history and likely future impact of *Bd* on African amphibians.

In the meantime, amphibian and wildlife disease biologists in Africa should shift their research paradigm from assuming *BdGPL* is indigenous to the continent, to the latest phylogenetically driven hypothesis that it is an introduced pathogen that may invade naïve host populations as it expands its distribution into new regions. There are relatively few historical records of *Bd* on the continent, which may or may not be records of *BdGPL*, therefore one research priority is to identify the mechanisms of spread of *BdGPL* within Africa and whether incursions into Africa are continuing. Where capacity is not yet available, research should be facilitated by collaboration between established laboratories with facilities to undertake necessary molecular techniques and researchers based in African nations or institutions with more limited scientific resources.

It has been hypothesized that *BdGPL* could have been spread globally from Africa in the early 20th century via the international trade in *Xenopus* spp. Frogs for biomedical purposes (Weldon *et al.*, 2004) with pet trade a more-recent pathway (Wombwell *et al.*, 2016; O'Hanlon *et al.*, 2018). It is notable, however, that the one known example of international spread of *Bd* (*BdCAPE*) from Africa was due to a conservation programme. Frogs endemic to South Africa (*Xenopus gilli*) were being captive-bred in the same facility in Jersey Zoo as Mallorcan midwife toads *Alytes muletensis*. It appears that the former infected the latter, so that when captive-bred midwife toads were released on Mallorca, they co-introduced *BdCAPE* (Walker *et al.*, 2008). Although the Mallorcan midwife toad conservation programme is considered to be a success, there is some evidence that *BdCAPE* is now restricting the population growth of the species in the wild (Doddington *et al.*, 2013).

Understanding variation of host responses among African amphibian species to infection with different lineages of *Bd* is a clear research gap. *Ex situ* exposure of Malagasy frogs to *BdGPL* has demonstrated extremely high susceptibility and rapid proliferation of infection, although trials were terminated with the onset of humane endpoints before lethality could be determined (Bletz *et al.*, 2015). Other African species have been found to succumb to lethal infection by *Bd*, including caecilians, such as *Geotrypetes seraphini* (from a *BdCAPE* strain), as well as dwarf clawed frogs *Hymenochirus curtipes* (unknown strain) albeit possibly confounded by stress from captivity in these cases (Gower, Doherty-Bone, *et al.*, 2013; Murphy *et al.*, 2015).

Research on other amphibian taxa that are possibly susceptible to infection is now needed, especially West African endemic species, and high elevation species of puddle frogs (*Phrynobatrachus* spp.), small tongued toads (*Werneria* spp.) and long-fingered frogs (*Cardioglossa* spp.) that have dramatically declined in the Cameroonian mountains, as well as other mountains where frog species have disappeared, such as Ethiopia (Gower, Aberra, *et al.*, 2013; Penner *et al.*, 2013; Hirschfeld *et al.*, 2016; Doherty-Bone & Gvoždík, 2017; Scheele *et al.*, 2019; Tchassem *et al.*, In press). A trait-based approach is likely to facilitate the prioritization of species for research into resilience to *Bd* infection (Gervasi *et al.*, 2017). In addition to long-term population monitoring with contemporaneous *Bd* surveillance, studies should take into account long-term deleterious effects, including impacts on population demographics where techniques like skeletochronology or less-invasive mark-recapture studies can reveal a reduced age structure in a *Bd*-impacted population (Scheele *et al.*, 2016; Lampo *et al.*, 2017). Sub-lethal effects of *Bd* infection should also be investigated, including impacts on growth rate and reproductive efforts (Roznik *et al.*, 2015; Campbell *et al.*, 2019). In addition to species that are susceptible, those that are tolerant to *Bd* infection, and that are likely reservoirs for reinfection of vulnerable species, should be considered (Woodhams *et al.*, 2011; Scheele *et al.*, 2016; Scheele *et al.*, 2017). Susceptibility should be assessed for species with high extinction risk to enable pro-active preparations for the event of disease incursion, such as the Mount Nimba toad *Nimbaphrynoides occidentalis* which is already threatened by mining (Penner *et al.*, 2013).

Africa's amphibian diversity is still being described and currently comprises of 886 species: 859 anurans, 23 caecilians and four salamanders (AmphibiaWeb, 2019). This diversity is subdivided by mountains, river basins, savanna and forests, the Highlands of Cameroon and Nigeria, the Congo Basin, the Albertine Rift, the Ethiopian Highlands, Eastern Arc Mountains, East African coastal forests and South Africa. Inclusion of Madagascar to this region adds a further 348 species (AmphibiaWeb, 2019). Of the total of 848 species on the African continent so far assessed by the IUCN, 212 are threatened with extinction (i.e., vulnerable, endangered, critically endangered, extinct in the wild). An additional 145 species (from 313 species assessed) are threatened on Madagascar, Comoros and Mayotte. Threats have been identified by the IUCN as habitat loss, overexploitation and climate change. Given the recent finding that *Bd* is an introduced pathogen, action is urgently needed to better understand the conservation risk it poses to amphibian diversity in Africa.

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References

- AmphibiaWeb. 2019. Berkeley, CA: University of California. Available at <https://amphibiaweb.org>. Date of access: 21 August 2019.
- Bletz, M.C., Rosa, G.M., Andreone, F., Courtois, E.A., Schmeller, D.S., Rabibisoa, N.H., Rabemananjara, F.C., ... Crottini, A. 2015. Widespread presence of the pathogenic fungus *Batrachochytrium dendrobatidis* in wild amphibian communities in Madagascar. *Scientific Reports*, 5, DOI: 10.1038/srep08633
- Bosch, J., Fernández-Beaskoetxea, S., Garner, T.W.J. & Carrascal, L.M. 2018. Long-term monitoring of an amphibian community after a climate change- and infectious disease-driven species extirpation. *Global change biology*, 24(6): 2622-2632. DOI: 10.1111/gcb.14092
- Briggs, C.J., Knapp, R.A. & Vredenburg, V.T. 2010. Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proceedings of the National Academy of Sciences*, 107(21): 9695-9700. DOI: 10.1073/pnas.0912886107
- Campbell, L., Bower, D.S., Clulow, S., Stockwell, M., Clulow, J. & Mahony, M. 2019. Interaction between temperature and sublethal infection with the amphibian chytrid fungus impacts a susceptible frog species. *Scientific Reports*, 9(1): 83. DOI:10.1038/s41598-018-35874-7
- Carvalho, T., Becker, C.G. & Toledo, L.F. 2017. Historical amphibian declines and extinctions in Brazil linked to chytridiomycosis. *Proceedings of the Royal Society B*, 284(1848): 20162254. DOI: 10.1098/rspb.2016.2254
- Cressey, E.R., Measey, G.J. & Tolley, K.A. 2015. Fading out of view: the enigmatic decline of Rose's mountain toad *Capensibufo rosei*. *Oryx*, 49(3): 521-528. DOI: 10.1017/S0030605313001051
- Doddington, B.J., Bosch, J., Oliver, J.A., Grassly, N.C., Garcia, G., Schmidt, B.R., Garner, T.W.J., ... Fisher, M.C. 2013. Context-dependent amphibian host population response to an invading pathogen. *Ecology*, 94(8): 1795-1804. DOI: 10.1890/12-1270.1
- Doherty-Bone, T.M. & Gvoždík, V. 2017. The Amphibians of Mount Oku, Cameroon: an updated species inventory and conservation review. *ZooKeys*, (643): 109-139. DOI: 10.3897/zookeys.643.9422
- Doherty-Bone, T.M., Ndifon, R.K., Nyingchia, O.N., Landrie, F.E., Yonghabi, F.T., Duffus, A.L.J., Price, S., ... Cunningham, A.A. 2013. Morbidity and mortality of the critically endangered Lake Oku clawed frog *Xenopus longipes*. *Endangered Species Research*, 21(2): 115-128. DOI: 10.3354/esr00514

- El Mouden, E., Slimani, T., Donaire, D., Fernández-Beaskoetxea, S., Fisher, M.C. & Bosch, J. 2011. First record of the chytrid fungus *Batrachochytrium dendrobatidis* in North Africa.
- Farrer, R.A., Weinert, L.A., Bielby, J., Garner, T.W.J., Balloux, F., Clare, F., Bosch, J., ... Fisher, M.C. 2011. Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proceedings of the National Academy of Sciences*, 108(46): 18732-18736. DOI:10.1073/pnas.1111915108
- Fisher, M.C., Ghosh, P., Shelton, J.M.G., Bates, K., Brookes, L., Wierzbicki, C., Rosa, C.M., ... Garner, T.W.J. 2018. Development and worldwide use of non-lethal, and minimal population-level impact, protocols for the isolation of amphibian chytrid fungi. *Scientific Reports*, 8(1): 7772. DOI: 10.1038/s41598-018-24472-2
- Gervasi, S.S., Stephens, P.R., Hua, J., Searle, C.L., Xie, G.Y., Urbina, J., Olson, D.H., ... Blaustein, A.R. 2017. Linking ecology and epidemiology to understand predictors of multi-host responses to an emerging pathogen, the amphibian chytrid fungus. *PLoS ONE*, 12(1): e0167882. DOI: 10.1371/journal.pone.0167882
- Goka, K., Yokoyama, J., Une, Y., Kuroki, T., Suzuki, K., Nakahara, M., Kobayashi, A., ... Hyatt, A.D. 2009. Amphibian chytridiomycosis in Japan: distribution, haplotypes and possible route of entry into Japan. *Molecular Ecology*, 18(23): 4757-4774. DOI: 10.1111/j.1365-294X.2009.04384.x
- Gower, D.J., Doherty-Bone, T.M., Abera, R.K., Mengistu, A., Schwaller, S., Menegon, M., De Sá, R.O., ... Loader, S.P. 2012. High prevalence of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) across multiple taxa and localities in the highlands of Ethiopia. *The Herpetological Journal*, 22(4): 225-233.
- Gower, D.J., Abera, R.K., Schwaller, S., Largen, M.J., Collen, B., Spawls, S., Menegon, M., ... Loader, S.P. 2013. Long-term data for endemic frog genera reveal potential conservation crisis in the Bale Mountains, Ethiopia. *Oryx*, 47(1): 59-69. DOI: 10.1017/S0030605311001426
- Gower, D.J., Doherty-Bone, T.M., Loader, S.P., Wilkinson, M., Kouete, M.T., Tapley, B., Orton, F., ... Garner, T.W.J. 2013. *Batrachochytrium dendrobatidis* infection and lethal chytridiomycosis in caecilian amphibians (Gymnophiona). *EcoHealth*, 10(2): 173-183. DOI: 10.1007/s10393-013-0831-9
- Greenspan, S.E., Lambertini, C., Carvalho, T., James, T.Y., Toledo, L.F., Haddad, C.F.B. & Becker, C.G. 2018. Hybrids of amphibian chytrid show high virulence in native hosts. *Scientific Reports*, 8(1): 9600. DOI: 10.1038/s41598-018-27828-w

- Griffiths, S.M., Harrison, X.A., Weldon, C., Wood, M.D., Pretorius, A., Hopkins, K., Fox, G., ... Antwis, R.E. 2018. Genetic variability and ontogeny predict microbiome structure in a disease-challenged montane amphibian. *The ISME Journal*, 12(10): 2506-2517. DOI: 10.1038/s41396-018-0167-0
- Hirschfeld, M., Blackburn, D.C., Doherty-Bone, T.M., Gonwouo, L.N., Ghose, S. & Rödel, M. 2016. Dramatic declines of montane frogs in a Central African biodiversity hotspot. *PLoS ONE*, 11(5): e0155129. DOI: 10.1371/journal.pone.0155129
- Hopkins, S. & Channing, A. 2003. Chytrid fungus in northern and western cape frog populations, South Africa. *Herpetological Review*, 34(4): 334.
- Hudson, M.A., Griffiths, R.A., Martin, L., Fenton, C., Adams, S.-L., Blackman, A., Sulton, M., ... Garcia, G. 2019. Reservoir frogs: seasonality of *Batrachochytrium dendrobatidis* infection in robber frogs in Dominica and Montserrat. *PeerJ*, 7: e7021. DOI: 10.7717/peerj.7021
- Hydeman, M.E., Longo, A.V., Velo-Antón, G., Rodriguez, D., Zamudio, K.R. & Bell, R.C. 2017. Prevalence and genetic diversity of *Batrachochytrium dendrobatidis* in Central African island and continental amphibian communities. *Ecology and Evolution*, 7(19): 7729-7738. DOI: 10.1002/ece3.3309
- Kielgast, J., Rödder, D., Veith, M. & Lötters, S. 2010. Widespread occurrence of the amphibian chytrid fungus in Kenya. *Animal Conservation*, 13(s1): 1-8. DOI: 10.1111/j.1469-1795.2009.00297.x
- Lampo, M., Señaris, C. & García, C.Z. 2017. Population dynamics of the critically endangered toad *Atelopus cruciger* and the fungal disease chytridiomycosis. *PLoS ONE*, 12(6): e0179007. DOI: 10.1371/journal.pone.0179007
- Lane, E.P., Weldon, C. & Bingham, J. 2003. Histological evidence of chytridiomycete fungal infection in a free-ranging amphibian, *Afrana fuscigula* (Anura: Ranidae), in South Africa. *Journal of the South African Veterinary Association*, 74(1): 20-21. DOI: 10.4102/jsava.v74i1.493
- Lips, K.R., Diffendorfer, J., Mendelson III, J.R. & Sears, M.W. 2008. Riding the wave: reconciling the roles of disease and climate change in amphibian declines. *PLoS Biology*, 6(3): e72. DOI: 10.1371/journal.pbio.0060072
- Longo, A.V. & Burrowes, P.A. 2010. Persistence with chytridiomycosis does not assure survival of direct-developing frogs. *EcoHealth*, 7(2): 185-195. DOI: 10.1007/s10393-010-0327-9

- Longo, A.V., Ossiboff, R.J., Zamudio, K.R. & Burrowes, P.A. 2013. Lability in host defenses: terrestrial frogs die from chytridiomycosis under enzootic conditions. *Journal of Wildlife Diseases*, 49(1): 197-199. DOI: 10.7589/2012-05-129
- Makange, M., Kulaya, N., Biseko, E., Kalenga, P., Mutagwaba, S. & Misinzo, G. 2014. *Batrachochytrium dendrobatidis* detected in Kihansi spray toads at a captive breeding facility (Kihansi, Tanzania). *Diseases of Aquatic Organisms*, 111(2): 159-164. DOI: 10.3354/dao02775
- McAloose, D., Pramuk, J., Raphael, B., Calle, P., Shellabarger, W., Odum, A., Garner, M., ... Duncan, A. 2008. Mortality summary (2000-2007) in endangered captive Kihansi spray toads (*Nectrophrynoides asperginis*). In: 57th annual international conference of the Wildlife Disease Association Edmonton.
- Miller, C.A., Taboue, G.C.T., Ekane, M.M.P., Robak, M., Clee, P.R.S., Richards-Zawacki, C., Fokam, E.B., ... Anthony, N.M. 2018. Distribution modeling and lineage diversity of the chytrid fungus *Batrachochytrium dendrobatidis* (Bd) in a central African amphibian hotspot. *PLoS ONE*, 13(6): e0199288. DOI: 10.1371/journal.pone.0199288
- Murphy, B.G., Hillman, C. & Groff, J.M. 2015. Chytridiomycosis in dwarf African frogs *Hymenochirus curtipes*. *Diseases of Aquatic Organisms*, 114(1): 69-75. DOI: 10.3354/dao02851
- O'Hanlon, S., Rieux, A., Farrer, R., Rosa, G., Waldman, B., Bataille, A., Kosch, T., ... Fumagalli, M. 2018. A 20th century out-of-Asia origin of a panzootic threat to global amphibian biodiversity. *Science*, 360: 621-627.
- Penner, J., Adum, G.B., McElroy, M.T., Doherty-Bone, T., Hirschfeld, M., Sandberger, L., Weldon, C., ... Rödel, M. 2013. West Africa - A safe haven for frogs? A sub-continental assessment of the chytrid fungus (*Batrachochytrium dendrobatidis*). *PLoS ONE*, 8(2): e56236. DOI: 10.1371/journal.pone.0056236
- Rachowicz, L.J., Hero, J., Alford, R.A., Taylor, J.W., Morgan, J.A., Vredenburg, V.T., Collins, J.P., ... Briggs, C.J. 2005. The novel and endemic pathogen hypotheses: competing explanations for the origin of emerging infectious diseases of wildlife. *Conservation Biology*, 19(5): 1441-1448. DOI: 10.1111/j.1523-1739.2005.00255.
- Reeder, N.M.M., Cheng, T.L., Vredenburg, V.T. & Blackburn, D.C. 2011. Survey of the chytrid fungus *Batrachochytrium dendrobatidis* from montane and lowland frogs in eastern Nigeria. *Herpetology Notes*, 4(1): 83-86.

- Roznik, E.A., Sapsford, S.J., Pike, D.A., Schwarzkopf, L. & Alford, R.A. 2015. Condition-dependent reproductive effort in frogs infected by a widespread pathogen. *Proceedings of the Royal Society B*, 282(1810): 20150694. DOI: 10.1098/rspb.2015.0694
- Scheele, B.C., Hunter, D.A., Brannelly, L.A., Skerratt, L.F. & Driscoll, D.A. 2017. Reservoir-host amplification of disease impact in an endangered amphibian. *Conservation Biology*, 31(3): 592-600. DOI: 10.1111/cobi.12830
- Scheele, B.C., Hunter, D.A., Banks, S.C., Pierson, J.C., Skerratt, L.F., Webb, R. & Driscoll, D.A. 2016. High adult mortality in disease-challenged frog populations increases vulnerability to drought. *Journal of Animal Ecology*, 85(6): 1453-1460. DOI: 10.1111/1365-2656.12569
- Scheele, B.C., Pasmans, F., Skerratt, L.F., Berger, L., Martel, A., Beukema, W., Acevedo, A.A., ... Catenazzi, A. 2019. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science*, 363(6434): 1459-1463.
- Seimon, T.A., Ayebare, S., Sekisambu, R., Muhindo, E., Mitamba, G., Greenbaum, E., Menegon, M., ... Plumptre, A.J. 2015. Assessing the threat of amphibian chytrid fungus in the Albertine Rift: past, present and future. *PLoS ONE*, 10(12): e0145841. DOI: 10.1371/journal.pone.0145841
- Smith, K.G., Weldon, C., Conradie, W. & Du Preez, L.H. 2007. Relationships among size, development, and *Batrachochytrium dendrobatidis* infection in African tadpoles. *Diseases of Aquatic Organisms*, 74(2): 159-164. DOI: 10.3354/dao074159
- Soto-Azat, C., Clarke, B.T., Poynton, J.C. & Cunningham, A.A. 2010. Widespread historical presence of *Batrachochytrium dendrobatidis* in African pipid frogs. *Diversity and Distributions*, 16(1): 126-131. DOI: 10.1111/j.1472-4642.2009.00618.x
- Talley, B.L., Muletz, C.R., Vredenburg, V.T., Fleischer, R.C. & Lips, K.R. 2015. A century of *Batrachochytrium dendrobatidis* in Illinois amphibians (1888–1989). *Biological Conservation*, 182: 254-261. DOI: 10.1016/j.biocon.2014.12.007
- Tarrant, J., Cilliers, D., Du Preez, L.H. & Weldon, C. 2013. Spatial assessment of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) in South Africa confirms endemic and widespread infection. *PLoS ONE*, 8(7): e69591. DOI: 10.1371/journal.pone.0069591
- Tchasse, A.M.F., Doherty-Bone, T.M., Kameni, M.M., Tapondjou, W.P., Tamesse, J.L. & Gonwouo, N.L. In press. What is driving declines of montane endemic amphibians? New insights from Mount Bamboutos, Cameroon. *Oryx*,

- Valenzuela-Sánchez, A., Schmidt, B.R., Uribe-Rivera, D.E., Costas, F., Cunningham, A.A. & Soto-Azat, C. 2017. Cryptic disease-induced mortality may cause host extinction in an apparently stable host–parasite system. *Proceedings of the Royal Society B*, 284(1863): 201711176. DOI: 10.1098/rspb.2017.1176
- Vredenburg, V.T., Felt, S.A., Morgan, E.C., McNally, S.V., Wilson, S. & Green, S.L. 2013. Prevalence of *Batrachochytrium dendrobatidis* in *Xenopus* collected in Africa (1871–2000) and in California (2001–2010). *PLoS ONE*, 8(5): e63791. DOI: 10.1371/journal.pone.0063791
- Walker, S.F., Bosch, J., James, T.Y., Litvintseva, A.P., Valls, J.A.O., Piña, S.I., García, G., ... Fisher, M.C. 2008. Invasive pathogens threaten species recovery programs. *Current Biology*, 18(18): R853-R854. DOI: 10.1016/j.cub.2008.07.033
- Weldon, C., De Villiers, A.L. & Du Preez, L.H. 2007. Quantification of the trade in *Xenopus laevis* from South Africa, with implications for biodiversity conservation. *African Journal of Herpetology*, 56(1): 77-83. DOI: 10.1080/21564574.2007.9635553
- Weldon, C., Channing, A., Misinzo, G. & Cunningham, A.A. 2020. Disease driven extinction in the wild of the Kihansi spray toad, *Nectophrynoides asperginis*. *African Journal of Herpetology*, 69(2): 151-164. DOI: 10.1080/21564574.2020.1752313
- Weldon, C., Du Preez, L.H., Hyatt, A.D., Muller, R. & Speare, R. 2004. Origin of the amphibian chytrid fungus. *Emerging Infectious Diseases*, 10(12): 2100-2105. DOI: 10.3201/eid1012.030804
- Wombwell, E.L., Garner, T.W.J., Cunningham, A.A., Quest, R., Pritchard, S., Rowcliffe, J.M. & Griffiths, R.A. 2016. Detection of *Batrachochytrium dendrobatidis* in amphibians imported into the UK for the pet trade. *EcoHealth*, 13(3): 456-466. DOI: 10.1007/s10393-016-1138-4
- Woodhams, D.C., Bosch, J., Briggs, C.J., Cashins, S., Davis, L.R., Lauer, A., Muths, E., ... Voyles, J. 2011. Mitigating amphibian disease: strategies to maintain wild populations and control chytridiomycosis. *Frontiers in Zoology*, 8(8): 1-23. DOI: 10.1186/1742-9994-8-8

Chapter 7

General discussion

The publications and manuscripts contained in this thesis all focus on different aspects of research with theoretical, field and experimental work aimed at increasing our understanding of the ecology of *Batrachochytrium dendrobatidis* (*Bd*). Enigmatic declines in amphibian populations started this research into the amphibian chytrid fungus in 1998 (Berger *et al.*, 1998), with a formal description of the causative organism in 1999 (Longcore *et al.*, 1999). Since its discovery researchers from around the globe have become involved in the study of amphibian chytrid, in large part due to the global presence of the fungus and the recognition that amphibians around the world may be at risk due to habitat degradation and climate change (Lips *et al.*, 2008; Rödder *et al.*, 2010). The spread of *Bd* within captive facilities (McAloose *et al.*, 2008; Walker *et al.*, 2008) also showed it is highly transmissible within confined habitats and that this ultimately needed to be quantified. The discovery of recombinant lineages (Schloegel *et al.*, 2012; O'Hanlon *et al.*, 2018) also highlighted the need to understand the circumstances necessary for these recombinants to be able to form, to better assess the probability for future emergence of novel lineages.

Chapter 2 of this thesis focussed on experimental work, with the objective of quantifying the transmission potential of two different genotypes of *Bd*. It has previously been proven that amphibian hosts can be infected with *Bd* in laboratory conditions (Farrer *et al.*, 2011; De Jong *et al.*, 2018) to determine the effect an infection with *Bd* has on the life expectancy of the amphibian hosts, as well as to define the symptoms related to infections. This was important as most field surveys would only give a momentary observation of population levels for the host and prevalence of *Bd*, or report on mortalities seen, but not the development of symptoms. Our transmission experiment was founded on field observations associated within the Orange River, where single sites were seen to host both *Bd*CAPE and *Bd*GPL; the two most prevalent lineages in South Africa (Weldon *et al.*, 2004; O'Hanlon *et al.*, 2018). O'Hanlon *et al.* (2018) also reported that one isolate obtained from these field surveys of the Orange River was a recombinant lineage based on whole genome sequencing, further highlighting the need to quantify the transmission capabilities of the lineages that needed to be present close to each other for the recombinant to form.

Our transmission experiment had three objectives: to quantify the transmission capabilities of *Bd*CAPE to uninfected individuals, to quantify the transmission capabilities of *Bd*GPL to uninfected individuals, and to determine if multi-lineage co-infection can be induced by co-housing individuals of different infecting lineage status. The hypovirulent lineage when

concerned with infection intensity and symptoms, *BdCAPE*, proved to be more transmissible than the hypervirulent lineage *BdGPL*. This was contrary to what would be expected in terms of other virulence characteristics that have been quantified for these lineages, such as infection intensity and zoospore production (Farrer *et al.*, 2011). This also showed that the lineages that are described as hypovirulent are still of conservation concern as they can potentially infect uninfected populations faster than the hypervirulent lineage, at least in the South African context. Although the invasion of new areas by fungi is often best done by clones from a single lineage (Morgan *et al.*, 2007), recombination is needed to maintain adaptability within the fungal population (Fisher *et al.*, 2012). The recombinants found in South Africa showed to be hybrids between *BdCAPE* and *BdGPL* (O'Hanlon *et al.*, 2018), and consequently, they are most likely to be found in areas where both these lineages occur. When field testing the use of lineage-specific primers (part of Chapter 5; Ghosh *et al.*, 2021) detected both *BdCAPE* and *BdGPL* on a single individual host, it became necessary to determine the co-infection dynamics, and whether this could be accomplished by co-housing individuals of different *Bd* lineage status. The experimental analysis showed that co-infection was not as readily obtainable as the infection of naïve hosts, and when it did occur, the co-infection was unidirectional, with only originally *BdCAPE* hosts becoming infected with *BdGPL*, and not the reverse. This is of significant impact, as the hypervirulent lineage, *BdGPL* may lead to extirpation of the host, and per implication its associated *BdCAPE* infection. This could then lead to the hypovirulent lineage becoming displaced, as in effect fewer hosts are available to infect with this lineage. The other consequence relates to host physiology, seeing as an infection with any *Bd* lineage has a metabolic impact on the host, co-infection with multiple lineages can increase this metabolic cost the infection has for the host, potentially inflating the threat. Co-infection also provides the ideal environment for genetic exchange between the different lineages, leading to potential recombination events, with unknown virulence characteristics and consequences for amphibian host populations.

In Chapter 3 these metabolic costs and influences of infection on a host were further explored again using the two predominant *Bd* lineages in South Africa. The objective of this experiment was to quantify the behavioural influences that a *Bd* infection has on tadpoles. We selected a South African host species that has been proven to be susceptible to infection (Tarrant *et al.*, 2013), and we used sophisticated behaviour tracking software from Noldus™, Netherlands (Ethovision XT14). This allowed for the measurement of several parameters related to the movement of the tadpoles, and the comparison between different infecting lineage statuses.

Bd has been shown to have morphological influences on the host, whether it be the abnormal posture and lethargy in post-metamorphic individuals (Daszak *et al.*, 1999), or the depigmentation and destruction of keratin in the mouthparts of tadpoles (Smith *et al.*, 2007),

or even adaptations in the cardiac muscle (Salla *et al.*, 2015). These morphological changes should theoretically be reflected in the changed behaviour of the host, in line with the relationship observed between morphology and escape behaviour in the lizard clade *Liolaemus* as described by Schulte II *et al.* (2004), and the changes seen during fish larvae development that hamper their behaviour in this developmental phase (Galis *et al.*, 1994). Feeding behaviour should be directly impacted by *Bd* infection in tadpoles as their mouthparts are susceptible to physical damage resulting from infection (Marantelli *et al.*, 2004). Physiologically tadpoles' behaviour should also be influenced by infection, as it places an additional metabolic load on the individual, with tadpoles already needing to change their behaviour during development to keep up with the normal need for oxygen in the body (Crowder *et al.*, 1998; Phillips, 2019). Consequently, it would be in these areas that one would expect changes in the behaviour of infected tadpoles, whether direct or indirectly related to *Bd*. It was already seen by Parris *et al.* (2006) that *Bd* infected tadpoles had lower activity levels when faced with a potential predator than uninfected tadpoles. Infected tadpoles in their study required both chemical and visual predator cues to elicit an equal response to uninfected tadpoles stimulated by only visual cues. This highlighted the influence *Bd* has on the anti-predator behaviour of tadpoles, affecting their potential survival, but did not account for the *Bd* lineage-dependent differences that may be seen. In our experiment, we were able to measure more parameters, and statistical effect size calculations allowed for the comparison of these parameters. Our objective was thus to elucidate the lineage-specific influence that may be found, especially with our field surveys showing that more than one lineage can be found in close proximity. Interestingly whereas Parris *et al.* (2006) found that *Bd* infected tadpoles tended to react less in the presence of a predator, our experiments showed lineage-specific reaction differences when we compared our predator detection trial to the baseline behaviour trial. In the baseline it was seen that *Bd*GPL infected animals spent more time stationary than moving per moving period, with no significant difference between the time spent moving per event, or time spent stationary between moving events for any lineage. In the predator trial the *Bd*CAPE animals showed a significant difference between these times, almost identical to the uninfected animals, with *Bd*GPL now showing longer moving times than times spent stationary. This different reaction of the tadpoles toward a chemical predator cue, based on the infecting lineage, can influence successful predation on these tadpoles in a natural environment. Interestingly, mean acceleration of the *Bd*CAPE infected tadpoles were highest throughout all the trials, with the uninfected individuals showing the second highest mean acceleration and *Bd*GPL having the lowest mean acceleration. This could impact their metabolic dynamics, as their need for energy changes based on their behavioural changes due to the infecting lineage.

The interaction between these different behaviours ultimately influences the survival fitness of the tadpole and from our experiment it is evident that behavioural influences need to be considered on a *Bd* lineage-specific level, and not merely a general *Bd* infection presence. In the past experimental work with *Bd* was often focused on the susceptibility of the host (Vazquez *et al.*, 2009; Garner *et al.*, 2011; Gervasi *et al.*, 2013), means of infecting toadlets (e.g. the influence of duration of exposure and the dosage magnitude of *Bd* on survival of the toadlets - Carey *et al.*, 2006), the influence of the passage history and maintenance of *Bd* cultures *in vitro* on virulence when used for *in vivo* experiments (Voyles *et al.*, 2014), or the changes in probable survival of the host when being infected at different life stages (Garner *et al.*, 2009; Farrer *et al.*, 2011). All these experiments always measured the mortality rate of individuals, seldomly including lineage, identified symptoms of infection and described the possible reasons for mortality in the host or circumstances that could be favourable for host recovery. These experiments only related different mortality rates and time till onset of symptoms to lineages, and not the detailed behavioural differences of asymptomatic tadpoles to the lineage of infection, where our experiments filled this crucial gap.

The objective of Chapter 4 was to use the data from a large-scale transect survey to determine the predicted environmental distribution of the different lineages of *Bd* found in South Africa. This also included a predicted distribution map for *Bd* in Africa based on a dataset compiled from peer-reviewed publications and our transect data. Based on previous models created by Tarrant *et al.* (2013) large sections of South Africa were predicted to have a low probability of being suitable for *Bd*, especially when considering the more arid western reaches of the country where suitability was restricted to areas associated with river systems. The field surveys that were done as part of Chapter 6 (Ghosh *et al.*, 2021) showed that within the Orange River system both lineages of *Bd* known for South Africa were present. When analysing the sites based on the *Bd* lineage present and the environmental conditions of the site, it became apparent that *Bd*GPL and *Bd*CAPE displayed different environmental preferences in this system. The isolation of a recombinant lineage from a single site within the system (O’Hanlon *et al.*, 2018) lead to the need to model the distribution of these two lineages within South Africa, using the extensive database collated from publications and our own field surveys, with the purpose of identifying other areas where the environmental conditions may be suitable for both *Bd*CAPE and *Bd*GPL, and consequently where possible recombinant lineages can be found.

Our initial models were constructed to show the potential distribution of *Bd*GPL and *Bd*CAPE in South Africa and showed that many of the sites that were sampled by us and included in the environmental characterisation database were located in areas that only showed a low to moderate likelihood for the respective lineages to be present. This was especially prominent

in the central region of South Africa, where both lineages were found. This showed that targeted sampling in predicted high likelihood areas may be misleading compared to the total distribution of a lineage. The inclusion of these sites with predicted lower likelihood in the database also allowed for much greater resolution on the predicted distribution map. Construction of the lineage-specific predicted distribution maps is also to our knowledge a first of its kind for South Africa. There has been much effort into modelling the predicted distribution of *Bd* in Africa (e.g. Miller *et al.*, 2018; Zimkus *et al.*, 2020), as well as other areas around the world, for example, Costa Rica (Puschendorf *et al.*, 2009) and Italy (Costa *et al.*, 2021), but these often only take presence or absence of *Bd* into account and not necessarily lineage. This may be due to the geographical location focussed on, as not many areas are known to have more than one lineage of *Bd*, as is the case for Africa. In the O'Hanlon *et al.* (2018) 42 isolates were shown for the African continent, with *BdCAPE* isolates originating from Cameroon and South Africa and *BdGPL* isolates originating from South Africa, Ethiopia and Uganda. Although the areas in central Africa where *BdCAPE* and *BdGPL* occur are relatively isolated from each other according to Zimkus *et al.* (2020), our model showed that there are areas of low to moderate likelihood of occurrence connecting these apparently separated lineage distributions. The fact that it was indeed these areas in South Africa that showed the presence of both lineages highlights the need for these areas to be included in surveillance and monitoring programs as these are also the areas where genetic exchange between lineages may occur, potentially leading to novel lineages of unknown virulence.

Chapter 5 pertains to the development of lineage-specific qPCR primers to be able to identify the lineage of infection from swabs collected in the field or as part of experiments. Being able to test for the lineage from field swabs has become extremely important when faced with the current global distribution of six distinct genotypes of *Bd* (O'Hanlon *et al.*, 2018). With *BdCAPE* and *BdGPL* being the most widely distributed and often associated with mortalities events globally (Farrer *et al.*, 2011; Jenkinson *et al.*, 2016; Byrne *et al.*, 2019), it made sense to focus on these lineages for the development of genetic diagnostic tools that would shorten the time, expertise and costs needed for the determination of lineage from field surveys. Before our work, it was only possible to determine lineage from whole genome sequencing, which required invasive tissue sampling of the specimen, cleaning of the tissue sample and sterile work environments (Fisher *et al.*, 2018), as far as possible. This presented challenges in the field, and cultures from isolation attempts had to be kept cool to prevent depletion of the nutrients prior to incubation in a formal laboratory. The benefit of only needing to swab the individual is that it is minimally invasive, requires minimal experience and is essentially the same protocol as has been done to conduct amphibian chytrid surveys since the first molecular diagnostic tool for *Bd* was developed (Boyle *et al.*, 2004). Less equipment is also

needed and the whole process is more economic than isolation attempts for whole genome sequencing.

The other benefit is that any laboratory that was already able to test for *Bd* presence using ITS-qPCR (Boyle *et al.*, 2004), would also be able to perform the lineage-specific qPCR test. This meant that the possibility to identify the lineages present became less restricted as more laboratories are capable of lineage identification at comparable costs to the standard ITS-qPCR assay. This new approach is also of great value in identifying areas where potential recombinant genotypes can be found (Schloegel *et al.*, 2012; O'Hanlon *et al.*, 2018) as these could only have resulted from multi-lineage infected habitats. Isolation attempts to create laboratory cultures of the different lineages for use in experimental work remains important, as this is the only way to determine the virulence characteristics and potential danger that the lineages may have towards the amphibian hosts. However, when using isolation attempts in concert with lineage-specific qPCR testing, resources can be targeted toward areas to increase the chances of success in obtaining viable lineages, especially if recombinants are to be the future target of field surveys. Another benefit of this new diagnostic tool is that prediction models can now be done based on lineage identity and not merely *Bd* presence (Chapter 4) which aids in the identification of overlapping zones of distribution. Previously the number of cultures that resulted from field isolation attempts would decrease due to unavoidable contamination, field equipment and financial limitations. Using swabs to identify both presence and lineage, and then targeting areas with a higher likelihood of success constitutes a better return on investment for the researcher. The development of these new testing tools will also be of great importance for experimental work in future, as co-infection risk and dynamics can now be studied in controlled, simulated environments (e.g., Chapter 2). These experiments are also of growing importance as our predictive models suggested that large areas of South Africa are suitable for both *Bd*CAPE and *Bd*GPL. The design of this genetic tool thus came at an opportune time as future research will have to be focused on the potential of recombinants forming and the risk they may pose to amphibian communities already hosting more than one lineage of *Bd*.

Chapter 6 was a review of the theories that have been accepted for almost two decades, i.e., that the origin of *Bd* thought to be Africa (Weldon *et al.*, 2004), with evidence in terms of multiple lineages present, wide distribution and rarity of cases where amphibian populations have declined due to chytridiomycosis. It was suggested that the export of *Xenopus laevis* from Africa may, in part, have been responsible for the global spread of *Bd* (Weldon *et al.*, 2007), but O'Hanlon *et al.* (2018) clarified that if the ancestral lineage of *Bd* is to consist of the genes from the various lineages, then *Bd* originated in Asia. Africa rarely saw the declines of amphibian populations due to chytridiomycosis in the absence of dramatic habitat changes.

There is however the case of the Kihansi Spray toad, *Nectophrynoides asperginis*, where habitat degradation played a crucial role, potentially instigating the decline, exacerbated by *Bd* infection (Weldon *et al.*, 2020). In South Africa, similar situations occurred with *Amietia fuscigula* populations (Hopkins & Channing, 2003; Lane *et al.*, 2003) and *Amietia hymenopus* populations (Griffiths *et al.*, 2018), with mortality events in the amphibian populations that cannot be attributed to the infection with *Bd* alone. This highlighted the need for the study of *Bd* to expand in Africa, even though the negative impact on amphibian populations seen elsewhere in the world is not mirrored on this continent.

The past hypotheses focussing on trade and export as a means of *Bd* dissemination cannot be overruled, as cases exist where this precise action has led to the restriction in populations due to the introduction of *Bd*, i.e., the Mallorcan midwife toad *Alytes muletensis* as an example (Walker *et al.*, 2008; Doddington *et al.*, 2013). Thus, when focussing on conservation programmes and the potential reintroduction of amphibians to areas they were previously extirpated, it still needs to consider the hypothesis that if *Bd* is introduced by trade or import, it can be potentially devastating to the hosts that are not tolerable to the introduced lineage, even if the lineages were previously thought to be hypovirulent in comparison to others such as *BdCAPE*. Chapter 6 also highlighted the need to study the reaction African amphibian species can have towards different *Bd* lineages, especially seeing as different species may not have co-evolved with all *Bd* lineages distributed throughout Africa. This led to the development of the experiments described in Chapters 2 and 3, as these experiments had different host species, and both *BdCAPE* and *BdGPL* exhibited different transmission potentials and behavioural influences on the host. The vast diversity found among amphibians on the African continent, based on the number of species alone, also necessitates the redesign of experiments to focus on the susceptibility of different species to different lineages of *Bd* with all lineages considered to be novel pathogens instead of the traditional thought approach of the endemic pathogen theory. Experiments and tools that were designed and executed as part of this thesis can provide valuable information in designing and implementing future conservation and monitoring programmes in Africa, especially when incorporating the discovery of recombinant lineages that are completely novel lineages for any amphibian population.

Conclusion

The animal disease research included in this thesis has a threefold impact, not just from an African amphibian perspective, but also on conservation planning, surveillance planning and experimental work. Using South Africa as a study site allows us to study a varied geographical landscape that offers the environmental conditions necessary for multiple lineages of *Bd* to survive. Using our predicted distribution models, future areas of concern can be highlighted, as well as inform stakeholders on the risks associated with potential anthropogenic assisted amphibian movement between the areas of different predicted *Bd* lineage presence. The large climate envelope of *Bd*GPL necessitates the continued monitoring and targeting of sites previously thought to be niches for the hypovirulent *Bd*CAPE. The possibility for *Bd*GPL to survive in a wider range of habitats than previously thought, as well as the possibility for co-infection of a single host or environment shows the need for increased biosecurity measures when surveillance programs target areas of different *Bd* lineage distributions. This increase is also needed since recombinant lineages have been found in South Africa, and the virulence and possibility of these recombinants to survive in different environments have not yet been assessed. The design of novel diagnostic tools allowing for the easier identification of lineages from field swabs also allows for better surveillance of areas with the specific goal of determining if the distribution of lineages change over time, or even if seasonality seen in the prevalence of *Bd* in general can also be seen in the prevalence of different lineages. This can be of great importance with ever-changing climates due to anthropogenic disturbances of habitats. Future experimental work can also now include more detailed co-infection studies, with the ability to identify the precise conditions needed for recombinant lineages to occur. Since the transmission potential for all lineages and co-infection dynamics can potentially be influenced by the different climate envelopes specific to the lineages, future experimental work can also aim at identifying the transmission and co-infection potential within the different climatic envelopes of the lineages used, allowing for models to be used to predict potential changes in the distribution of the different lineages in the future.

References

- Berger, L., Speare, R., Daszak, P., Green, D.E., Cunningham, A.A., Goggin, C.L., Slocombe, R., ... Parkes, H. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences*, 95(15): 9031-9036. DOI: 10.1073/pnas.95.15.9031
- Boyle, D.G., Boyle, D.B., Olsen, V., Morgan, J.A.T. & Hyatt, A.D. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms*, 60: 141-148. DOI: 10.3390/s130708551
- Byrne, A.Q., Vredenburg, V.T., Martel, A., Pasmans, F., Bell, R.C., Blackburn, D.C., Bletz, M.C., ... Rosenblum, E.B. 2019. Cryptic diversity of a widespread global pathogen reveals expanded threats to amphibian conservation. *Proceedings of the National Academy of Sciences*, 116(41): 20382-20387. DOI: 10.1073/pnas.1908289116
- Carey, C., Bruzgul, J.E., Livo, L.J., Walling, M.L., Kuehl, K.A., Dixon, B.F., Pessier, A.P., ... Rogers, K.B. 2006. Experimental exposures of Boreal toads (*Bufo boreas*) to a pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*). *EcoHealth*, 3(1): 5-21. DOI: 10.1007/s10393-005-0006-4
- Costa, A., Dondero, L., Allaria, G., Sanchez, B.N.M., Rosa, G., Salvidio, S. & Grasselli, E. 2021. Modelling the amphibian chytrid fungus spread by connectivity analysis: towards a national monitoring network in Italy. *Biodiversity and Conservation*: 1-19. DOI: 10.1007/s10531-021-02224-5
- Crowder, W.C., Nie, M. & Ultsch, G.R. 1998. Oxygen uptake in bullfrog tadpoles (*Rana catesbeiana*). *Journal of Experimental Zoology*, 280(2): 121-134. DOI: 10.1002/(SICI)1097-010X(19980201)280:2%3C121::AID-JEZ3%3E3.0.CO;2-Q
- Daszak, P., Berger, L., Cunningham, A.A., Hyatt, A.D., Green, D.E. & Speare, R. 1999. Emerging infectious diseases and amphibian population declines. *Emerging Infectious Diseases*, 5(6): 735-748. DOI: 10.3201/eid0506.990601
- De Jong, M.S., Van Dyk, R. & Weldon, C. 2018. Antifungal efficacy of F10SC veterinary disinfectant against *Batrachochytrium dendrobatidis*. *Medical Mycology*, 56(1): 60-68. DOI: 10.1093/mmy/myx023
- Doddington, B.J., Bosch, J., Oliver, J.A., Grassly, N.C., Garcia, G., Schmidt, B.R., Garner, T.W.J., ... Fisher, M.C. 2013. Context-dependent amphibian host population response to an invading pathogen. *Ecology*, 94(8): 1795-1804. DOI: 10.1890/12-1270.1

- Farrer, R.A., Weinert, L.A., Bielby, J., Garner, T.W.J., Balloux, F., Clare, F., Bosch, J., ... Fisher, M.C. 2011. Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proceedings of the National Academy of Sciences*, 108(46): 18732-18736. DOI:10.1073/pnas.1111915108
- Fisher, M.C., Henk, D.A., Briggs, C.J., Brownstein, J.S., Madoff, L.C., McCraw, S.L. & Gurr, S.J. 2012. Emerging fungal threats to animal, plant and ecosystem health. *Nature*, 484(7393), DOI: 10.1038/nature10947
- Fisher, M.C., Ghosh, P., Shelton, J.M.G., Bates, K., Brookes, L., Wierzbicki, C., Rosa, C.M., ... Garner, T.W.J. 2018. Development and worldwide use of non-lethal, and minimal population-level impact, protocols for the isolation of amphibian chytrid fungi. *Scientific Reports*, 8(1): 7772. DOI: 10.1038/s41598-018-24472-2
- Galis, F., Terlouw, A. & Osse, J.W.M. 1994. The relation between morphology and behaviour during ontogenetic and evolutionary changes. *Journal of Fish Biology*, 45: 13-26. DOI: 10.1111/j.1095-8649.1994.tb01081.x
- Garner, T.W.J., Rowcliffe, J.M. & Fisher, M.C. 2011. Climate change, chytridiomycosis or condition: an experimental test of amphibian survival. *Global Change Biology*, 17(2): 667-675. DOI: 10.1111/j.1365-2486.2010.02272.x
- Garner, T.W.J., Walker, S., Bosch, J., Leech, S., Rowcliffe, J.M., Cunningham, A.A. & Fisher, M.C. 2009. Life history tradeoffs influence mortality associated with the amphibian pathogen *Batrachochytrium dendrobatidis*. *Oikos*, 118(5): 783-791. DOI: 10.1111/j.1600-0706.2008.17202.x,
- Gervasi, S.S., Urbina, J., Hua, J., Chestnut, T., Relyea, R.A. & Blaustein, A.R. 2013. Experimental evidence for American bullfrog (*Lithobates catesbeianus*) susceptibility to chytrid fungus (*Batrachochytrium dendrobatidis*). *EcoHealth*, 10(2): 166-171. DOI: 10.1007/s10393-013-0832-8
- Ghosh, P.N., Verster, R., Sewell, T.R., O'Hanlon, S.J., Brookes, L.M., Rieux, A., Garner, T.W.J., ... Fisher, M.C. 2021. Discriminating lineages of *Batrachochytrium dendrobatidis* using quantitative PCR. *Molecular Ecology Resources*, 21: 1452-1459. DOI: 10.1111/1755-0998.13299
- Griffiths, S.M., Harrison, X.A., Weldon, C., Wood, M.D., Pretorius, A., Hopkins, K., Fox, G., ... Antwis, R.E. 2018. Genetic variability and ontogeny predict microbiome structure in a disease-challenged montane amphibian. *The ISME Journal*, 12(10): 2506-2517. DOI: 10.1038/s41396-018-0167-0

- Hopkins, S. & Channing, A. 2003. Chytrid fungus in northern and western cape frog populations, South Africa. *Herpetological Review*, 34(4): 334.
- Jenkinson, T.S., Betancourt Román, C.M., Lambertini, C., Valencia-Aguilar, A., Rodriguez, D., Nunes-de-Almeida, C.H.L., Ruggeri, J., ... James, T.Y. 2016. Amphibian-killing chytrid in Brazil comprises both locally endemic and globally expanding populations. *Molecular Ecology*, 25(13): 2978-2996. DOI:10.1111/mec.13599
- Lane, E.P., Weldon, C. & Bingham, J. 2003. Histological evidence of chytridiomycete fungal infection in a free-ranging amphibian, *Afrana fuscigula* (Anura: Ranidae), in South Africa. *Journal of the South African Veterinary Association*, 74(1): 20-21. DOI: 10.4102/jsava.v74i1.493
- Lips, K.R., Diffendorfer, J., Mendelson III, J.R. & Sears, M.W. 2008. Riding the wave: reconciling the roles of disease and climate change in amphibian declines. *PLoS Biology*, 6(3): e72. DOI: 10.1371/journal.pbio.0060072
- Longcore, J.E., Pessier, A.P. & Nichols, D.K. 1999. *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia*, 91(2): 219-227. DOI: 10.1080/00275514.1999.12061011
- Marantelli, G., Berger, L., Speare, R. & Keegan, L. 2004. Distribution of the amphibian chytrid *Batrachochytrium dendrobatidis* and keratin during tadpole development. *Pacific Conservation Biology*, 10(3): 173-179. DOI: 10.1071/PC040173
- McAloose, D., Pramuk, J., Raphael, B., Calle, P., Shellabarger, W., Odum, A., Garner, M., ... Duncan, A. 2008. Mortality summary (2000-2007) in endangered captive Kihansi spray toads (*Nectrophrynoides asperginis*). In: 57th annual international conference of the Wildlife Disease Association Edmonton.
- Miller, C.A., Taboue, G.C.T., Ekane, M.M.P., Robak, M., Clee, P.R.S., Richards-Zawacki, C., Fokam, E.B., ... Anthony, N.M. 2018. Distribution modeling and lineage diversity of the chytrid fungus *Batrachochytrium dendrobatidis* (Bd) in a central African amphibian hotspot. *PLoS ONE*, 13(6): e0199288. DOI: 10.1371/journal.pone.0199288
- Morgan, J.A.T., Vredenburg, V.T., Rachowicz, L.J., Knapp, R.A., Stice, M.J., Tunstall, T., Bingham, R.E., ... Taylor, J.W. 2007. Population genetics of the frog-killing fungus *Batrachochytrium dendrobatidis*. *Proceedings of the National Academy of Sciences*, 104(34): 13845-13850. DOI: 10.1073/pnas.0701838104
- O'Hanlon, S.J., Rieux, A., Farrer, R.A., Rosa, G.M., Waldman, B., Bataille, A., Kosch, T.A., ... Fisher, M.C. 2018. Recent Asian origin of chytrid fungi causing global amphibian declines. *Science*, 360: 621-627. DOI: 10.1126/science.aar1965

- Parris, M.J., Reese, E. & Storfer, A. 2006. Antipredator behavior of chytridiomycosis-infected northern leopard frog (*Rana pipiens*) tadpoles. *Canadian Journal of Zoology*, 84(1): 58-65. DOI: 10.1139/Z05-175
- Phillips, J. 2019. *The Biomechanics and Evolution of Air-Breathing in Anuran Tadpoles*. University of Connecticut. (Master's Theses).
https://opencommons.uconn.edu/gs_theses/1378.
- Puschendorf, R., Carnaval, A.C., VanDerWal, J., Zumbado-Ulate, H., Chaves, G., Bolaños, F. & Alford, R.A. 2009. Distribution models for the amphibian chytrid *Batrachochytrium dendrobatidis* in Costa Rica: proposing climatic refuges as a conservation tool. *Diversity and Distributions*, 15(3): 401-408. DOI: 10.1111/j.1472-4642.2008.00548.x
- Rödger, D., Kielgast, J. & Lötters, S. 2010. Future potential distribution of the emerging amphibian chytrid fungus under anthropogenic climate change. *Diseases of Aquatic Organisms*, 92(2): 201-207. DOI: 10.3354/dao02197
- Salla, R.F., Gamero, F.U., Ribeiro, L.R., Rizzi, G.M., Medico, S.E.D., Rissoli, R.Z., Vieira, C.A., ... Costa, M.J. 2015. Cardiac adaptations of bullfrog tadpoles in response to chytrid infection. *Journal of Experimental Zoology*, 323A(7): 487-496. DOI: 10.1002/jez.1945
- Schloegel, L.M., Toledo, L.F., Longcore, J.E., Greenspan, S.E., Vieira, C.A., Lee, M., Zhao, S., ... Daszak, P.J., T. Y. 2012. Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. *Molecular Ecology*, 21(21): 5162-5177. DOI: 10.1111/j.1365-294X.2012.05710.x
- Schulte II, J.A., Losos, J.B., Cruz, F.B. & Núñez, H. 2004. The relationship between morphology, escape behaviour and microhabitat occupation in the lizard clade *Liolaemus* (Iguanidae: Tropidurinae*: Liolaemini). *Journal of Evolutionary Biology*, 17(2): 408-420. DOI: 10.1046/j.1420-9101.2003.00659.x
- Smith, K.G., Weldon, C., Conradie, W. & Du Preez, L.H. 2007. Relationships among size, development, and *Batrachochytrium dendrobatidis* infection in African tadpoles. *Diseases of Aquatic Organisms*, 74(2): 159-164. DOI: 10.3354/dao074159
- Tarrant, J., Cilliers, D., Du Preez, L.H. & Weldon, C. 2013. Spatial assessment of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) in South Africa confirms endemic and widespread infection. *PLoS ONE*, 8(7): e69591. DOI: 10.1371/journal.pone.0069591
- Vazquez, V.M., Rothermel, B.B. & Pessier, A.P. 2009. Experimental infection of North American plethodontid salamanders with the fungus *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms*, 84(1): 1-7. DOI: 10.3354/dao02026

- Voyles, J., Johnson, L.R., Briggs, C.J., Cashins, S.D., Alford, R.A., Berger, L., Skerratt, L.F., ... Rosenblum, E.B. 2014. Experimental evolution alters the rate and temporal pattern of population growth in *Batrachochytrium dendrobatidis*, a lethal fungal pathogen of amphibians. *Ecology and Evolution*, 4(18): 3633-3641. DOI: 10.1002/ece3.1199
- Walker, S.F., Bosch, J., James, T.Y., Litvintseva, A.P., Valls, J.A.O., Piña, S.I., García, G., ... Fisher, M.C. 2008. Invasive pathogens threaten species recovery programs. *Current Biology*, 18(18): R853-R854. DOI: 10.1016/j.cub.2008.07.033
- Weldon, C., De Villiers, A.L. & Du Preez, L.H. 2007. Quantification of the trade in *Xenopus laevis* from South Africa, with implications for biodiversity conservation. *African Journal of Herpetology*, 56(1): 77-83. DOI: 10.1080/21564574.2007.9635553
- Weldon, C., Channing, A., Misinzo, G. & Cunningham, A.A. 2020. Disease driven extinction in the wild of the Kihansi spray toad, *Nectophrynoides asperginis*. *African Journal of Herpetology*, 69(2): 151-164. DOI: 10.1080/21564574.2020.1752313
- Weldon, C., Du Preez, L.H., Hyatt, A.D., Muller, R. & Speare, R. 2004. Origin of the amphibian chytrid fungus. *Emerging Infectious Diseases*, 10(12): 2100-2105. DOI: 10.3201/eid1012.030804
- Zimkus, B.M., Baláž, V., Belasen, A.M., Bell, R.C., Channing, A., Doumbia, J., Fokam, E.B., ... Penner, J. 2020. Chytrid pathogen (*Batrachochytrium dendrobatidis*) in African amphibians: a continental analysis of occurrences and modeling of its potential distribution. *Herpetologica*, 76(2): 201-215. DOI: 10.1655/0018-0831-76.2.201

Supplemental information

Chapter 4: Environmental fitness of *Batrachochytrium dendrobatidis* lineages increases potential for recombination

Jack-knife test results for the predicted distribution models pertaining to Figures 2, 3-A and 3-B. Note that without variable indicates the effect removing the specific parameter will have on the models, with only variable indicates the performance of the model using the specific parameter in isolation and with all variables indicate the AUC score if all variables are used.

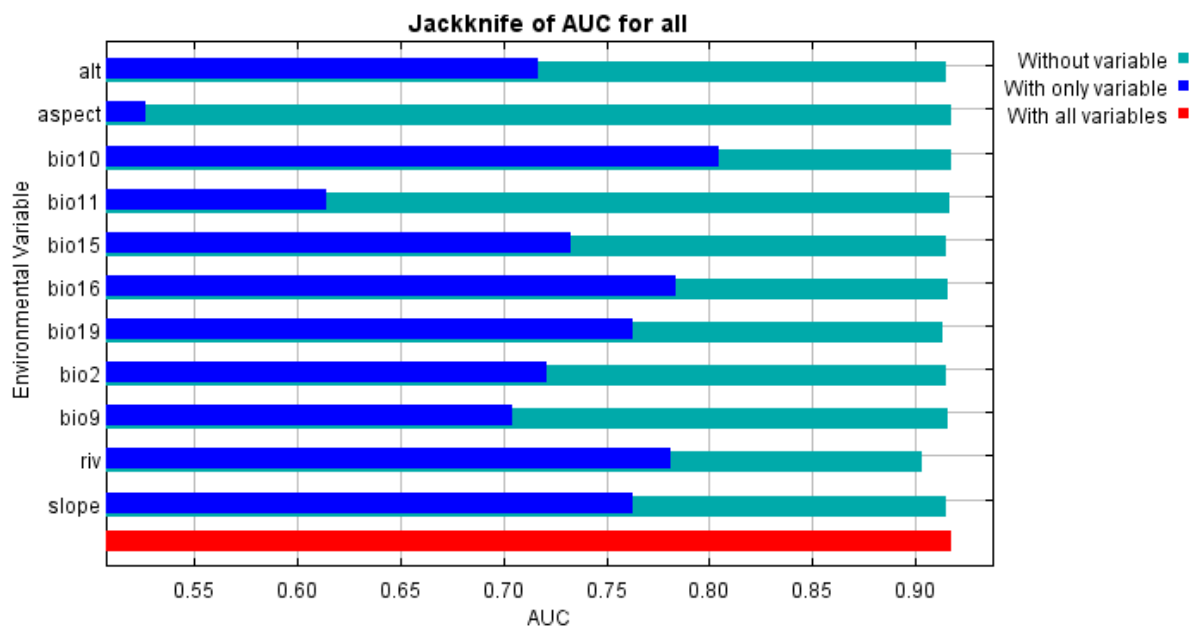


Figure S1: Jack-knife analyses of the environmental variables contributing to the final prediction model of *Bd* in Africa

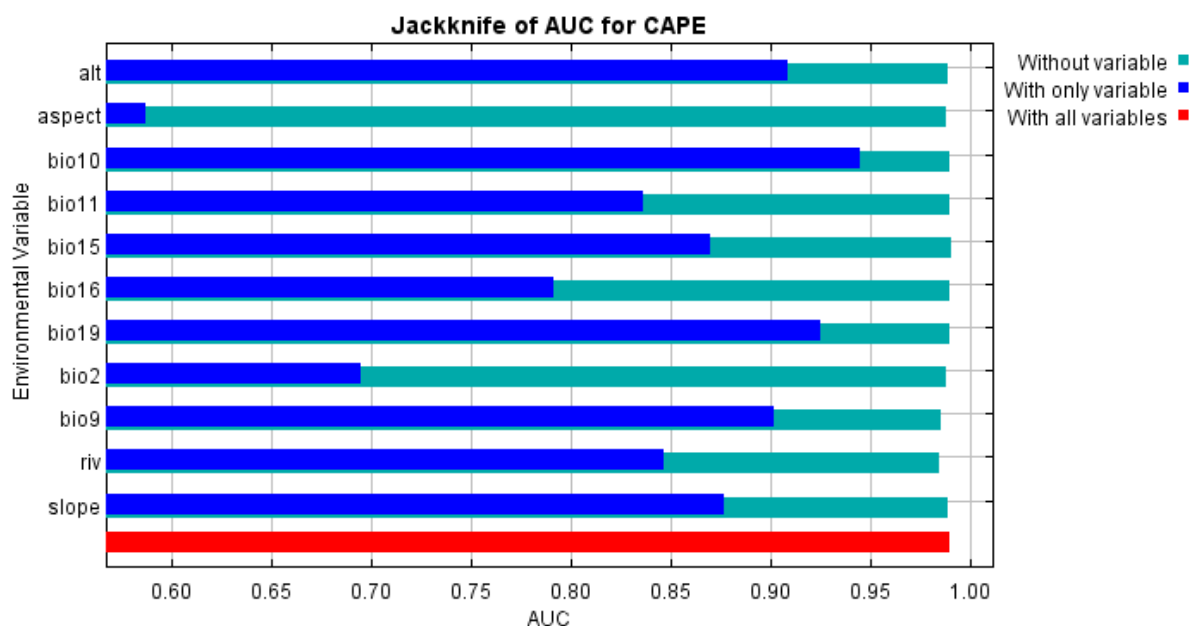


Figure S2: Jack-knife analyses of the environmental variables contributing to the final prediction model of *Bd*CAPE in South Africa

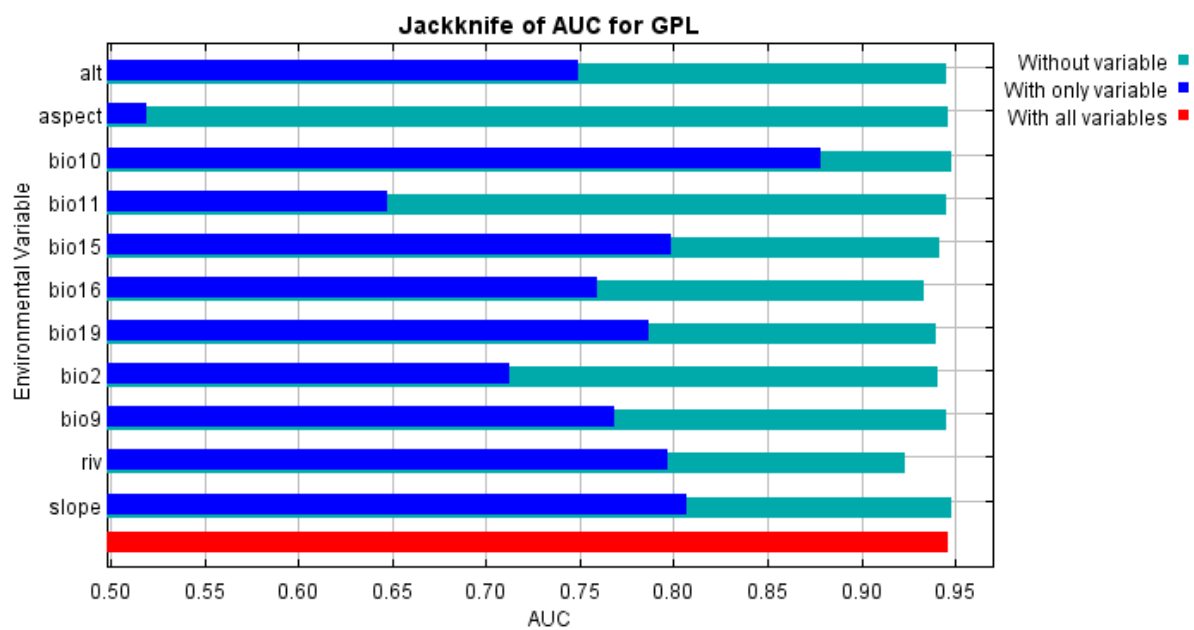


Figure S3: Jack-knife analyses of the environmental variables contributing to the final prediction model of *BdGPL* in South Africa

Chapter 5: Supplemental Information as it appeared in Molecular Ecology Resources for the article titled **Discriminating Lineages of *Batrachochytrium dendrobatidis* using quantitative PCR**

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Materials and Methods

Bd lineage-specific qPCR reaction mix and cycling conditions

The optimised multiplex reaction mix for a single 25mL reaction (with a starting concentration of 100mM for all primers and probes) was as follows: 12.5mL TaqMan Fast Advanced MasterMix (ThermoFisher Scientific, Massachusetts, USA); 0.125mL each of *Bd*GPL-specific forward and reverse primers; 0.225mL each of *Bd*-CAPE specific forward and reverse primers; 0.03125mL *Bd*GPL-specific TaqMan MGB probe; 0.09375mL of *Bd*-CAPE specific TaqMan MGB probe; 0.2mL Bovine Serum Albumin (BSA); 6.475mL filtered water.

The cycling conditions were two minutes at 50°C, followed by 10 minutes at 95°C, then 15 seconds at 95°C and one minute at 62°C, cycled 40 times. The Ct threshold was set at DRn = 0.1 for all reactions.

Risk Assessment of Chytridiomycosis to European Amphibian biodiversity (RACE) swabbing protocol

Use tubed sterile dry swabs with a fine tip (if possible). Ideally use gloves when handling amphibians and change gloves between each animal. Alternatively, thoroughly wash/disinfect hands between each animal however this will not prevent cross-contamination of samples with DNA. Hold the animal firmly by its back legs. For caudatans, hold the animal on either side of

its abdomen. Firmly run the swab over the back and legs ten times. You will need to press quite hard on the skin as you swab, as you are trying to remove any fungal zoospores or sporangia embedded on the amphibian's skin. Rotate the swab to ensure the entire surface has come into contact with the skin. Next, swab the back legs and toes ten times per limb, then flip the animal over to swab the pelvic patch and insides of the back legs ten times. Pay particular attention to the swabbing the back legs, toes and pelvic patch, because this is where *Bd* is most likely to be found. Release the animal and store the swab at four degrees. Ambient temperature is fine for a few hours. Give the swab a clear number either in permanent marker or pencil. In a separate data file record for each swab: location (as specific as possible); species (if known); collector's name; date; any additional information that may be relevant e.g. male/female/juvenile/body condition).

RACE DNA extraction protocol

In a BioSafety ACDP Class 2 cabinet or equivalent, fill one 1.5mL Eppendorf tube with approximately 0.03g Zirconium/silica microbeads for each sample. Pipette 60mL of Prepman ultra (ThermoFisher Scientific, Massachusetts, USA) into each Eppendorf for swab samples and 50mL of Prepman ultra for tissue samples. If processing swab samples, snap the end of each swab off into an individual Eppendorf tube directly. If processing tissue samples, add approximately 1-4mm² of tissue, depending on the thickness. Large tissue samples should be dissected for processing. Add the tissue to an Eppendorf tube. Homogenize the samples using a bead beater for 45 seconds, then centrifuge for 30 seconds at 14500rpm. Repeat the homogenisation and centrifugation. Incubate the samples at 100°C for 10 minutes, followed by 2 minutes cooling at ambient temperature. Centrifuge for 3 minutes at 14500 rpm. Collect as much supernatant as possible and store in a fresh sterile 0.5mL Eppendorf tube.

Preparation of DNA quantitation standards

All work with live *Bd* isolate cultures should be carried out in a BioSafety ACDP Class 2 cabinet or equivalent. To prepare DNA quantitation standards, transfer 500mL of *Bd* culture suspended in Tryptone Gelatin hydrosolate Lactose (TGhL) broth to a TGhL 1 % agar plate. Allow the plate to dry, then para-film it and incubate inverted at 18°C for approximately 5 days. When large numbers of zoospores have been produced, wash the plates with 1mL of TGhL broth and incubate them for 10 minutes at ambient temperature. Pipette the broth off the plate and collect the supernatant. Repeat this process at least twice. Calculate the concentration of live zoospores in the supernatant using a haemocytometer, then dilute down to the appropriate

concentration of zoospores with more TGhL broth. Transfer 1mL of the solution into a 1.5mL sterile Eppendorf tube and centrifuge for 10 minutes at 14500 rpm. Remove the supernatant, being careful not to disturb the pellet, and then add 50mL of Prepman Ultra. After this point, follow the RACE protocol for DNA extraction.

Table S1: Isolates used for DNA quantitation standards

Isolate	Lineage	Source location	Latitude	Longitude	Year isolated	Host species
TF5a1	<i>BdCAPE</i>	Mallorca	39.86	-2.84	2007	<i>Alytes muletensis</i>
IA042	<i>BdGPL</i>	Spain	40.85	-3.96	2004	<i>Alytes obstetricans</i>

Table S2: Isolates used for lineage-specific qPCR specificity testing panel

Isolate	Lineage	Source location	Year isolated	Host species
KBO_319	<i>BdASIA-1</i>	South Korea	2014	<i>Bombina orientalis</i>
KBO_317	<i>BdASIA-1</i>	South Korea	2014	<i>Bombina orientalis</i>
KBO_327	<i>BdASIA-1</i>	South Korea	2014	<i>Bombina orientalis</i>
TRBOOR_11	<i>BdASIA-1</i>	Trade	2011	<i>Bombina variegata</i>
0739	<i>BdCH</i>	Switzerland	2007	<i>Alytes obstetricans</i>
ACON	<i>BdCH</i>	Switzerland	2007	<i>Alytes obstetricans</i>
CLFT-065	<i>BdASIA2/BRAZIL</i>	Brazil	2013	<i>Hylodes japi</i>
UM142	<i>BdASIA2/BRAZIL</i>	Brazil	2009	<i>Lithobates catesbeianus</i>
SA4c	<i>BdCAPE</i>	South Africa	2010	<i>Amietia delalandii</i>
TF5a1	<i>BdCAPE</i>	Mallorca	2007	<i>Alytes muletensis</i>
SA1d	<i>BdCAPE</i>	South Africa	2010	<i>Hadramophryne natalensis</i>
TRGSCM.12	<i>BdCAPE</i>	Trade	2007	<i>Geotrypetes seraphini</i>
NTA6	<i>BdCAPE</i>	Cameroon	2012	<i>Ophisthothylax immaculatus</i>
RC5.1	<i>BdCAPE</i>	France	2010	<i>Lithobates catesbeianus</i>
MG1	<i>BdCAPE</i>	South Africa	2008	<i>Amietia hymenopus</i>
MG8	<i>BdGPL</i>	South Africa	2008	<i>Amietia delalandii</i>
SP10	<i>BdGPL</i>	Sardinia	2010	<i>Discoglossus sardus</i>
TORO11	<i>BdGPL</i>	Spain	2011	<i>Alytes obstetricans</i>
UKSHBC12	<i>BdGPL</i>	UK	2012	<i>Bufo calamita</i>
AP15	<i>BdGPL</i>	Sardinia	2010	<i>Discoglossus sardus</i>
MODS27.3	<i>BdGPL</i>	Sardinia	2010	<i>Discoglossus sardus</i>
JEL423	<i>BdGPL</i>	Panama	2004	<i>Hylomantus lemur</i>
BLI1	<i>BdGPL</i>	Switzerland	2010	<i>Alytes obstetricans</i>
JEL274	<i>BdGPL</i>	USA	1999	<i>Bufo boreas</i>
BEW2	<i>BdGPL</i>	Switzerland	2010	<i>Alytes obstetricans</i>
JEL270	<i>BdGPL</i>	USA	1999	<i>Lithobates catesbeianus</i>
UKMABC12	<i>BdGPL</i>	UK	2012	<i>Bufo calamita</i>

picosAo1	BdGPL	Spain	2010	<i>Alytes obstetricans</i>
ADMALA	BdGPL	Spain	2012	<i>Alytes dickilleni</i>
C2A	BdGPL	Spain	2002	<i>Alytes obstetricans</i>
L2203	BdGPL	Montserrat	2009	<i>Leptodactylus fallax</i>
BR1	BdGPL	Switzerland	2011	<i>Alytes obstetricans</i>
JEL261	BdGPL	Canada	1999	<i>Lithobates catesbeianus</i>
UKBELV12	BdGPL	UK	2012	<i>Lissotriton vulgaris</i>
BAV_OBER_1	BdGPL	Germany	2012	<i>Alytes obstetricans</i>
BAV_OBER_2	BdGPL	Germany	2013	<i>Alytes obstetricans</i>
BAV_OBER_3	BdGPL	Germany	2013	<i>Alytes obstetricans</i>
ETH2	BdGPL	Ethiopia	2011	<i>Leptopelis spp.</i>
ETH4	BdGPL	Ethiopia	2011	<i>Afrivalus enseticola</i>
UG3	BdGPL	Uganda	2012	<i>Amietophrynus spp.</i>
IA043	BdGPL	Spain	2004	<i>Alytes obstetricans</i>
IA_12No1	BdGPL	Spain	2012	<i>Alytes obstetricans</i>
PUITS_12No3	BdGPL	France	2012	<i>Alytes obstetricans</i>
Puits_12No4	BdGPL	France	2012	<i>Alytes obstetricans</i>
Puits_12No5	BdGPL	France	2012	<i>Alytes obstetricans</i>
Puit_12No7	BdGPL	France	2012	<i>Alytes obstetricans</i>
PUITS_11	BdGPL	France	2011	<i>Alytes obstetricans</i>
LHURS_11No11	BdGPL	France	2011	<i>Alytes obstetricans</i>
LHURS_11No7	BdGPL	France	2011	<i>Alytes obstetricans</i>
IA_12No3	BdGPL	Spain	2012	<i>Alytes obstetricans</i>
IA042	BdGPL	Spain	2004	<i>Alytes obstetricans</i>
IA_11	BdGPL	Spain	2011	<i>Alytes obstetricans</i>

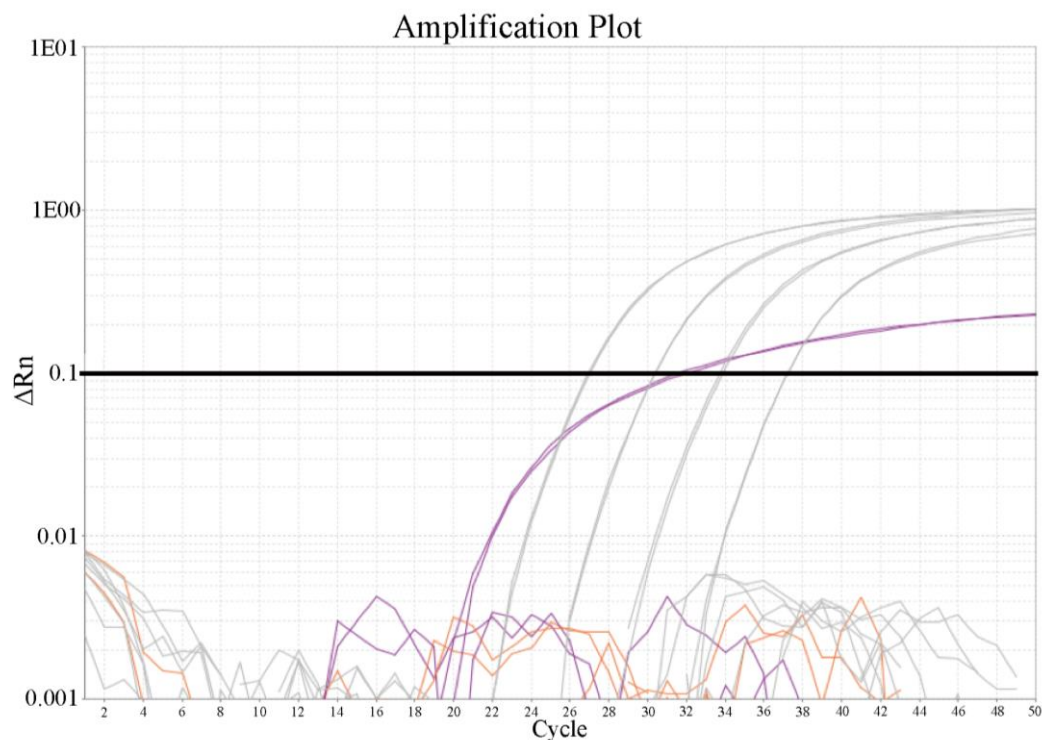


Figure S1: qPCR fluorescence amplification plot for isolate UM142 (purple) with *BdGPL*-specific primers and probes and DNA quantitation standards for *BdGPL* of 1000 GE, 100 GE, 10 GE and 1 GE (grey)