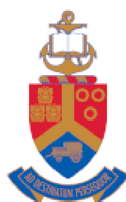

Exposure of wildlife to anthrax in Kruger and Etosha National Parks and the effect of haemoparasite coinfections

By

Sunday Ochonu Ochai

Submitted in fulfilment of the requirements for the degree of Master of Science Veterinary Science Tropical Diseases in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria

Supervisor:

Prof. Henriette van Heerden

Co-supervisor:

Dr. Wendy Turner

2020

Declaration

I, Sunday Ochonu Ochai, do solemnly declare that this work submitted for the degree of Masters in Veterinary Science is my original work and has not been submitted by me anywhere else for the purpose of a degree.

Sunday Ochonu Ochai

23/10/2020
Date

Table of Contents

Declaration ii
Table of Contents iii
List of Figures v
List of Tables viii
List of Abbreviations ix
Dedication xi
Acknowledgement xii
Abstract xiv
Chapter 1 INTRODUCTION 1
1.1 Aim 4
1.2 Objectives 4
Chapter 2 LITERATURE REVIEW 5
2.1 Anthrax Aetiology, Distribution, and Transmission 5
2.2 Anthrax Toxins and Pathogenesis 6
2.3 Clinical signs and lesions 7
2.4 Host immunity against *B. anthracis* 8
2.5 Anthrax distribution in Kruger and Etosha National Parks 9
2.6 Effects of environmental factors in the persistence of anthrax spores 10
2.7 Diagnosis and Identification of *Bacillus anthracis* 11
2.7.1 Enzyme-Linked Immunosorbent Assay (ELISA) 12
2.7.2 Toxin Neutralisation Assay (TNA) 14
2.8 Haemoparasites 15
2.8.1 Tick-Borne Diseases (TBDs) in Wildlife 16
2.8.2 Theileriosis in African Wild Equids and Antelopes 16
2.8.3 Babesiosis in African Equids and Wild Antelopes 17
2.8.4 Anaplasmosis in African Equids and Wild Antelopes 17
2.8.5 Ehrlichiosis (Heartwater) in African Wild Equids and Antelopes 17
2.9 Diagnosis and Identification of Haemoparasites 18
2.9.1 Microscopy 18
2.9.2 Serology 18
2.9.3 Nucleic Acid detection 19
2.9.3.1 Reverse Line Blot Hybridization 19
Chapter 3 MATERIALS AND METHODS 20
3.1 Study Area and Animals 20
3.1.1 Study Area 20
3.1.2 Selection of Host Study Species 22

3.1.3 Study animal and sample collection 23
3.2 Immunological methods 24
3.2.1 Protective Antigen (PA) Enzyme-Linked Immunosorbent Assay 24
3.2.2 Toxin neutralisation assay (TNA). 25
3.3 Statistical analyses 26
3.3.1 Poisson regression analysis of anti-PA titres between KNP and ENP, areas and interaction between park and area 26
3.3.2 Multivariable logistic regression of the association of anthrax serological status 26
3.3.2 Multivariable logistic regression analysis of the association of the proportion of animals that neutralized the anthrax lethal toxin 26
3.3.5 Analysis of the distribution of the *Bacillus anthracis* neutralisation titres (NT) in the wildlife species 27
3.3.6 Correlation between ELISA and TNA titres and their binary outcome 27
3.4 Reverse Line Blot hybridisation assay (RLB) 28
3.4.1 PCR and Hybridisation of PCR products on membrane 31
3.4.2 Haemoparasite analysis and Correlation between Parasite burden and the Neutralisation Titre 50 (NT50) 33
Chapter 4 RESULTS 34
4.1. Results of anti-PA ELISA Optical densities (ODs) zebra, kudu, impala and wildebeest in KNP and ENP 34
4.1.1 Multivariable logistic regression analysis of the association of anthrax serological status 36
4.2 Toxin Neutralisation assay 39
4.2.1 Results of the proportion of animals that neutralized the anthrax lethal toxin 39
4.2.2 Results of the analysis of the distribution of the *Bacillus anthracis* neutralisation titres (NT) in the wildlife species 41
4.2.3 Result of comparison of the two tests and correlation analysis 44
4.3 Analysis of the Reverse Line Blot (RLB) hybridization results 46
4.3.1 Results of logistic regression analysis 51
4.3.2 Results of the correlation analysis 52
Chapter 5 DISCUSSION, CONCLUSION AND RECOMMENDATION 55
5.1 Discussion 55
5.2 Conclusion 67
5.3 Future Recommendations 68
References 69
APPENDIX 85

List of Figures

Figure 2-1 Figure illustrating how proteins combine to form anthrax exotoxins. 7

Figure 2-2 Mechanism of action of anthrax toxin in the host. The protective antigen (PA) binds to one of the two anthrax toxin receptors (ATR) and becomes proteolytically activated. The resultant PA-heptamer then binds to the lethal factor (LF) leading to induction of the death of macrophages by unsettling the pathways of mitogen-activated protein kinase kinases (MAPKK) or binds the oedema factor (EF). This deactivates the phagocytes of the host by inducing a continuous activation of the animal's cyclic adenosine monophosphate (cAMP) following a rapid conversion of the cells adenosinetriphosphate (ATP), ultimately leading to oedema. The anthrax exotoxins are then endocytosed and translocated into the host's cell where it destroys the host immune mechanism. Obtained from https://en.wikipedia.org/wiki/Anthrax_toxin. 7

Figure 2-3 Image demonstrating the process of performing an indirect ELISA. Microtitre plate is coated with the antigen. Sample containing antibody is added and allowed to bind to the antigen. A secondary labelled antibody is added and allowed to bind. Then a substrate is added to allow for colour detection.......... 14

Figure 2-4 Describing the concept of the Toxin Neutralisation Assay: Previously incubated antibody and lethal toxin are added to already seeded cells and neutralisation or death of cells are measured utilizing a colorimetric agent which shows the presence of antibodies that neutralizes the lethal toxin is present in the cells. 15

Figure 3-1 Etosha National Park (ENP) and Kruger National Park (KNP) in southern Africa, showing study sites where anthrax outbreaks occur endemically or rarely. Both study species (kudu and zebra) occur in all four sites (depicted with animal figures) and the secondary host species that is Impala (occurring in the northern part of KNP) and Wildebeest (occurring in the central part of ENP). The most common anthrax host species in each endemic region is shown in red and the secondary host species are depicted in orange. 20

Figure 3-2 Map of Etosha National Park, Namibia showing the elevation level of the three sections of the park. 21

Figure 3-3 Map of Kruger National Park, South Africa showing the elevation level of each section and loality of the park. Adopted from https://www.sanparks.org/assets/images/conservation/scientific_new/savanna_arid/knp-elevation.jpg 22

Figure 4-1 Bar charts representing anthrax ELISA titres (optical density) across four wild animal species from Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia. The data represents Means ± standard error of the means. Letters that differ above the graphs are significantly different at

a significance level of 0.05. Similar letters above the graph indicate lack of statistically significant difference in the mean OD results between endemic and non-endemic areas for the corresponding park and animal species. 35

Figure 4-2 Bar graph showing the proportion of wild animals that were seropositive for anti-PA antibodies, as determined using enzyme-linked immunosorbent assay (ELISA). The four wild animal species (kudu, zebra, Impala and wildebeest) were sampled in Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia. Similar letters above the graphs indicate lack of statistically significant difference in the proportion of seropositive animals between endemic and non-endemic areas for the corresponding national park and animal species, as determined using the chi-square test, at a significance level of 0.05. Apart from kudu whose sample sizes in endemic and non-endemic areas in KNP were 19 and 18 respectively, the sample size of animals in each of the other sub-categories was 20. 37

Figure 4-3 Image showing anthrax suitability map on the left of Steenkamp et al (2018) coinciding with areas where animals tested positive for antibodies against Bacillus anthracis protective antigen (PA) antibodies in the southern part of Kruger National.. 38

Figure 4-4 Bar graph representing anthrax toxin neutralisation across four wild animal species from Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia. On the Y-axis is the proportion of animals whose antibodies in sera protected 50% of cells in culture from the toxin. The cells in the culture were of the J774A.1 cell line. Similar letters above the graphs mean that there was no significant difference between endemic and non-endemic areas for the corresponding national park and animal species, as determined using the chi-square test, at a significance level of 0.05. Apart from kudu whose sample sizes in endemic and non-endemic areas in KNP were 19 and 18 respectively, all other subcategories each had a sample size of 20 animals. 40

Figure 4-5 Bar chart showing Bacillus anthracis toxin neutralisation assay (TNA) titres among four wild animal species in Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia. The Y-axis represents means ± standard error of the means. Similar letters above graphs imply no significant difference in the mean TNA titres, as determined using the independent samples T-test, at a significance level of 0.05. 43

Figure 4-6 scatter plots and box and whiskers representing anthrax toxin neutralization and ELISA ODs across four wild animal species from Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia. The TNA status indicates if there was neutralization or not and the samples below the set cut-off are considered as negatives. TNA negative sera are seen below the dotted horizontal lines in each plot while ELISA negative samples can be seen behind the vertical dotted lines of each plot. 45

Figure 4-7 Prevalence of different genera of haemoparasites in Kudu, Zebra (Kruger and Etosha National parks), Impala (Kruger National park) and Wildebeest (Etosha National park) using the reverse line blot hybridization. 48

Figure 4-8 Bar charts representing Ehrlichia/Anaplasma, Therileria/Babesia, Theileria and babesia genera-specific signals without corresponding species-specific signals across four wild animal species from Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia. The data represents the percentage of samples that had only genus-specific signals out of the total population sampled in each park and each region (endemic and non-endemic) of the parks. 49

Figure 4-9 Prevalence of different species of haemoparasites in kudu, zebra (Kruger and Etosha National parks), impala(Kruger National park) and wildebeest (Etosha National park) identified using the reverse line blot hybridization for Theileria, Anaplasma and Ehrlichia species. 50

List of Tables

Table 3-1 Reference list Oligonucleotide probes fixed on the Biodyne C membrane for the detection of rictkettsiales and protozoan DNA. 30

Table 3-2 Thermocycling schedule for Babesia/Theileria and Ehrlichia/Anaplasma touchdown PCR 32

Table 4-1 Poisson regression analysis for the significance of the average anti-Bacillus anthracis titres in wild animals, as predicted by national park and locality....... 35

Table 4-2 Anthrax ELISA ODs in wild animal species, based on two national parks and endemic status of wildlife habitat 36

Table 4-3 Multivariable logistic regression analysis for the association between exposure to *Bacillus anthracis* in wild animals in two national parks in southern Africa, and predictor variables 37

Table 4-4 Multivariable logistic regression analysis for the association between the frequency of *Bacillus anthracis* toxin neutralisation in wild animals in two national parks in southern Africa, and the identified predictor variables 40

Table 4-5 Negative binomial regression analysis for the significance of differences in anti-*Bacillus anthracis* toxin neutralisation scores from Kudu and Zebra 42

Table 4-6 *Bacillus anthracis* toxin neutralisation assay (TNA) titres in wild animal species based on two national parks and the endemic status of wildlife habitat 42

Table 4-7 Comparison of *Bacillus anthracis* TNA titres between two national parks in southern Africa, based on wild animal species 43

Table 4-8 Comparison of results from ELISA and TNA binary outcome in four wild animal species from Kruger and Etosha National Parks in South Africa and Namibia respectively... 44

Table 4-9 Final logistic regression analysis for the association between infection with tick-borne haemoparasites and the predictors of infection in wild animal populations in two national parks in South Africa and Namibia 52

Table 4-10 Parasite diversity by Neutralisation 50 (NT50) cross tabulation 54

List of Abbreviations

µL	Mcrolitre
µM	Micromolar
Ab	Antibody
Ag	Antigen
ATP	Adenotriphosphate
ATR	Anthrax Toxin Receptor
cAMP	Cyclic adenosine monophosphate
CO2	Carbon dioxide
CVL	Central Veterinary Laboratory
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
DNA	Doexyribonucleic acid
DVTD	Department of Veterinary Tropical Diseases
ECACC	European Collection of Authenticated Cell Cultures
EDTA	Ethylenediaminetetraacetic acid
EEI	Etosha Ecological Institute
EF	Oedema factor
ELISA	Enzyme-linked immunosorbent assay
ENP	Etosha National Park
ET	Oedema toxin
FCS	Fetal calf serum
HCl	Hydrogen chloride
IFAT	Immunoflourescence Antibody Test
IgG	Immunoglobulin G
IMHA	Indirect microhemagglutination test
kDa	Kilodalton
Km	Kilometres
KNP	Kruger National park
LD50	Lethal dose 50%
LF	Lethal factor
LT	Lethal toxin
LT	Lethal toxin
MAPKK	Mitogen-activated protein kinase kinases
Mm	Milimetre
MTT	3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaHCO	Sodium bicarbonate
NT50	50 % neutralization
OBP	Onderstepoort biological company
°C	Degree Celsius
OD	Optical Density
PAG	Protein A/G
PBS	Phosphate buffered saline
PBST	PBS with Tween
PBSTM	PBST with skimmed milk powder
PCR	Polymerase chain reaction
PGDA	Poly-gamma-D-glutamic acid
ProtA	Protein A
ProtG	Protein G
p-value	Probability values
pXO1	Plasmid XO 1

pXO2	Plasmid XO 2
qPCR	Quantitative PCR
RLB	Reverse Line Blot
RPMI	Roswell park memorial institute
SDS	Sodium Dodecyl Sulfate
SNP	Single Necleotide Polymorphism
Spp	Species
SSVS	Skukuza State Veterinary Services
TBDs	Tick-Borne Diseases

Dedication

I dedicate this work to God Almighty for His provision and protection. And to my Late parents; Mr and Mrs Samuel Ochonu Ochai. I will eternally be grateful and always make you proud.

Acknowledgement

All gratitude to God Almighty for His sustenance, provision and guidance throughout this work. May your name be praised.

My sincere and heartfelt appreciation to my amiable supervisor Prof. Henriette van Heerden. Your endless support, meekness and limitless kindness are too numerous to iterate. Thank you for all the emotional, academic and financial assistance you offered to me. I will forever be grateful.

To my co-supervisor, Dr Wendy Turner, I wish to extend my deepest appreciation for all that you are and all that you have given to me. The opportunity to work under your tutelage was a life-impacting experience I will not forget in a hurry. I have learnt quite a lot from your friendliness, promptness and of course, your wealth of knowledge and experience.

I wish to especially appreciate and acknowledge the Mandela Rhodes Foundation for believing in me and giving me this platform. Thank you so much for the scholarship and leadership training, I assure you that I will not relent in my quest to make Africa a better place. Also, I wish to appreciate the National Science Foundation grant that sponsored my research, I am truly grateful. I also wish to appreciate all the staff of Skukuza state veterinary office and Etosha ecological institute for their support.

I wholeheartedly wish to appreciate Dr Charles Byaruhanga for his tutelage and support during my data analysis and also to Huang Yen-Hua for his enormous support. I also appreciate the enormous support of Dr Raksha Bhoora.

My special appreciation goes to Dr Ayesha Hassim, for being a true “Iya” to us all, to Dr Solomon Jauro for being a brother indeed, to Drs Francis and Agatha Kolo for their hospitality and Dr Betty Ledwaba for all her support. I also wish to appreciate Dr Hassan Ibrahim for being a good friend, Drs David Lazarus and Yusuf Ngoshe for their endless support. I also extend my thanks to Dr Barbara, Dr Bosco and all other members of the ABC research group.

I wish to sincerely appreciate Dr Jannie Crafford for his endless support, tutelage and expertise, I have learnt a lot from you and I won’t stop learning.

To all the staff and students of DVTD, I am most grateful for all your kindness and enormous support throughout this study period.

I wish to convey my appreciation to Anna-marie Bossman, Milana Troskie, Ilse Voster, Karen Ebersohn and Rebone Mahlare for their support and training in the lab.

Special appreciation to my siblings Zion Ochai (Man), Christopher Ochai (Astom), Emmanuel Ochai (Majesty), Peter Ochai (Pato) and Paschal Ochai (Bouncer), thank you for all the support, prayers and love. I also wish to appreciate “our wives”; Chundung Ochai, Nkesi Ochai and Funke Ochai and all my nephews and nieces (Ochi, Sophie, Alex, Nathan, Michel, Victor and Victoria).

To Mummy Ogenyi, Dr Fati and Mrs Ighogboja for their enormous support throughout this study period.

To my friends, Rinret Wetle, Godwin Edache, Ufuoma Ighogboja, Faith Okeke, Oche Itodo, Ugochukwu Ojiegbu, Debby Ogenyi, Ikenna Isu. I appreciate all your love and show of care.

Abstract

Exposure of wildlife to anthrax in Kruger and Etosha National Parks and the effect of haemoparasite coinfections

By

Sunday Ochonu Ochai

Supervisor: Henriette van Heerden

Department of Veterinary Tropical Diseases, University of Pretoria

Co-supervisor: Wendy Turner

U.S. Geological Survey, Wisconsin Cooperative Wildlife Research Unit, Department of Forest and Wildlife Ecology, University of Wisconsin-Madison.

Degree: Master of Science Veterinary Science Tropical Diseases

Anthrax has a global distribution and it is enzootic in Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia. These parks share some similarities such as hosts species and both have endemic and non-endemic areas, but host species differ in their susceptibility between the parks. We measured the presence of anti-PA antibodies in zebra and kudu from both parks using ELISA and also their ability to neutralize anthrax lethal toxin vis-à-vis haemoparasite coinfections detected with reverse line blot probes of *Theileria*, *Babesia*, *Anaplasma* and *Ehrlichia*. It was found that kudus in KNP had higher titres and proportion (95%) of positive animals than ENP (40%). ENP zebras had higher titres and proportions of positive animals (83%) than those of KNP (63%). Animals in anthrax endemic areas in KNP had higher titres than those in non-endemic areas, but this was not so in ENP. ENP kudus and KNP zebras showed better and higher proportion of neutralization. Animals positive to haemoparasites (*Theileria, Babesia, Ehrlichia* and *Anaplasma*) showed a significant difference between the kudu in KNP (100%) compared to 70% in ENP, while all ENP zebra (100%) tested positive compared to 84.6% KNP zebra. An increase in toxin neutralization was significantly associated with less likelihood of infection with haemoparasites in zebras. In summary, this study shows that rarity is largely a function of resistance toward anthrax, which could have emanated from both the dose of infection and the interval between exposures and the interplays of other infections in the host.

Chapter 1
INTRODUCTION

Anthrax is a zoonosis that affects a myriad of species, including humans, although its most susceptible hosts are mammalian herbivores (W.H.O., 2008). Anthrax is caused by the gram-positive, capsule-forming, endospore-forming *Bacillus anthracis* bacterium. This pathogen must kill its animal host in a bid to further spread. After the death of the host and the shedding of infectious materials containing the vegetative part of the organism, the bacilli begins to undergo sporulation forming endospores. The endospores can persevere in the soil for numerous years until uptake (normally ingestion) by a susceptible host and germinate forming vegetative cells. This is followed by further propagation and increase of cells (Sterne, 1959, W.H.O., 2008, World Health Organization 2015) producing toxins that ultimately leads to the death of the host (Leppla, 1982). In a broader sense, there is a progression of the disease which occurs either as acute or peracute septicaemia following incubation of 2-8 days. The variation in the incubation period could occur due to the infectious dose intake and exposure intervals (Klein et al., 1962, W.H.O., 2008, Beyer and Turnbull, 2009). Due to the acute and peracute nature of anthrax, diagnosis is mainly based on, molecular identification, microscopy and culture and not on serology (Gates et al., 2001, Lembo et al., 2011, Bagamian et al., 2013).

Environmental factors like rainfall, humidity, precipitation, temperature and the type of soil affect the survival of the *B. anthracis* endospore. Generally, anthrax endemic regions are found in places with warmer temperatures, even though there are pieces of evidence that outbreaks can occur in places close to the Arctic circle (Minett and Dhanda, 1941). This happens mostly in summer when daylight can get to about 24 hours, as such, heating the spores of *B. anthracis* survive in soils that are alkaline and also have an abundance of calcium (Minett and Dhanda, 1941, Van Ness and Stein, 1956, Van Ness, 1971), as well as moderately high moisture content (W.H.O., 2008, Hugh-Jones and Blackburn, 2009). As anthrax is non-contagious, dissemination of *B. anthracis* relies on various vectors and factors like soil, water, vegetation and host grazing behaviour ,(de-Vos, 1990, Lindeque and Turnbull, 1994, de-Vos and Bryden, 1996, Hugh-Jones and de Vos, 2002, Saile and Koehler, 2006, W.H.O., 2008, Hampson et al., 2011, Turner et al., 2014), flies (de-Vos, 1990, Blackburn et al., 2010, Fasanella et al., 2010, Beyer et al., 2018), scavengers such as vultures (Pienaar, 1967, Houston and Cooper, 1975, Lindeque and Turnbull, 1994, Hugh-Jones and de Vos, 2002), jackals and hyena (Lindeque and Turnbull, 1994, Bellan et al., 2013). *Bacillus anthracis* thus has a lifecycle consisting of soil/environment, endospore, vegetative cells producing toxins, disseminating vectors and hosts. In a natural system, the latter consist of resistant and susceptible hosts whose immunity is also under pressure of other pathogens as well as endo-and ectoparasites.

Anthrax is endemic in Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia. Southern Africa, including KNP and ENP, is considered the origin of anthrax (Keim et al., 1997). These two parks have anthrax endemic and nonendemic regions. In the endemic region of ENP, deaths of plains zebra (*Equus quagga*) and other herbivores climax at the closing of the raining season while African elephant (*Loxodonta africana*) deaths climax during the late dry season, though cases in all species can be observed sporadically through the year (Ebedes, 1976b, Lindeque and Turnbull, 1994, Beyer et al., 2012, Turner et al., 2013). Seasonal outbreaks have been linked to differences in host foraging behaviour altering exposure rates (Turner et al., 2013, Havarua et al., 2014, Cizauskas et al., 2014) and and seasonal immune trade-offs (Cizauskas et al., 2015). Anthrax primarily affects grazing and mixed-feeding herbivores in ENP. Of all recorded cases, 52% are plains zebras, 22% are blue wildebeest (*Connochaetes taurinus*) (both grazers) (Turner et al., 2013), and only 1.7% are browsers which include kudu (*Tragelaphus strepsiceros*) and impala (*Aepyceros melampus*) (Havarua et al., 2014). In KNP, anthrax is endemic in the northern part of KNP, Pafuri in South Africa (Hugh-Jones and de Vos, 2002). The main host species in KNP over time has been greater kudu (*Tragelaphus strepsiceros*), a browser contributing up to 75% of recorded cases from 1960-1990s (de-Vos and Bryden, 1996). Anthrax was historically associated with dry seasons or droughts in KNP, occurring in explosive outbreaks on a roughly decadal cycle (Pienaar, 1960, Pienaar, 1961, de-Vos, 1990, de-Vos and Bryden, 1996, Hugh-Jones and de Vos, 2002). Since 2008, lesser outbreaks have occurred annually and mainly in the wet season, and primarily affecting impala (*Aepyceros melampus*), a mixed grazing-browsing species (Basson et al., 2018). Exposure of browsing species has been hypothesized to occur via blowflies (*Chrysomya* spp.) feeding on anthrax infected carcasses and then depositing *B. anthracis* spores onto the leaves of trees/shrubs near the carcass (Braack and De Vos, 1990, Blackburn et al., 2014, Basson et al., 2018). Plains zebra and wildebeest have been relatively rare host species, contributing at most 10% of cases in outbreaks (personal communication with Skukuza State Veterinary Services). These national parks are an ideal environment to investigate the pathogens, environmental factors, dissemination, host factors as well as the influence of ectoparasites and haemoparasites on the susceptibility of the hosts to anthrax (Horak et al., 2003, Dondona et al., 2012).

Haemoparasites such as Rickettsiales of the genera *Anaplasma* and *Ehrlichia* (Dumler et al., 2001) and protozoans like *Babesia* and *Theileria* are pathogens that are transmitted by ticks (Mehlhorn and Schein, 1985, Duh et al., 2008). These protozoans can infect and destroy red blood cells and/or the white cells of different wildlife and livestock (Duh et al., 2008), and hence may affect susceptibility to anthrax mortality. Hosts including humans and animals alike are often infected with more than one pathogen at a particular moment (Christensen et al., 1987,

Lello and Hussell, 2008). These interplays could be in the form of protozoa-bacteria, virus-bacteria, helminth-protozoa, protozoa-protozoa interactions amidst other interactions. These interactions often modulate and/ or alter the immunity of the host and hence increasing susceptibility of the host to one or the other pathogen (Chen et al., 2005) or increase the severity of other pathogenic organisms (Borkow et al., 2001). With the knowledge of such interactions between microparasites in the body of the host, an in-depth understanding of the immunological subtleties of the relationship of a co-infection with haemoparasites and the ability of an animal host to neutralize anthrax lethal toxin is imperative.

Anthrax is considered a part of the natural ecosystem of KNP and ENP and is primarily unmanaged. The long history of unmanaged anthrax in these systems, with no evidence of an increase in cases over time, suggests that anthrax in these areas is at or near an endemic equilibrium. Both parks contain high and low incidence areas, allowing the study of host resistance evolution in sub-populations with and without regular pathogen exposure. These two parks share some similarities in that they share many of the same hosts species and both have low and high incidence areas within the parks, but a striking difference between the parks is that a susceptible species (kudu) in KNP is a rarely affected species in ENP and susceptible species (zebra) in ENP is a rare affected species in KNP. This difference, therefore, calls for intra- and interspecific host comparisons between parks and between high/low incidence areas within parks.

With abounding knowledge of how pathogens evolve, it has, therefore, become imperative to study within-host infection and between-host transmission for envisaging virulence progression and epidemiological subtleties (Mideo et al., 2008, Restif and Graham, 2015, Cressler et al., 2016). The changeability in anthrax ecology worldwide has served as an impediment for the blending of knowledge and outbreak forecasting (Mullins et al., 2013) and therefore, identifying the factors responsible for the various forms and disease outbreak patterns warrants substantial attention. No research of this nature in literature has been conducted before now to measure and compare the variability in the exposure status and protection level of animals in different areas (e.g. KNP and ENP) and other factors such as coinfections with haemoparasites. It has, therefore, become necessary to investigate the immunological dynamics of anthrax infection in the two national parks with a bid to understanding if rarity and susceptibility are a function of low/no exposure or higher resistance respectively. Additionally, our methodology seeks to reveal the understanding of host-specific immunological differences vis-a-viz haemoparasite coinfections among systems by comparing two areas that differ in anthrax dynamics yet share host species.

1.1 Aim

The study aims at understanding the exposure to anthrax in Kruger and Etosha National Parks and the effect of haemoparasite co-infection.

1.2 Objectives

1. To ascertain antibodies present in hosts in KNP and ENP using *B. anthracis*-specific anti-protective antigen (PA) ELISA antibody titers, and make comparisons:
   a. between endemic vs. non-endemic populations within parks, and
   b. between parks where a given species is considered to be a more (ENP: zebra, KNP: kudu) or less (ENP: kudu, KNP: zebra) susceptible host, and
   c. among the three host species examined in each park (ENP: zebra, kudu, wildebeest; KNP: kudu, zebra, impala).
2. To measure neutralizing antibodies between parks where a given species is considered to be a more (ENP: zebra, KNP: kudu) or less (ENP: kudu, KNP: zebra) susceptible host, and within parks between zebra and kudu
3. To measure the association between haemoparasite diversity and the ability of the animals to neutralize anthrax lethal toxins.

Chapter 2
LITERATURE REVIEW

2.1 Anthrax Aetiology, Distribution, and Transmission

Anthrax is a multispecies animal disease, but the most susceptible hosts are herbivorous mammals. While primarily a fatal disease in herbivorous wildlife and livestock, humans are susceptible to anthrax infections and human cases occur largely due to the handling of dead carcasses, infected meat, hides and skin (Kamal et al., 2011, World Health Organization 2015). Other routes of human infections include via injections by heroin addicts (Ringertz et al., 2000), inhalation exposure to infected animal products, during outbreaks in animal populations, or when used as a biological weapon (Jernigan et al., 2002).

Bacillus anthracis is the bacterium that causes anthrax and emanates from the phylum Firmicutes, family Bacillaceae and belongs to the group referred to as the *B. cereus* group. The B. *cereus* group consist of 8 Bacillus species (*B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, *B. cytotoxicus*, and *B. toyonensis)* that have closely related phylogeny. *B. anthracis* is nearly affiliated to *B. cereus sensu stricto*, *B. mycoides, B. psuedomycoides, B. weihestephanesis* and *B. thuringiensis* because they share some genetic similarities but vary phenotypically (Radnedge et al., 2003, Rasko et al., 2005). These organisms also differ in their aetiology, pathogenesis, clinical manifestations and also host preferences (Drobniewski, 1993, Rasko et al., 2005, Pilo and Frey, 2011).

Bacillus anthracis is an aerobic, non-motile, gram-positive rod-shaped (bamboo-like) bacterium that produces endospores. This bacterium occurs in two forms; the spore form and the vegetative form (Vilas-Bôas et al., 2007). The spore form occurs in an oxygen sufficient environment which is mostly outside the body of the host and is capable of enduring severe climatic conditions for an extended period owing to their morphological makeup (Leppla, 1982, Turnbull et al., 1998). Upon exposure to the spores, germination occurs at the site of infection before being drained by the regional or associated lymph nodes. Depending on the form of the disease, the vegetative cells grow and replicate at the initial site of entry of spores either in the Peyer's patches or the respiratory-related lymphoid organs (Glomski et al., 2007). After this, they spread to the lymphatic system before going into the bloodstream which is then elicited by further proliferation of the bacilli leading to bacteremia(Glomski et al., 2007). The vegetative form occurs in the oxygen-deficient environment and occurs strictly within the host (W.H.O., 2008). The vegetative form of *B. anthracis* is the growth form, responsible for the production of a combination of fatal toxins that are accountable for the different clinical indications as well as the lesions associated with anthrax (Lincoln and Fish, 1970, Hoover et al., 1994).

2.2 Anthrax Toxins and Pathogenesis

The virulence factors of *B. anthracis* are encoded on two plasmids namely pX01 which is largely responsible for the production of the toxins, while the pX02 synthesizes the poly-y-D-glutamic acid capsule (Makino et al., 1989, Okinaka, 1999). The pX01 is made up of genes, responsible for the protective antigen (PA), lethal factor (LF) and the oedema factor (EF) proteins. These proteins are grouped in two forms namely the A and the B form. The A form which consists of the EF or LF bears the enzymatic activity (Leppla, 1982, Moayeri and Leppla, 2004, Moayeri and Leppla, 2009) (Figure 2.1). The B component consisting of PA, which is the receptor-binding component of the two toxins namely lethal toxin (LT) and oedema toxin (ET) and the courier of LF and EF respectively into the host cells (Smith et al., 1955, Leppla, 1982, Barth et al., 2004, Moayeri and Leppla, 2009). The poly-γ-D-glutamic acid (PGDA) capsule protects the bacilli from the effect of the immune system owing largely to its ability to cause a very low immune response and its effect on phagocytes (Makino et al., 1989, Little and Ivins, 1999, Moayeri et al., 2015). The PA combines with LF to produce lethal toxin, which is responsible for the induction of the death of macrophages by unsettling the pathways of mitogen-activated protein kinase kinases (Turk, 2007) (Figure 2.1 and 2.2). The PA combines with the EF to produce the oedema toxin (ET) that deactivates the phagocytes of the host by inducing a continuous activation of the animal's cyclic adenosine monophosphate (cAMP) pathway (Turk, 2007) (Figure 2.1; 2.2). It is these complexes (ET and LT) that work to bring about the reduction in the phagocytic effect of the body's defence mechanism, as well as alteration of the blood clotting system and rise in the permeability of the membranes (Hoover et al., 1994, W.H.O., 2008).

This PGDA capsule directly attaches to the peptidoglycan surface of host cells, which has been suggested to potentiate the effect of the lethal toxin (Scorpio et al., 2007, Jang et al., 2011). PGDA has been incriminated in the pathogenesis of anthrax and recent studies have demonstrated that this is achieved by its ability to target the cytokine pathway and in turn inhibiting the host immune response (Smith et al., 1955, Makino et al., 2002, Jelacic et al., 2014, Jeon et al., 2015). The number of pX02 in a cell is closely related to the level of virulence (Pannifer et al., 2001). Some studies have reported that animals that are resistant to lethal toxin are more susceptible to challenge by anthrax spores and that the inverse is also true (Lincoln et al., 1967, Welkos et al., 1989, Terra et al., 2010). The prevalence of the lethal toxin-susceptible macrophages over the resistant ones yielded better protection in mice infected by *B. anthracis* (Terra et al., 2010). In another report, it was shown that there is a relationship between deaths in rats caused by LT and the susceptibility of macrophages to the effect of the lethal toxin (Newman et al., 2010).

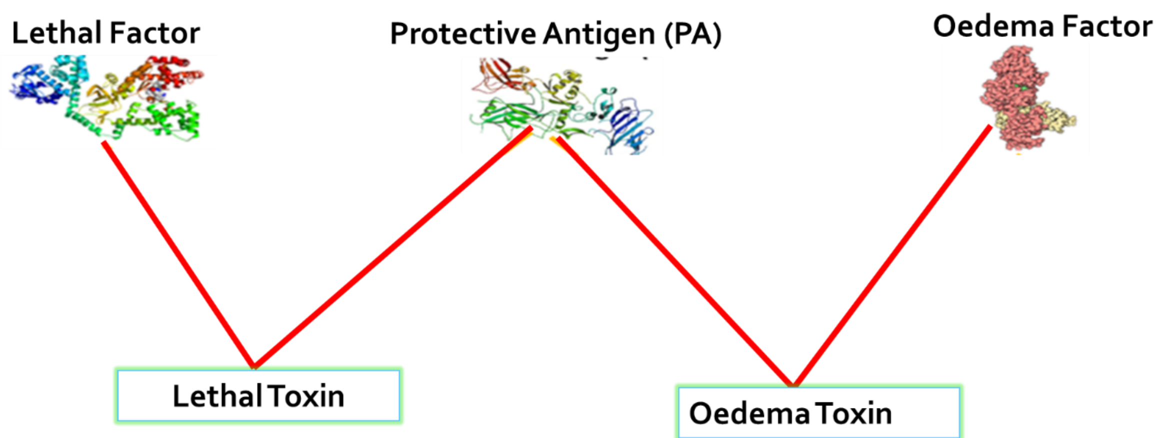

Figure 2-1 Figure illustrating how proteins combine to form anthrax exotoxins.

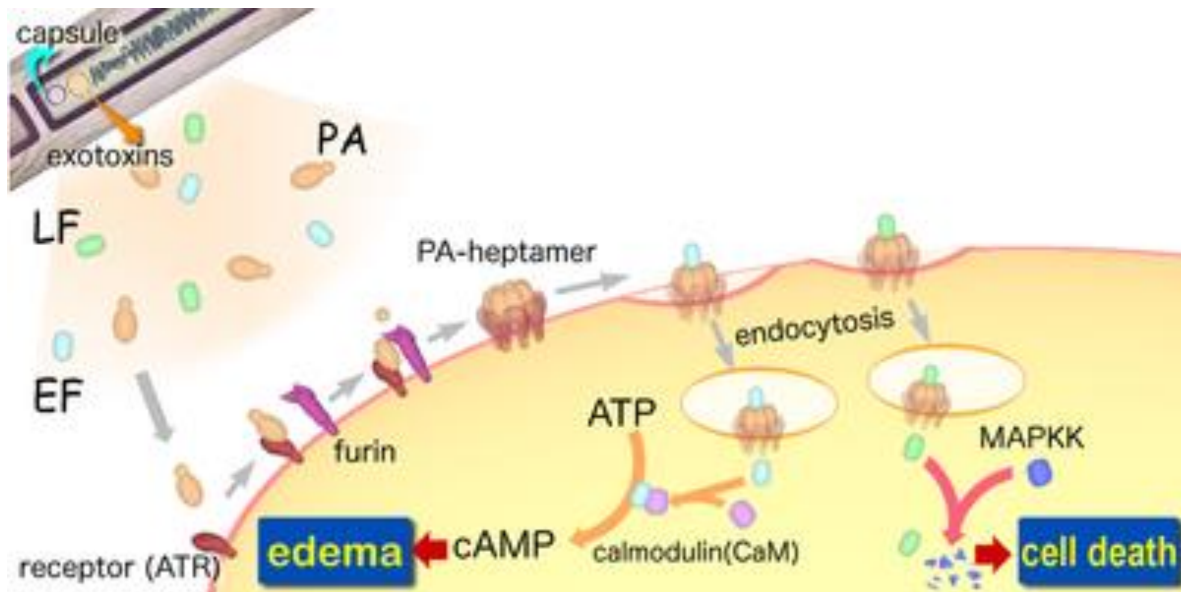

Figure 2-2 Mechanism of action of anthrax toxin in the host. The protective antigen (PA) binds to one of the two anthrax toxin receptors (ATR) and becomes proteolytically activated. The resultant PA-heptamer then binds to the lethal factor (LF) leading to induction of the death of macrophages by unsettling the pathways of mitogen-activated protein kinase kinases (MAPKK) or binds the oedema factor (EF). This deactivates the phagocytes of the host by inducing a continuous activation of the animal's cyclic adenosine monophosphate (cAMP) following a rapid conversion of the cells adenosinetriphosphate (ATP), ultimately leading to oedema. The anthrax exotoxins are then endocytosed and translocated into the host's cell where it destroys the host immune mechanism. Obtained from https://en.wikipedia.org/wiki/Anthrax_toxin.

2.3 Clinical signs and lesions

The pathological manifestations of anthrax depend on the form of the disease which could be defined based on the portal of entry of the organism. Although, other studies have shown that once the disease is established, it is quite difficult to differentiate between the forms through post-mortem identification of lesions (Glomski et al., 2007, Easterday et al., 2020). The three commonly identified forms of anthrax are cutaneous, inhalational and gastrointestinal forms.

Studies have demonstrated that inoculation of the skin with spores, results in germination and establishment of oedema-like regions containing capsules of the organism within 2-4 hours (Cromartie et al., 1947). Widespread oedema and shock have also been reported as clinical presentations of cutaneous anthrax in humans (Turnbull, 2008). The inhalational form of anthrax is characterised by lesions in the lungs as well as the enlargement of regional lymph nodes, this, in turn, leads to some of the signs associated with this form of anthrax (Gleiser et al., 1963). Signs such as dysphagia, hemoptysis, dyspnea and other signs are commonly associated with this form (Gleiser et al., 1963). Other lesions include enlargement of the spleen and shortly after death there occurs oozing of blood (with impaired clotting factors) from natural orifices (Gleiser et al., 1963, Hugh-Jones and de Vos, 2002).orifices (Gleiser et al., 1963, Hugh-Jones and de Vos, 2002). Gross pathologic lesions associated with the gastrointestinal type of this disease include haemorrhage, necrosis and inflammation of the small intestine (Nieberle and Cohrs, 1967).

2.4 Host immunity against *B. anthracis*

Development of antibodies following natural or experimental infection with any or a combination of the three proteins (PA, LF and EF) of the pXO1 component of *B. anthracis* has been demonstrated using enzyme-linked immunosorbent assay (ELISA) (Ivins and Welkos, 1988, Turnbull et al., 1992, Hanson et al., 2006, Lembo et al., 2011, Cizauskas et al., 2014). PA is the most crucial part of the components of the pX01 and strains of *B. anthracis* lacking the pX01 plasmid fail to confer immunity as they become avirulent to animals except for mice (Leppla et al., 2002). For the conferment of immunity to occur against anthrax, the host must be able to resist the establishment of disease or stall the progression (Mahlandt et al., 1966). To stall the progression of the disease it becomes important for the host to have specific antibodies against PA. The deleterious effect of *B. anthracis* and pathogenesis has been attributed to the ability of the PA to bind to both LF and EF and the subsequent uptake (translocation) into the host cell's internal environment (Leppla et al., 2002). PA is considered the most important immunogen in most anthrax vaccines (Ivins et al., 1994) and antibodies against PA have been shown to inhibit the germination of spores and enhance the activities of macrophages against the spores (Welkos et al., 2001). Anti-PA antibodies have been detected in several wildlife species following sublethal exposures to anthrax spores (Turnbull et al., 1992, Lembo et al., 2011, Cizauskas et al., 2014). Antibodies to EF and LF have been reported in some species of animals (Turnbull et al., 1992) but the extent to which the EF and/or LF confer immunity and how they provide protection is not fully understood (Taft and Weiss, 2008). Mahlandt et al. (1966) showed that both EF and LF have an augmenting effect on the protective ability of anti-PA antibodies in some laboratory animals. Vaccination with antigens containing regions for the binding of LF and PA have been

shown to yield better and broader protection as against using only PA (Baillie et al., 2010) and using EF as an adjuvant has been shown to have a potentiating effect on the activities anti-PA IgG response in mice immunised with PA (Quesnel-Hellmann et al., 2006).

The toxin neutralisation assay (TNA), a cell-based assay, is used to measure the capability of the anti-PA antibodies to neutralize the cytotoxic effect of LT and also the ET-induced rise in intracellular cAMP (Ngundi et al., 2010). The presence of neutralising antibodies against anthrax have been reported in vaccine studies (Hering et al., 2004, Taft and Weiss, 2008, Ngundi et al., 2010, Ndumnego et al., 2013) and also in natural exposure to anthrax (Dumas et al., 2020).

2.5 Anthrax distribution in Kruger and Etosha National Parks

With anthrax being endemic in the northernmost part of KNP in South Africa (Hugh-Jones and de Vos, 2002), the most susceptible host species has been greater kudu, which contributes up to 75% of recorded cases from 1960-1990s (de-Vos and Bryden, 1996). Outbreaks in KNP occur in almost decadal cycles and this has been associated with dry seasons or droughts (Pienaar, 1960, Pienaar, 1961, de-Vos, 1990, de-Vos and Bryden, 1996, Hugh-Jones and de Vos, 2002). Although, this trend seems to have shifted a bit with the impala, a mixed grazing-browsing herbivore being affected most during annual outbreaks and mainly in the wet season (Basson et al., 2018). Grazers which constitute of zebra and wildebeest are reported to contribute only a paltry 10% of anthrax related deaths in this park (personal communication with Skukuza State Veterinary Services). It has been suggested that browser exposures occur by means of these browsers browsing on leaves contimanted with *B. anthracis* spores deposited by blowflies

Diagnosis of anthrax in ENP was first reported in 1964, even though grey literature suggests the existence of this disease long before its first report (Ebedes, 1976a). Through nearly 50 years of investigation, cases in ENP exhibit marked seasonality. As stated earlier, the primary host in ENP is the plains zebra contributing about 52% of all the deaths in the Park (Turner et al., 2013). The death of the plains zebra and other herbivores in the central part of ENP peaks at the end of the wet season and African elephant deaths climax at the end of the dry season (Ebedes, 1976b, Lindeque and Turnbull, 1994, Beyer et al., 2012, Turner et al., 2013). Another grazer that contributes significantly to anthrax deaths is the blue wildebeest contributing about 22% of the total anthrax mortalities in the park (Turner et al., 2013). Browsers in this park such as kudu, impala and others account for only 1.7% of the deaths (Havarua et al., 2014). Contact between herbivores and *B. anthracis* spores in the environment is not unconnected to the fact that spores promote grass growth (Ganz et al., 2014, Turner et al., 2014) and nutrients from animal carcasses improve grass quality and productivity (Turner et al., 2014). These positive effects on vegetation attract grazing herbivores to infectious carcass sites 1-2 years after death, where they graze on

grasses polluted with spores of *B. anthracis* (Turner et al., 2014). Thereafter, host-pathogen contact is likely to be reduced, since attraction ceases and spore concentrations decay with time (Turner et al., 2014, Turner et al., 2016). Older carcass sites may serve as "vaccinator" sites, exposing grazing hosts to a low dose, sub-lethal infections , and may promote the development of host resistance.

In herbivorous wildlife, anthrax transmission from one infected host to a susceptible host occurs via the death of the infected host and release of long-lived *B. anthracis* spores into the environment and onward ingestion. It has been demonstrated how *B. anthracis* relies on various factors and vectors for its dissemination. The primary transmission mode in herbivorous wildlife and livestock is through ingestion of spore-contaminated vegetation (Turnbull, 2008, Turner et al., 2014, Turner et al., 2016). Anthrax can also be spread by the activities of scavengers mostly hyenas (*Crocuta crocuta*), wild dogs (*Lycaon pictus*), lions (*Panthera leo*), birds such as vultures (different species), ravens (*Corvus corax*) as well as other scavengers such as the wild cats (*Felis lybica*) (Pienaar, 1967, Ebedes, 1976a, Turnbull and Snoeyenbos, 1989, W.H.O., 2008). When an animal dies, the carcass is often opened by scavengers, this exposes the vegetative bacilli to the aerobic environment and encourage sporulation, thus making the spores domiciled in the soil available for subsequent ingestion and spread (Pienaar, 1967, Hugh-Jones and de Vos, 2002). Bellan et al. (2013) on the other hand showed that the vegetative cells were able to escape into the environment even in the absence of vertebrate scavengers. They reported no significant difference in the spore counts with or without scavengers. Other vectors such as flies have also been incriminated in the dissemination of anthrax especially in the browsing population of animals in the wild. A linkage has been established between the dissemination of anthrax and high concentrations of blowflies with anthrax outbreaks in southern Africa (Braack and De Vos, 1990). Blowflies feed on carcasses and deposit *B. anthracis* on close by leaves and shrubs on which they animals browse (Braack and De Vos, 1990, Basson et al., 2018). This blowfly infection pathway has been postulated to be the main means of transmission for wildlife species such as kudu, impala and other animals (Blackburn et al., 2010, Hampson et al., 2011, Basson et al., 2018). Various environmental factors also contribute to the transmission and propagation of anthrax.

2.6 Effects of environmental factors in the persistence of anthrax spores

There are several reports on the relationship between anthrax and some environmental players such as soil composition, temperature, humidity and pH. In a study conducted in the temperate, boreal, and arctic North, there was a close association between warming temperature changes and increasing anthrax suitability, as well as a relationship between increasing suitability and

water-soil balance that supported slightly to modest water stress (Walsh et al., 2018). Previous findings have shown a significant effect of seasonal difference on the occurrences of anthrax in different regions (Chikerema et al., 2012, Nsoh et al., 2016). According to Nsoh et al. (2016), most of the epidemics happen at the closing of the dry season and the beginning part of the rainy season which could force animals to feed closer to the ground and as such, increasing the likelihood of these animals acquiring anthrax. Outbreaks in regions of KNP appear to be influenced by rainfall and other water activities of the location (de-Vos and Bryden, 1996). In support of the above mentioned claim, this postulates that disease usually occurs during the end of winter and dry environmental situations are good for anthrax survival in KNP (Steenkamp et al., 2018). Although outbreaks have also been associated with floods which have been suggested to be due to the excavation of the spores (Lewerin et al., 2010). Studies have been conducted to illustrate how precipitation, temperature and soil type influence anthrax occurrences, and it was shown that annual precipitation of 100 mm and above and the temperature of $\geq 15.5^{\circ}$C support the presence of anthrax spores (Van Ness, 1971, Blackburn et al., 2007, Steenkamp et al., 2018). Soil pH greater than 6.1 has been demonstrated to be a key factor in determining the survival of the spores in any location (Van Ness and Stein, 1956, Nsoh et al., 2016).

2.7 Diagnosis and Identification of *Bacillus anthracis*

Most common confirmatory diagnoses of anthrax are usually by means of culture and post-mortem identification of lesions and signs that are classical to this disease (Bagamian et al., 2013). However, relying only on the mortality reports or the discovery of carcasses in the field often lead to a misrepresentation of the species affected and as well as the severity of exposure (Bellan et al., 2012). Surveillance of anthrax in wildlife species is very uncommon except in the face of an outbreak (Bagamian et al., 2013). Serology which operates on the principle of antigen binding to the specific antibody is rarely used in the antemortem diagnosis of pathogenic and progressive zoonotic diseases such as anthrax (Jacobson, 2007). Measuring antibodies to the protective antigen is the most reliable means of ascertaining previous exposure status to anthrax (Turnbull et al., 1992).

Previous studies have shown that wild animals, especially carnivores, are regularly exposed to anthrax with evidence of recovery (Turnbull and Snoeyenbos, 1989). Even though Pienaar (1967) described that lions can succumb to anthrax infection, this is infrequent. Very scarce information is available on the immunity to anthrax conferred by natural means and the degree to which herbivorous livestock and wildlife acquire and recover from anthrax infection. Herbivores have been reported to succumb more to anthrax and as such do not produce antibodies against it except for a few species such as wood bison (*Bison bison*) and the African buffalo that show medium to

a higher presence of antibodies to anthrax (Rijks, 1999, Lembo et al., 2011, Hugh-Jones and de Vos, 2002). Zebras showed a high level of susceptibility in both Serengeti and ENP (Turnbull and Snoeyenbos, 1989, Lembo et al., 2011). However, in another study conducted in ENP where they utilised enzyme-linked immunosorbent assay (ELISA) to measure the seroprevalence of anthrax, it was demonstrated that zebras, elephants, and springboks had antibodies to the protective antigen and zebras were able to seroconvert (Cizauskas et al., 2014). ELISA is the most widely used serological technique and owing to its versatility, it can be utilised in different ways.

2.7.1 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA, also known as enzyme immunoassay is a technique that depends on the use of plates specifically designed for the detection and quantification of antibodies as well as other substances (Voller et al., 1974). The enzyme-linked technique of detection of antigen-antibody (Ag-Ab) interaction was first brought to fore by Engvall and Perlmann as a replacement for the radioisotope technique of detection (Engvall and Perlmann, 1971). The ELISA for the recognition of the antibodies to *B. anthracis* was first modified and adopted by Anna Johnson-Winegar (Johnson-Winegar, 1984, Turnbull et al., 1992). First, an an antigen is restricted on a firm surface of a plate, followed by the addition of the sample antibody which then binds with a secondary antibody that is connected to an enzyme and this conjugated enzyme reaction is measured by incubating with a chromogene substrate (ThermoFisher Scientific 2019). Horseradish peroxidase (HRP) and alkaline phosphatase are the most commonly used enzymes conjugated with the secondary antibodies (Voller et al., 1974, Payment and Descoteaux, 1978, Rennard et al., 1980). ELISA in different forms has been used over the years since its advent and of the most common forms are the sandwich, direct, and indirect ELISA.

Sandwich ELISA is a less used type of ELISA but is very effective for the detection of antigens in a sample. This technique quantifies the antigen sandwiched between two layers of antibodies referred to as the capture and detection antibody (Claire, 2020). In brief, the technique involves binding the capture antibody on a solid surface; a flat bottom 96 or 12 wells microtitre plate. After washing the plate, the sample containing the antigen is added. This is followed by a wash step after which the secondary antibody (detection antibody) is added and allowed to bind. This is followed by the addition of a substrate and the colour change is measured. The advantage of this technique is that it provides fast and specific recognition of antigen concentration in a sample that is not known (Verma et al., 2013). The major setback of this technique is that the antigen to be quantified must possess a minimum of two antigenic epitopes able to bind to antibody, this is because a minimum of two antibodies are present in the sandwich (Verma et al., 2013).

The direct form of this assay against *B. anthracis* allows for the identification of the antigen (Ag) that is already fixed on the polystyrene plate using attachment of a conjugated specific antibody (Ab) (Van Weemen and Schuurs, 1971, Engvall and Perlmann, 1971, Crowther, 2000). The aforementioned interaction is then measured by the addition of a chromogen which produces a varying degree of chroma depending on the concentration of the Ag and Ab (Engvall, 2010). This form of ELISA is often regarded as the simplest and fastest of the three and it's highly resourceful when monoclonal antibodies are conjugated or Ag that are clearly defined are utilised (Crowther, 2000). This technique is though, not without limitations especially owing to the facts that it has low sensitivity, rigidity in its application and it is only efficient when the sample has a high concentration of the antigen (Aydin, 2015).

The Indirect form of this assay was established on the foundation of measuring the presence of specific antibodies by introducing a secondary antibody conjugated with an enzyme (Lindström and Wager, 1978). That is to say, the formed Ag-Ab complex is identified through the use of an enzyme-labelled secondary antibody (conjugate); thus, the term indirect ELISA. Following the initial immobilization of the antigen on the polystyrene surface, the primary antibody is then added to form the complex (Figure 2.3). This is then trailed by the addition of the conjugate. Subsequently, the substrate (chromophore) is then added to produce the needed colour change which correlates to the complexes formed (Figure 2.3). The chroma increases with higher concentrations of complexes and decreases with lower concentrations (Lindström and Wager, 1978, Crowther, 2000). This system of ELISA affords the comparative advantage that many antibodies can be examined using one non-species specific conjugate such as Protein A/G (PAG) which is chimeric construct or protein A (ProtA) and protein G (ProtG) (Eliasson et al., 1988, Crowther, 2000). A key problem with this system of ELISA is the problem of cross-reactivity owing to the varying extent of non-specificity in antigen binding (Crowther, 2000, Gan and Patel, 2013).

There is a paucity of information regarding the use of ELISA in wildlife herbivorous species, with few studies conducted at Serengeti in Tanzania and ENP in Namibia.(Turnbull and Snoeyenbos, 1989, Lembo et al., 2011, Cizauskas et al., 2014). This is probably as a result of the absence of conjugated secondary antibodies that are specific to each species and other difficulties associated with such studies. Kelly et al. (1993) measured the binding ability of antibodies to different commercial conjugates, the authors tested the binding activity of protA and a chimeric construct of PAG in 4 laboratory animals, 10 domestic and 16 wildlife species. It was shown that crocodile and birds (chicken, goose, ostrich) reacted weakly to both protA and PAG, while the impala, waterbuck (*Kobus ellipsiprymnus*), kudu, wildebeest, bushbuck (*Tragelaphus scriptus*)

and horse (*Equus caballus*) showed weak reactivity to protA and strong reactivity to PAG. But other studies revealed that impala reacted weakly to PAG (Stöbel et al., 2002, Smith, 2017) and also to protA and protG (Feir et al., 1993). The elephant, rhinoceros (*Diceros bicornis*), giraffe (*Giraffa spp*) and mouse (*Mus musculus*) showed moderate reactions to PA and strong reaction to PAG. The non-human primates as well as dogs, pigs and other laboratory animals showed strong reactivity to both conjugates. It was therefore concluded that the chimeric PAG has a wider spectrum of reactivity across a large number of wildlife animals (Kelly et al., 1993). It has been reported that kudu showed strong binding reactivity with PAG (Stöbel et al., 2002), this is in contrast with the work performed by Smith (2017) who reported that kudu did not react to PAG. Furthermore, zebra bound sturdily to protein A and G as well as PAG as described by previous studies ((Feir et al., 1993, Stöbel et al., 2002, Kramsky et al., 2003, Smith, 2017).

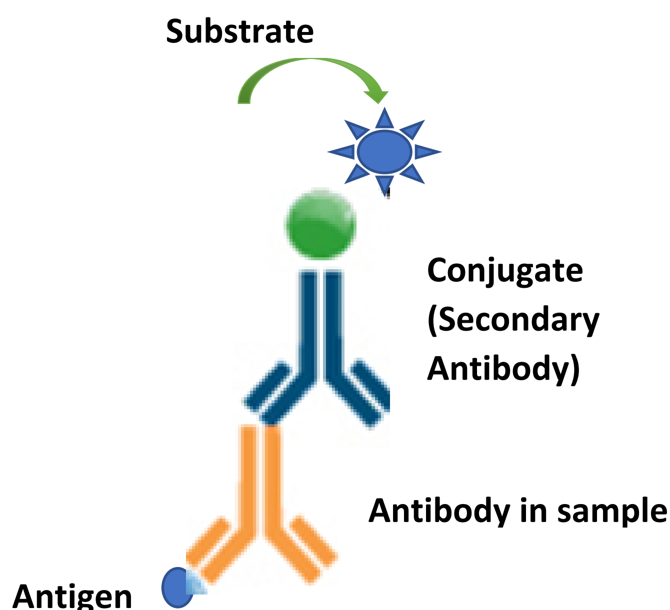

Figure 2-3 Image demonstrating the process of performing an indirect ELISA. Microtitre plate is coated with the antigen. Sample containing antibody is added and allowed to bind to the antigen. A secondary labelled antibody is added and allowed to bind. Then a substrate is added to allow for colour detection

2.7.2 Toxin Neutralisation Assay (TNA)

This assay is becoming increasingly important especially in *B. anthracis* for measuring the level of protection of the antibodies against the toxin(s). The toxin neutralisation assay (TNA) is designed for the measurement of neutralizing antibodies against epitopes on the protective antigen (PA) and the lethal factor (LF) (Hering et al., 2004). This assay is done *in vitro* to ascertain the capability of the antibodies specific to anthrax to protect mice macrophage cells (J774A.1) from the cytopathic effect of the anthrax lethal toxin (Figure 2.4). A colorimetric indicator such as 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) which

involves the conversion of the MTT into a dye indicating either growth or death of the cells has been commonly used for this assay (Hansen et al., 1989) (Figure 2.4). It has been shown in previous studies that lethal toxin is responsible for death in animals. The lethal toxin also possesses the ability to cause in vitro lysis of some cell lines, as was demonstrated in mice to measure monoclonal and polyclonal antibodies to neutralize the anthrax PA (Friedlander, 1986). Hering et al. (2004) have validated this assay in different forms. An assortment of the lethal toxin-sensitive cell lines have been utilized in TNA. Hanna et al. (1993) utilized the RAW264 cell line where they demonstrated the sensitivity and roles of macrophages in anthrax. The macrophage cell line J774A.1 has also been used for several studies owing to their sensitivity to the lethal toxin (Friedlander, 1986, Hering et al., 2004).

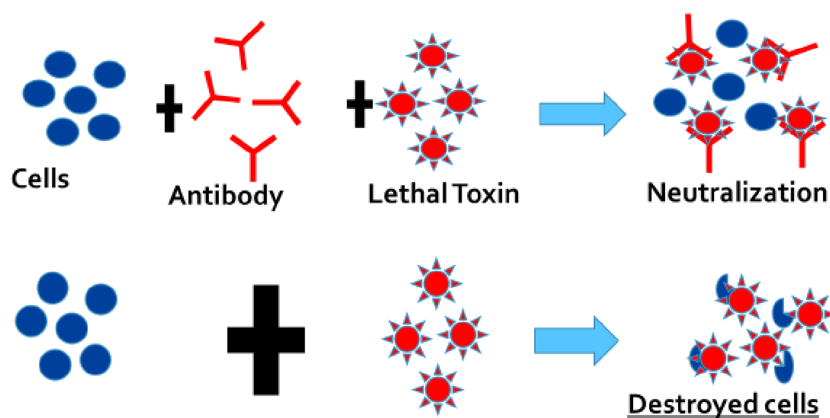

Figure 2-4 Describing the concept of the Toxin Neutralisation Assay: Previously incubated antibody and lethal toxin are added to already seeded cells and neutralisation or death of cells are measured utilizing a colorimetric agent which shows the presence of antibodies that neutralizes the lethal toxin is present in the cells.

2.8 Haemoparasites

Most humans and animals alike are often infected with more than one pathogen at a particular moment (Christensen et al., 1987, Lello and Hussell, 2008). These interplays could be in the form of protozoa-bacteria, virus-bacteria, helminth-protozoa, protozoa-protozoa interactions amidst other interactions. These interactions often modulate and/ or alter the immunity of the host and hence increasing susceptibility of the host to one or the other pathogen (Chen et al., 2005) or increase the severity of other pathogenic organisms (Borkow et al., 2001). Co-infection with tick-borne diseases (TBDs) has also been shown to exacerbate diseases and leading to higher fatality (Munson et al., 2008).

2.8.1 Tick-Borne Diseases (TBDs) in Wildlife

Tick-transmitted diseases pose serious threats to livestock farming and wildlife populations (Mbati et al., 2002, Nijhof et al., 2005). Although wild animals often serve as reservoirs and often present with no symptoms (Worthington and Bigalke, 2001), debilitating outbreaks do occur in the wildlife population due to factors such as naivety of a newly introduced member, the burden of the parasites and stresses coming from translocation (Nijhof et al., 2005).

Ticks are very important invertebrate organisms that transmit several pathogenic and non-pathogenic microorganisms (Parola and Raoult, 2001). They belong to the order *Acari*, which emanates from the class *Arachnida* and phylum *Arthropoda* (Walker et al., 2003). These organisms have damaging effects on their host as they feed on the blood of their hosts by attaching to their skins and through that vector various microparasites from an infected to a healthy host (Jongejan and Uilenberg, 2004). Ticks cause diseases in humans and also transmit various microorganisms such as Rickettsiales (*Anaplasma* and *Ehrlichia*), protozoans (*Theileria* and *Babesia*), viruses and other microorganisms (Jongejan and Uilenberg, 2004). Haemoparasites have a global distribution affecting both livestock and wildlife in different parts of the world and especially Africa (Carmichael and Hobday, 1975, Smith and Parker, 2010). *Theileria*, *Babesia*, *Anaplasma* and *Ehrlichia* are regarded as very important haemoparasites of livestock and wildlife in most African countries, resulting in wide financial losses and are of immense veterinary importance (Uilenberg, 1995, Anderson et al., 2013). These haemoparasites are transmitted by ticks and they often infect the red cells and white cells of their hosts (Dumler et al., 2001, Dumler et al., 2005) causing theileriosis, babesiosis, anaplasmosis and ehrlichiosis.

2.8.2 Theileriosis in African Wild Equids and Antelopes

Theileriosis caused by the protozoans of the genera *Theileria* is significant and capable of causing fatal infections in African antelopes and equids. However, some wild even-toed animals are considered as reservoirs of *Theileria* species that are not pathogenic and are often asymptomatic unless stressed (Nijhof et al., 2005, Pfitzer et al., 2011, Brothers et al., 2011), these animals can also suffer debilitating theileriosis with attendant effects on their populations (Oosthuizen et al., 2009, Steyl et al., 2012). Lethal infections have been described in some antelopes such as the kudu, sable (*Hippotragus niger*) and gray duiker (*Sylvicapra grimmia*) (Nijhof et al., 2005), eland (*Taurotragus oryx*) (Grootenhuis et al., 1979), roan (*Hippotragus equinus*) (Steyl et al., 2012) and wildebeest (Dondona et al., 2012). Theileriosis has also been reported in wild equids such as the zebra (Lampen et al., 2009). In a study done by Hawkins et al. (2015), they demonstrated that of the 87 blood samples tested, 72% (N=71) of donkeys (*Equus africanus asinus*) and 100% (N=16) of Grevy's zebras (*Equus grevyi*) tested positive for *T. equi*. Some of the pathogenic

Theileria spp. of the African antelopes include but are not limited *Theileria* sp. (sable) (Nijhof et al., 2005, Steyl et al., 2012), *Theileria* sp. (gray duiker) (Nijhof et al., 2005), and *Theileria* sp. (kudu) (Nijhof et al., 2005). *T. equi* is considered highly pathogenic in equids (Laus et al., 2015).

2.8.3 Babesiosis in African Equids and Wild Antelopes

Babesiosis is a very important disease in livestock, wildlife and humans which is caused by haemoprotozoans in the genus *Babesia* (Thomas et al., 1982, Bock et al., 2004, Gray, 2006). A wide number of antelopes and equids are susceptible to babesiosis. Equine babesiosis has been reported in horses (*Equus ferus*), zebra, donkey (*Equus africanus asinus*) and mules (*Equus asinus* × *Equus caballus*) (Bhoora, 2010, Rothschild, 2013, Onyiche et al., 2019). Babesiosis has also been reported from blood samples of these antelopes: kudu, impala, wildebeest, tsessebe (*Damaliscus lunatus*), eland (Carmichael and Hobday, 1975), roan (Oosthuizen et al., 2009) and sable (Oosthuizen et al., 2008) and red deer (*Cervus elaphus*) (Zintl et al., 2011).

2.8.4 Anaplasmosis in African Equids and Wild Antelopes

Anaplasmosis is a condition caused by the haemoparasite *Anaplasma*. Species in this genus are known to cause devastating disease in cattle leading to great economic loss with 99% of cattle in South Africa being at risk (de Waal, 2000). Anaplasmosis has a wide distribution, occurring in both the tropics and temperate regions of the world (Soulsby and Mönnig, 1982) and is especially common in the southern parts of Sub-Saharan Africa and is endemic in South Africa (de Waal, 2000).

Kuttler (1984) reviewed various serological evidence of antibodies to *Anaplasma*, from natural and experimental infections. So many reports have been made about the prevalence of anaplasmosis in various wildlife species. In a study conducted in Kenya, the blue wildebeest had a 96.5% prevalence (N=58), eland had a combined prevalence of 94.4% (N=18); plain zebra, 72.7% (N=11); impala, 100% (N=7); and Thomson's gazelle (*Eudorcas thomsonii*), 75% (N=8) (Ngeranwa et al., 2008). It has also been reported in black wildebeest (*Connochaetes gnou*) (Neitz, 1935b). Anasplasmosis is, therefore, an important disease in wildlife.

2.8.5 Ehrlichiosis (Heartwater) in African Wild Equids and Antelopes

Ehrlichiosis is a rickettsial haemoparasite that affects livestock (Van Winkelhoff and Uilenberg, 1981), wildlife and humans (Groen et al., 2002). This disease is instigated by the rickettsia of the genus *Ehrlichia* (Dumler et al., 2001) and is extensively distributed across the world and noteworthily endemic in Africa (Provost and Bezuidenhout, 1987). Heartwater has been reported in various African wildlife species.

King'ori et al. (2019) reported a prevalence of 5.8% (N=66) in elephants in Kenya. Very little information is available about natural infections with *E. ruminantium* in African wildlife. Susceptibility of African and non-African wildlife has been described in several species that were infected experimentally. Some of which include white tailed-deer (*Odocoileus virginianus*) in the United States of America (Dardiri et al., 1987), blesbuck and black wildebeest (Neitz, 1935a), kudu, blue wildebeest, eland and giraffe (*Giraffa camelopardalis*) (Peter et al., 1998), impala, tsessebee and sable (Peter et al., 1999) and donkeys (Halajian et al., 2018).. *E. ruminantium* has also been reported in apparently healthy wildlife. Kock et al. (1995) reported a prevalence of 60% (N=28) in tsessebee, 33% (N = 3) in impala from bone marrow samples in Zimbabwe. Prevalence of 29% (N=65) and 75.9% (N=58) were reported in black and white rhinoceros respectively from parks in Zimbabwe (Kock et al., 1992).

2.9 Diagnosis and Identification of Haemoparasites

Several methods have been employed in the diagnosis of haemoparasites in blood samples of susceptible animals. These techniques involve procedures that detect and identify the parasites in blood films on slides (microscopy), detection of antibodies specific to these parasites (serology), and identification of the DNA component of these pathogens (Real-time PCR, Reverse Line Blot Hybridization).

2.9.1 Microscopy

This technique remains the most widely and potent means for the identification of the morphometry of haemoparasites. The identification of a dense body at the centre or margin of an erythrocyte indicates the presence of *A. centrale* and *A. marginale* respectively (Kocan et al., 2004). The *Babesia* parasite present intraerythrocytic presence as a pair of the parasite centrally located at a smaller angle between $>90^{o}$ and $<180^{o}$ (Bock et al., 2004). The *Ehrlichia* species appear as clumps of organisms close to the nucleus of the cytoplasm of the host cells (Sreekumar et al., 1996). The microscopic identification of *Theileria* infection though widely used for the rapid diagnosis, it is marred by the diffulty associated with distinguishing the schizonts and piroplasms of the numerous *Theileria* species (Norval et al., 1992)

2.9.2 Serology

The frequently employed serological techniques used for the detection of antibodies which indicate past of present infections include the ELISA, and the indirect fluorescent antibody test (IFAT). ELISA and IFAT have been used for the recognition of the *Babesia* species (de Echaide et al., 1995). Also, various other serological techniques have been utilised for the recognition of other haemoparasites (*Theileria, Babesia, Ehrlichia* and *Anaplasma*).

2.9.3 Nucleic Acid detection

Different techniques like the conventional PCR (Gale et al., 1996), reverse line blot hybridisation and also the quantitative PCR (Bhoora, 2010, Chaisi et al., 2017) have been used for the detection of the DNA of both ricketsiales and protozoans from the blood samples of infected animals.

2.9.3.1 Reverse Line Blot Hybridization

This technique involves the use of a membrane fixed with genera and/or species-specific oligonucleotide probes for the identification of single or multiple genera and/or species. It employs a combination of the conventional PCR and the hybridisation of the amplified PCR products. This technique has been modified over the years.

Chapter 3
MATERIALS AND METHODS

3.1 Study Area and Animals

3.1.1 Study Area

This study compared anthrax dynamics in two large, flagship national parks located nearly 2,000 km apart in southern Africa. Etosha National Park (ENP, 22,915 km^2), Namibia and Kruger National Park (KNP, 19,485 km^2), South Africa. Within our study areas, the ecosystems of these parks are classified as arid savannas, with annual rainfall less than 650 mm (Huntley, 1982). ENP is largely flat with some mountains in the far eastern and western part of the park (Figure 3.2). KNP on the other hand has varying elevations with pafuri (the endemic region) having lower elevation surrounded by higher elevations (Figure 3.3).

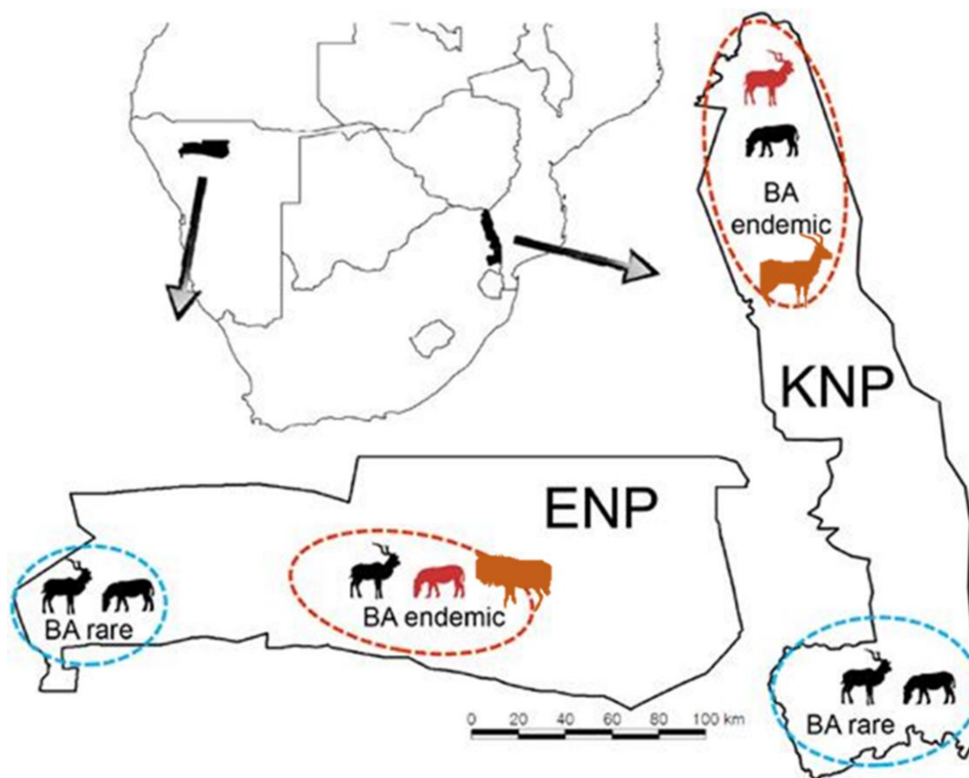

Figure 3-1 Etosha National Park (ENP) and Kruger National Park (KNP) in southern Africa, showing study sites where anthrax outbreaks occur endemically or rarely. Both study species (kudu and zebra) occur in all four sites (depicted with animal figures) and the secondary host species that is Impala (occurring in the northern part of KNP) and Wildebeest (occurring in the central part of ENP). The most common anthrax host species in each endemic region is shown in red and the secondary host species are depicted in orange.

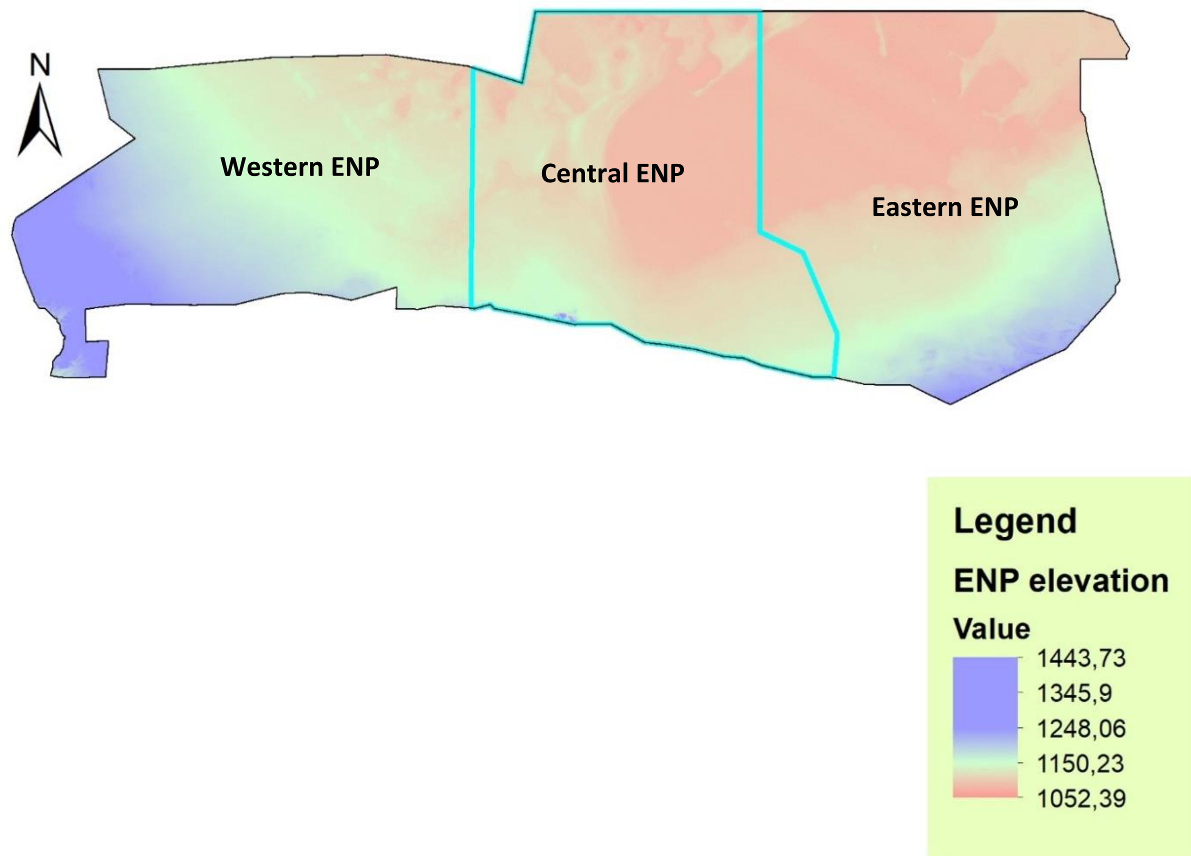

Figure 3-2 Map of Etosha National Park, Namibia showing the elevation level of the three sections of the park.

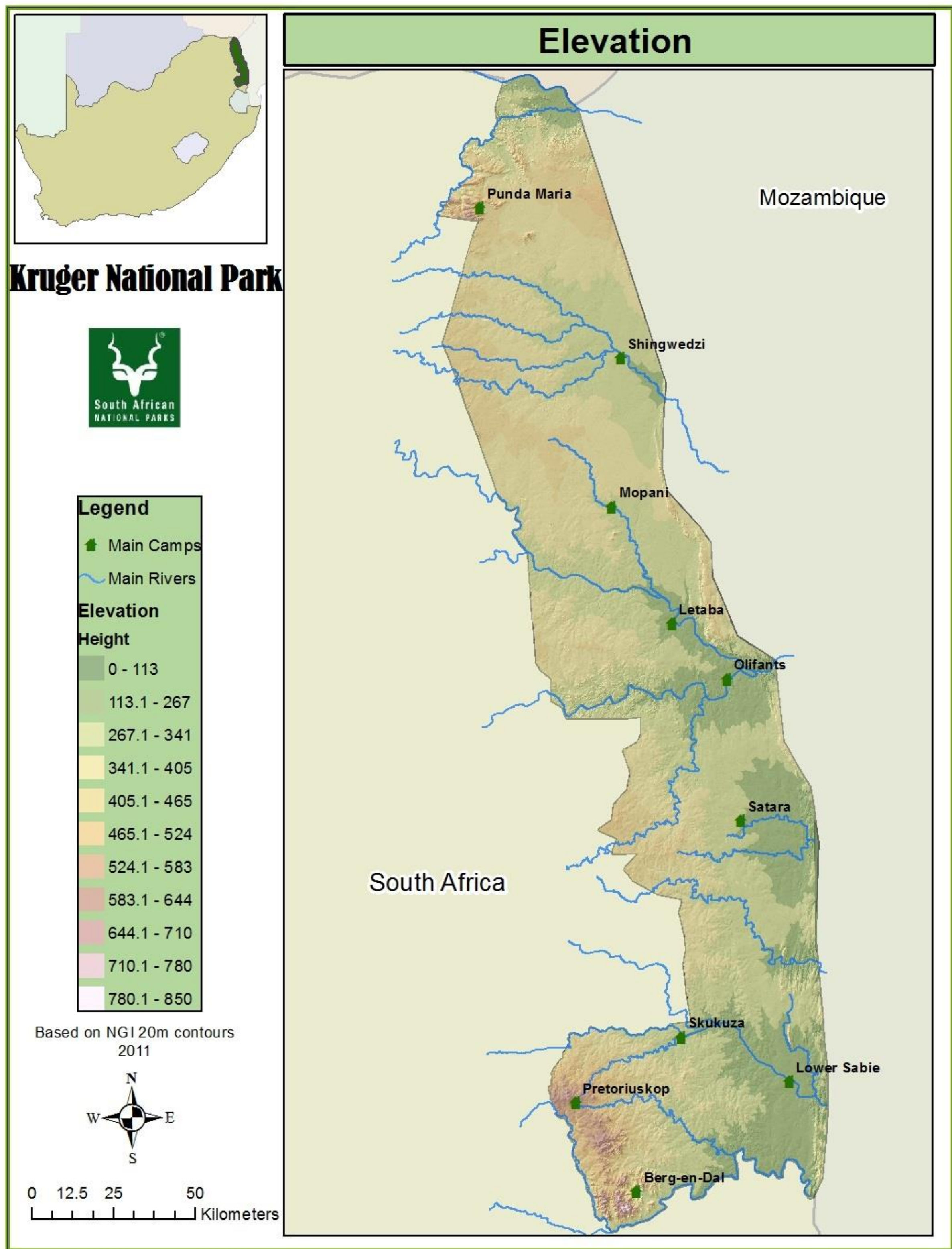

Figure 3-3 Map of Kruger National Park, South Africa showing the elevation level of each section and loality of .the park. Adopted from. https://www.sanparks.org/assets/images/conservation/scientific_new/savanna_arid/knp-elevation.jpg

3.1.2 Selection of Host Study Species

Over noted history, plains zebra and greater kudu have been the primary host species in ENP and KNP, respectively (de-Vos, 1990, Turner et al., 2013, Havarua et al., 2014). Kudu has suffered

tremendous selective pressure from anthrax, with some outbreaks resulting in nearly 100% mortality (Clegg et al., 2007). These two species make a perfect model for comparison as both species are present in both systems and a primary anthrax host species in one park is rare in the other. Previous studies (Lembo et al., 2011, Cizauskas et al., 2014), give us more insight into differences in transmission and host resistance to determine if the differences in cases noted between species and areas are due to lack of exposure or higher resistance. The grazing versus browsing transmission pathways occurs at different timescale, which may have important effects on disease dynamics, pathogen diversity, and host resistance. The browsing-based transmission should occur shortly after host death before rainfall or leaf loss by deciduous trees/shrubs prevent further browser exposure. Grazing-based transmission occurs only upon regeneration of vegetation at a carcass site, and continues for years, with exposure dose decaying over time. These two time scales could indicate that zebra is more likely to be exposed to sub-lethal doses of *B. anthracis* spores than kudu, a hypothesis we will explore in this study. In addition to zebra and kudu, we studied a secondary host species in the endemic area of each park, i.e., blue wildebeest in central ENP, and impala in northern KNP. These species have not contributed as many cases over time as the main host species, but are critical toward understanding the ecology of this multi-host disease.

3.1.3 Study animal and sample collection

Samples for serological and molecular analyses were obtained from live animal captures. Animals were captured and blood was collected from zebra (N=20) and kudu (N=19) in northern KNP (from Pafuri down to Shingwedzi) and non-endemic southern KNP(from Skukuza to Crocodile bridge) (zebra N=20, kudu N=18) regions, and also from Kudu and Zebra in the central region (Endemic) and western part (nonendemic) of ENP (N = 20/species/region = 80 individuals), from impala (in northern KNP N=20) and wildebeest (eastern ENP = 20). DNA from the 196 blood samples (from kudu, zebra, impala and wildebeest) from both KNP and ENP was extracted using the Invitrogen Purelink® Genomic DNA mini kit (Invitrogen, USA) as prescribed by the manufacturer. The extracted samples were eluted in 100 µL of the provided elution buffer and stored in the freezer until utilisation.

All ethical approvals were obtained from the University of Pretoria Research Ethics Committee, Animal Ethics Committee and the Department of Agriculture, Forestry and Fisheries (DAFF) in South Africa. Animals were immobilized following the "standard operating procedures (section 2.1.11) for the capture, transportation and maintenance in holding facilities of wildlife" by certified veterinarians and SANpark regulations. The sampling consisted of blood collection through venipuncture (4 ml EDTA and 10 ml serum vacutainer tubes from each animal). Samples

were pre-processed at the laboratories of Skukuza State Veterinary Services (SSVS) and the Etosha Ecological Institute (EEI) and stored at SANparks Wildlife centre in KNP and EEI and the Central Veterinary Laboratory (CVL) in Namibia. Transport permits were obtained from the Department of Agriculture, Forestry and Fisheries (DAFF) in South Africa and Directorate of Veterinary Services, Namibia. ELISA and TNA tests were performed at the Department of Veterinary Tropical Diseases (DVTD) after inactivation as directed in section 20 approval. The inactivation was done at a temperature of 56± 2° for 20± 2 minutes. using the Accu block™ digital Dry Bath (Labnet International, Inc, USA)

DNA extraction was performed at SSVS and EEI laboratories from EDTA blood samples of each sampled animal.

Two representative animals of each species (kudu, impala, zebra and wildebeest), were captured from a non-anthrax region in South Africa, collared and vaccinated with the Sterne Live Spore vaccine (Onderstepoort Biological Products, South Africa). Blood samples were collected from the animals before vaccination and that served as the negative control. Each animal was vaccinated with 1 ml of Sterne spore vaccine intramuscularly as prescribed by the manufacturer. These animals were then recaptured after a month and serum samples were collected which served as the positive control.

3.2 Immunological methods

3.2.1 Protective Antigen (PA) Enzyme-Linked Immunosorbent Assay

The sera from all the animals from both parks were assessed for the presence of specific antibodies against the anthrax PA as described by (Yu et al., 2002, Ndumnego et al., 2013, Cizauskas et al., 2014), although, some little alterations were made to suit the protocol. Microtitre plates containing 96 wells (Thermo Scientific™ Pierce 96-well Plates-Corner, USA) were encrusted with 0.5 µg of antigen (PA) (List Biological Laboratories Inc., USA) in the coating buffer (bicarbonate buffer) per well and left overnight at 4°C to incubate. The microtitre plates coated with the antigen were washed 2 times with wash buffer (PBS + 0.05% Tween (hereafter will be called PBST)) with Biorad PW40 washer (Mamesla-Coquette, France). After which the coated plates were blocked with the blocking buffer (200 µL) containing PBST and 5% skimmed milk powder (PBSTM) and then incubated for 1 hour at room temperature. Subsequently, the plates were washed twice followed by adding the test sera, positive, negative controls and dilution buffer containing PBSTM to each well. These sera were put in duplicates in a dilution of 1:40 across the microtitre plates and incubate for 30 minutes on a rotatory incubator (Environmental Shaker-Incubator ES-20, Biosan Ltd, Germany). Afterwards, the plates were washed 5 times and 100 µL of recombinant protein A/G (for zebra and wildebeest) and protein

G (for impala and kudu) (Pierce® Protein A/G, USA and Invitrogen Protein G, USA) both conjugated to horseradish peroxidase using the dilution of 1:10000 in PBSTM was added to each well and allowed to incubate for 30 minutes on the shaker-incubator. The plates were washed 5 more times, after which the ABTS substrate (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) (Thermo Scientific 1-Step ABTS, USA) was added and incubated in the dark for 45 minutes, and the absorbance was read at 405 nm using the Biotek powerwave XS2 reader (USA). The outcome of the ELISA was determined using the OD values of the mean plus 3 times the standard deviation of known negative samples of the representative species captured from the non-anthrax region in South Africa vaccinated with the Sterne Live Spore vaccine (Onderstepoort Biological Products, South Africa).

3.2.2 Toxin neutralisation assay (TNA).

The TNA was conducted *in vitro* using J774A.1 mouse macrophage cell line (ECACC cat no 91051511) with modifications as described by (Hering et al., 2004, Ndumnego et al., 2018). 100,000 cells (counted using the Neubauer counting chamber) in the media (Dulbecco's modified eagle media and 10% foetal bovine serum) were seeded per well in 96-well cell culture plates having a flat bottom (Corning ™ 96-well clear TC-treated MIC, Corning incorporated, Germany) and incubated at 37°C and 5% CO_2 for 24 hours. The test sera in duplicates were diluted two-fold with a start dilution of 1:50 in the media containing the PA 500 ng/mL and the lethal factor (LF) 400 ng/mL (List Biological Laboratories Inc., USA). The mixture was then incubated for 1 hour at 37°C and transferred it to the previously seeded cells and incubated for 3 hours. In addition to the test sera that were added in duplicates, each plate had 3 wells left blank without cells, 3 wells for the toxin control and 2 wells for medium control. Each batch also contained a single dilution for the positive controls for each animal species followed by adding 25 μL of 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Invitrogen, USA) into every well and incubated in a CO_2 incubator at 37°C for 2 hours. Subsequently, a mixture of 90% isopropyl alcohol, 0.5% SDS, 25 mM HCl was added to all the wells to lyse the cells by gently pipetting for several times to further mix the dye followed by 5 minutes incubation at room temperature. The plates were read at an absorbance of 570 nm (Biotek power wave XS2 reader, USA). Afterwards, the neutralisation was calculated as:

$$NT = \frac{Sample - Toxin\ control}{(Medium\ control - Toxin\ control} X\ 100$$

The neutralisation titres (NT50) were gotten using the Gen5 analysis software (Biotek Instruments, USA). These were presented as the reciprocal value of the highest dilution of the serum at which the antibodies protected 50% of the macrophages.

3.3 Statistical analyses

3.3.1 Poisson regression analysis of anti-PA titres between KNP and ENP, areas and interaction between park and area

Poisson regression analysis was performed to determine if there was a significant difference in anti-PA antibodies titres (ODS determined by ELISA) between national parks (KNP, ENP) and areas (endemic Vs non-endemic), and an interaction between national park and area. The analysis was done separately for kudu and zebra. Data from wildebeest and impala were not included in the analysis because these were sampled only from endemic areas in ENP and KNP, respectively, but the average anti-*B. anthracis* antibody titres for each species were determined.

3.3.2 Multivariable logistic regression of the association of anthrax serological status

Logistic regression analysis was performed to determine the association between the presence of anti-PA antibodies in animals (as determined using ELISA) and the potential risk factors; national park, area, and interaction between national park and area. The analysis was done separately for kudu and zebra, and the serological status was considered as a binary outcome (positive or negative). Given that wildebeest and impala were sampled only from endemic area in Etosha National Park and Kruger National Park respectively, these were not included in the analysis, but their respective proportion of exposure to infection was determined. The logistic regression included a stepwise elimination procedure to obtain the most adequate model as determined by the Akaike Information Criteria (AIC). The Hosmer-Lemeshow (χ^2) was used as a measure of the goodness-of-fit test.

A comparison was also done to determine the difference in exposure to *B. anthracis* in wild animals between the endemic area and non-endemic area within each national park, separately for the host species (kudu, zebra), using the chi-square test.

A comparison was made between Steenkamp et al. (2018) B. anthracis suitability map and the map generated from the anti-PA ELISA titres.

3.3.2 Multivariable logistic regression analysis of the association of the proportion of animals that neutralized the anthrax lethal toxin

Logistic regression analysis was conducted to determine significant predictors for the frequency of *B. anthracis* toxin neutralisation (determined by toxin neutralisation assay) in wild animal populations in Etosha and Kruger National Parks. The variables considered were: area (endemic, non-endemic), national park (Kruger, Etosha) and host species (kudu, zebra), the interaction between park and area, the interaction between park and host species, the interaction between host species and area, and interaction between park, area and host species. The serological status was considered as a binary outcome (positive or negative). The logistic regression included a

stepwise elimination procedure to obtain the most adequate model as determined by the Akaike Information Criteria (AIC). The Hosmer-Lemeshow (χ^2) was used as a measure of the goodness-of-fit test. Given that Wildebeest and Impala were sampled only from endemic area in Etosha National Park and Kruger National Park respectively, these were not included in the analysis, but the respective proportion of exposure to infection was determined.
We further compared the frequency of *B. anthracis* toxin neutralisation in wild animals between endemic area and non-endemic area within each national park, separately for the host species (kudu, zebra), using the chi-square test.

3.3.5 Analysis of the distribution of the *Bacillus anthracis* neutralisation titres (NT) in the wildlife species

Measures of variation (mean, 1st and 3rd quartiles, maximum) were used to determine the distribution of the TNA titres in kudu, zebra, impala and wildebeest in endemic and non-endemic areas in KNP and ENP. A negative binomial regression analysis was performed to test for significant differences in anti-*B. anthracis* toxin neutralisation mean scores between national parks (KNP, ENP) and area (endemic Vs non-endemic), host species (Kudu, Zebra), and the interactions: national park*area, national park*host species, host species*area, national park*area*host species. Data from Wildebeest and Impala were not included in the analysis because these were sampled only from endemic areas in ENP and KNP, respectively, but the average anti-*B. anthracis* TN titres for each species were determined. The independence samples t-test was then employed to determine the statistical significance of differences in titres between national parks, or between endemic status (endemic, non-endemic), in each animal species. Given that wildebeest and impala were sampled only from endemic areas in ENP and KNP respectively, their TNA titres were not included in the inferential analysis.

3.3.6 Correlation between ELISA and TNA titres and their binary outcome

The extent of the agreement was determined using Cohen's kappa (*k*) test (Landis and Koch, 1977) between the binary outcome of anti-PA ELISA and TNA results for anthrax in wildlife (kudu=77, zebra=80, wildebeest= 20 and Impala=20) for the hypothesis, kappa $\neq 0$, means that the agreement between anti-PA ELISA and TNA is different from chance agreement. The strength of agreement was assessed based on the criterion by Landis and Koch (1977), where <0=poor; 0.01-0.20=slight; 0.21-0.40=fair; 0.41-0.60=moderate; 0.61-0.80=substantial; 0.81-1.00=almost perfect. The data were analysed using R Console version 3.2.1 (R Core Team, 2017) at a 5% significance level.

The Pearson correlation (r) test was used to determine the linear dependence between ELISA titres and TNA values. The Spearman's rank correlation was also used, after log transformation, given that the data were not normally distributed. The Cohen's standard (Cohen, 2003) was used to evaluate the strength of the relationship, where 0.10 to 0.29=small association, 0.30 to 0.49 =medium association and ≥0.50=large association.

3.4 Reverse Line Blot hybridisation assay (RLB)

The RLB hybridisation assay was used to detect *Anaplasma, Ehrlichia, Babesia* and *Theileria* species in DNA extracted from EDTA-preserved blood samples as previously described by (Bekker et al., 2002, Nijhof et al., 2005). *Theileria/Babesia* (T/B) specific forward primers RLB F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') (Whitehead Scientific (Pty) Ltd, South Africa) and biotin-conjugated reverse primers RLB-R2 (5'-biotin-CTA AGA ATT TCA CCT CTG ACA GT-3') (Whitehead Scientific (Pty) Ltd, South Africa) were used for the amplification of the V4 portion of the pathogens and the amplification of the V1 portion, the *Anaplasma/Ehrlichia*-specific primers; the EHR F (5'-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3') (Whitehead Scientific (Pty) Ltd, South Africa) forward primers and the reverse primers EHR R (5'-biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3') (Whitehead Scientific (Pty) Ltd, South Africa) were utilised (Bekker et al., 2002).

An RLB membrane (Biodyne C, Separation Scientific, Johannesburg, South Africa) was made to fit the mini blotter (Immunetics, UK). The membrane was activated for 10 minutes using a 16% solution of 1-ethyl-3-(-3-dimethylamininopropyl) carbodiimide (Separation Scientific, South Africa) and then rinsed. After placing the membrane in the mini blotter, the Oligonucleotide probes were bound to the membrane by diluting each probe to a concentration of 2 pmol/µL with a solution of 500mM NaHCO (Sigma Aldrich Pty, South Africa) at pH 8.4 and 0.2 L was added to the membrane already placed in the mini blotter and incubated for 1 minute. The specific *Theileria/Babesia* (T/B) and *Ehrlichia/Anaplasma* (E/A) genus and species probes are shown in Table 3.1. After a 2-minute incubation, inactivation of the membrane was done for 8 minutes using a solution 0.1 M NaOH (Sigma Aldrich Pty, South Africa) that was newly made. The membrane was then washed at 60°C for 5 minutes with a 100 mL solution of 2X SSPE (Thermo Scientific™, South Africa) and 0.1%SDS (Sigma Aldrich Pty, South Africa).

A biodyne® C membrane was made by cutting a piece of the membrane according to the size of the mini blotter apparatus. This membrane was then activated in 10 mL newly made 16% EDAC (1-ethyl-3-(-3-dimethylamininopropyl) carbodiimide) (DNA Thunder™, Separation Scientific, South Africa) for 10 minutes. 0.5 M NaHCO (Sigma, South Africa) was added to Individual probe with a pH 8.4 to make a final concentration of 2 pmol/µL and 200 µL was loaded onto the

membrane using a mini blotter apparatus (Immunetics, UK). The genera and species-specific probes included on the membrane are shown in Table 3.1. *Babesia bovis* and *Anaplasma centrale* species-specific probes were included in separate wells to serve as positive controls for troubleshooting purposes. The membrane was inactivated at room temperature for 8 minutes with 0.1M recently prepared NaOH (Sigma, South Africa) on a shaker. The inactivated membrane was then washed in 100 mL 2X SSPE (ThermoFisher Scientific, South Africa)/0.1% SDS (Sigma, South Africa) at 60°C for 5 minutes.

Table 3-1 Reference list Oligonucleotide probes fixed on the Biodyne C membrane for the detection of rictkettsiales and protozoan DNA.

Genus/Species-specific probes	Sequences of probes (5′ to 3′)	Reference
Anaplasma bovis	GTA GCT TGC TAT GRG AAC A	(Bekker et al., 2002)
Anaplasma platys	CGG ATT TTT GTC GTA GCT TGC TAT GAT	(Sirigireddy and Ganta, 2005)
Anaplasma centrale	TCG AAC GGA CCA TAC GC	(Georges et al., 2001)
Anaplasma marginale	GAC CGT ATA CGC AGC TTG	(Bekker et al., 2002)
Anaplasma sp. Omatjenne	CGG ATT TTT ATC ATA GCT TGC	(Bekker et al., 2002)
Anaplasma phagocytophilum	TTG CTA TAA AGA ATA ATT AGT GG	(Schouls et al., 1999)
Babesia genus-specific probe 1	ATT AGA GTG TTT CAA GCA GAC	Nijhof (unpublished)
Babesia genus-specific probe 2	ACT AGA GTG TTT CAA ACA GGC	Nijhof (unpublished)
Babesia bicornis	TTG GTA AAT CGC CTT GGT C	
Babesia bigemina	GTA GTT GTA TTT CAG CCT CG	(Stoltsz et al., 2020)
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG	(Gubbels et al., 1999)
Babesia caballi	GTG TTT ATC GCA GAC TTT TGT	(Butler et al., 2008)
Babesia canis	TGC GTT GAC CGT TTG AC	(Matjila et al., 2004)
Babesia divergens	ACT RAT GTC GAG ATT GCA C	
Babesia felis	TTA TGC GTT TTC CGA CTG GC	(Bosman et al., 2007)
Babesia gibsoni	CAT CCC TCT GGT TAA TTT G	(Bosman et al., 2007)
Babesia leo	ATC TTG CTT GCA GCT T	(Bosman et al., 2007)
Babesia lengau	CTC CTG ATA GCA TTC	(Bosman et al., 2007)
Babesia microti	GRC TTG GCA TCW TCT GGA	
Babesia occultans	CCT CTT TTG GCC CAT CTC GTC	(He et al., 2011)
Babesia rossi	CGG TTT GTT GCC TTT GTG	(Matjila et al., 2004)
*Babesia sp. (*sable*)*	GCT GCA TTG CCT TTT CTC C	(Oosthuizen et al., 2008)
Babesia vogeli	AGC GTG TTC GAG TTT GCC	(Matjila et al., 2004)
Ehrlichia/Anaplasma group-specific probe	GGG GGA AAG ATT TAT CGC TA	(Bekker et al., 2002)
Ehrlichia canis	TCT GGC TAT AGG AAA TTG TTA	(Schouls et al., 1999)
Ehrlichia chaffeensis	ACC TTT TGG TTA TAA ATA ATT GTT	(Schouls et al., 1999)
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG	(Bekker et al., 2002)
Theileria/Babesia group-specific probe	TAA TGG TTA ATA GGA RCR GTT G	(Gubbels et al., 1999)
Theileria genus-specific probe	ATT AGA GTG CTC AAA GCA GGC	Nijhof (unpublished)
Theileria annae	CCG AAC GTA ATT TTA TTG ATT TG	(Matjila et al., 2008)
Theileria bicornis	GCG TTG TGG CTT TTT TCT G	
Theileria buffeli	GGC TTA TTT CGG WTT GAT TTT	(Gubbels et al., 1999)
*Theileria sp. (*buffalo*)*	CAG ACG GAG TTT ACT TTG T	(Oura et al., 2004)
Theileria equi	TTC GTT GAC TGC GYT TGG	(Butler et al., 2008)
*Theileria sp. (*kudu*)*	CTG CAT TGT TTC TTT CCT TTG	(Nijhof et al., 2005)
Theileria lestoquardi	CTT GTG TCC CTC CGG G	(Nagore et al., 2004)
Theileria mutans	CTT GCG TCT CCG AAT GTT	(Gubbels et al., 1999)
Theileria ovis	TTG CTT TTG CTC CTT TAC GAG	(Bekker et al., 2002)
Theileria parva	GGA CGG AGT TCG CTT TG	
*Theileria sp. (*sable*)*	GCT GCA TTG CCT TTT CTC C	(Nijhof et al., 2005)
Theileria separata	GGT CGT GGT TTT CCT CGT	(Schnittger et al., 2004)
Theileria taurotragi	TCT TGG CAC GTG GCT TTT	(Gubbels et al., 1999)
Theileria velifera	CCT ATT CTC CTT TAC GAG T	(Gubbels et al., 1999)

Degenerate positions R signifies A/G while W signifies A/T

3.4.1 PCR and Hybridisation of PCR products on membrane

A final volume of 25 µL for a single reaction was made containing 0.5X Platinum Quantitative PCR SuperMix-UDG, 8pmol of each forward and reverse primer of T/B and E/A, 9.5µl of molecular grade water and 2.5 µL of the DNA sample. Two separate master mixes were made for T/B and E/A species. A Thermocycler (Gene Amp®, PCR System 9700, Life Technologies™, South Africa) was utilised using the template in Table 3.2.

Table 3-2 Thermocycling schedule for Babesia/Theileria and Ehrlichia/Anaplasma touchdown PCR

Number of cycles	Time	Temperature
1 cycle	3 minutes	37ºC
1 cycle	10 minutes	94ºC
	20 seconds	94ºC
2 cycles	30 seconds	67ºC
	30 seconds	72ºC
	20 seconds	94ºC
2 cycles	30 seconds	65ºC
	30 seconds	72ºC
	20 seconds	94ºC
2 cycles	30 seconds	63ºC
	30 seconds	72ºC
	20 seconds	94ºC
2 cycles	30 seconds	61ºC
	30 seconds	72ºC
	20 seconds	94ºC
2 cycles	30 seconds	59ºC
	30 seconds	72ºC
	20 seconds	94ºC
40 cycles	30 seconds	57ºC
	30 seconds	72ºC
1 cycle	7 minutes	72ºC
		4ºC

The hybridisation of the membrane and PCR products was achieved as done by (Nijhof et al., 2005). The membrane fixed with T/B and E/A specific probes were first activated for 5 minutes at 25ºC under gentle shaking in 2× SSPE/0.1% SDS.

The PCR products were diluted by the addition of 1.7× SSPE/0.08% SDS to the PCR reaction and mixture were denatured for 10 minutes in the thermocycler and immediately put to cool on ice. The activated membrane was put in the mini blotter with the slots vertical to the line design of the probes applied. The slots were filled with the diluted PCR products according to the sample list and the slots without PCR products were filled with 2X SSPE/0.1% SDS to prevent the flow of products from one lane to the next and the membrane was hybridised at 42ºC for 60 minutes on a level surface. The membrane was washed 2 times with already heated 2X SSPE/0.5% SDS at an interval of 10 minutes at 50°C between each wash. The membrane was then incubated with 1.9× SSPE/0.49% SDS (pre-heated) and 0,0016 U streptavidin-POD (peroxidase labelled) conjugate (1.25 U) for 30 minutes at 42°C under gentle shaking inside the incubator. The membrane was then washed twice in preheated 2× SSPE/0.5% SDS for 10 minutes at 42ºC

incubator under gentle shaking. The membrane was washed 2 times with 2× SSPE for 5 minutes at room temperature under gentle shaking. Thereafter, the membrane was incubated for 10 minutes with 10 mL chemiluminescence detector (ECL) (5 mL ECL1 + 5 mL ECL2) under gentle shaking for 1 minute at room temperature. The membrane was exposed to an X-ray film (Kodak X-OMAT™ Blue XB-1, Separation Scientific, South Africa) and positive reactions were evident with black spots on the film. The membrane was then stripped for future use using 1% SDS heated for 30 minutes at 80ºC, after which it was washed for 15 minutes using 20 mM EDTA with a pH8 at room temperature.

3.4.2 Haemoparasite analysis and Correlation between Parasite burden and the Neutralisation Titre 50 (NT50)

First, a descriptive analysis was done to determine the prevalence and proportions of animals that tested positive for any of the haemoparasites. The student t-test was conducted to determine the statistical difference between groups. The Pearson correlation (r) test was used to determine the linear dependence between parasite diversity (total number of positive parasite genera) and NT50 values. The Spearman's rank correlation was also used, after log transformation of the NT50, given that the data were not normally distributed.

Multivariable logistic regression analysis was conducted to determine the association between tick-borne haemoparasite infection and potential risk factors; national park, area, toxin neutralisation, and the interaction between national park and area. The analysis was done separately for each of the host species (zebra, kudu) and each parasite genus (Theileria, Babesia, Ehrlichia, Anaplasma). The RLB status of haemoparasites was considered as a binary outcome (positive or negative). A stepwise elimination procedure was followed, to obtain the most adequate model for each analysis, as determined by the least Akaike Information Criteria (AIC). The Hosmer-Lemeshow ($\chi 2$) was used as a measure of the goodness-of-fit test.

The Pearson Chi-square test was also done to measure the association between the parasite diversity (total number of haemoparasite genera found in an animal) and the neutralizing ability of the animals. Two categories were created for each parameter; the "parasite diversity" was classified into "low" (animals with 0-2 parasites genera) and "high" (animals with >2 parasites genera). This classification was done based on the total parasite genera present which was 4. While the "neutralisation status" was categorized into "neutralisation" (animals that neutralized the lethal toxin) and "no neutralisation"(animals without any neutralisation). The analysis was done considering a level of significance of 5%.

Chapter 4
RESULTS

4.1. Results of anti-PA ELISA Optical densities (ODs) zebra, kudu, impala and wildebeest in KNP and ENP

Poisson regression showed that kudus in KNP had significantly higher anti-PA ELISA antibody titres (1.498 ± se 0.136) than those in ENP (0.641 ± se 0.073) (p=0.0004), but the difference was not significant for the area in national parks (p=0.14) (Table 4.1). Zebras showed neither significant difference in average antibody titres between KNP and ENP nor between endemic and non-endemic area (Table 4.1). The measure of interaction between national park and area, for ELISA antibody titres, was not significant for kudus (p=0.16) and zebras (p=0.53) (not shown in the table). The Poisson models fitted well the data for both kudu and zebra (p=1.0), as determined by goodness-of-fit tests. The average OD value for the 20 impalas sampled in the endemic area in KNP and the 20 wildebeest sampled in the endemic area in ENP were 0.314 ± 0.043se and 0.321 ± 0.051se, respectively (Figure 4.1 and Table 4.2).

When areas of different endemic status were compared for ELISA titres, there was a significant difference for kudu ($p<0.05$) and zebra ($p<0.05$) in KNP, but not for ENP for both kudu and zebra ($p>0.05$) (Table 4.2). Details of antibody titres for the host species segregated by area in each national park are shown in Figure 4.1 and Table 4.2.

Table 4-1 Poisson regression analysis for the significance of the average anti-Bacillus anthracis titres in wild animals, as predicted by national park and locality

Variable	Category	No. of animals sampled	Mean ELISA titres (ODs) ± se	*p*-value
Kudu				
Area	Non-endemic	38	0.872 ± 0.105	
	Endemic	39	1.229 ± 0.139	0.14
National park	ENP	40	0.641 ± 0.073	
	KNP	37	1.498 ± 0.136	0.0004[a]
Zebra				
Area	Non-endemic	40	0.552 ± 0.08	
	Endemic	40	0.668 ± 0.06	0.50
National park	KNP	40	0.553 ± 0.07	
	ENP	40	0.668 ± 0.08	0.51

Serum samples were collected from Kudus and Zebras in Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia; Area = Endemic Vs Non-endemic. OD, optical density; ELISA, enzyme-linked immunosorbent assay; Se, standard error of the mean. [a] Significant difference in ELISA antibody titres in Kudus between two national parks.

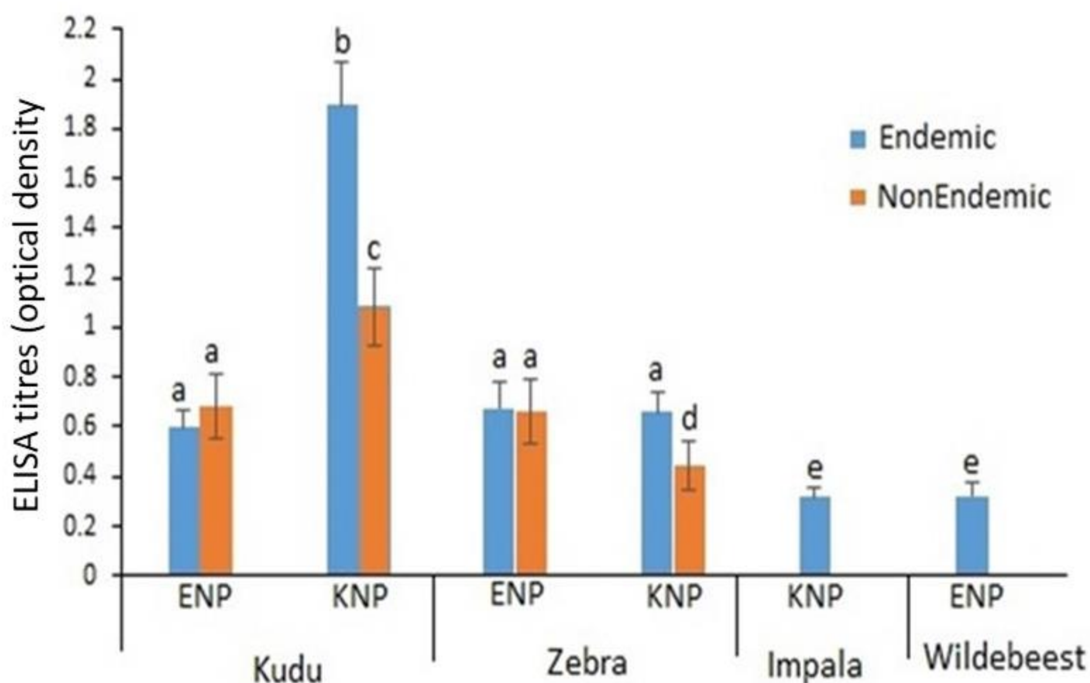

Figure 4-1 Bar charts representing anthrax ELISA titres (optical density) across four wild animal species from Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia. The data represents Means ± standard error of the means. Letters that differ above the graphs are significantly different at a significance level of 0.05. Similar letters above the graph indicate lack of statistically significant difference in the mean OD results between endemic and non-endemic areas for the corresponding park and animal species.

Table 4-2 Anthrax ELISA ODs in wild animal species, based on two national parks and endemic status of wildlife habitat

Animal species	National park	Endemic status	Mean ELISA titres (ODs) ±se	no. of animals	*p*-value
Kudu	ENP	Endemic	0.599 ± 0.065	20	0.646
		Non-endemic	0.682 ± 0.132	20	
	KNP	Endemic	1.893 ± 0.180	19	0.0008
		Non-endemic	1.082 ± 0.157	18	
Zebra	ENP	Endemic	0.677 ± 0.099	20	0.434
		Non-endemic	0.659 ± 0.130	20	
	KNP	Endemic	0.661 ± 0.082	20	0.016
		Non-endemic	0.445 ± 0.102	20	
Impala	KNP	Endemic	0.314 ± 0.043	20	
Wildebeest	ENP	Endemic	0.321 ± 0.051	20	

4.1.1 Multivariable logistic regression analysis of the association of anthrax serological status

Results of the multivariable logistic regression analysis for kudu data showed that only the factor "national park" was a significant determinant for exposure to *B. anthracis*. Kudus in Kruger National Park were significantly associated with higher odds of exposure to *B. anthracis* (odds ratios [OR]=19.4, *p*=0.005) than those in Etosha National Park (Table 4.3). The area of a national park was eliminated from the final regression model (using stepwise AIC analysis), and showed no effect on exposure to *B. anthracis* in Kudus (*p*=0.36); endemic areas = 33/39 (85%) Vs non-endemic areas = 29/38 (76%) (data not shown in table). For zebra, animals in Etosha National Park had three times higher odds of exposure to *B. anthracis* than those in Kruger, and zebras in endemic areas (85%, 34/40) were four times more likely to be exposed than those in non-endemic areas (Table 4.3). The interaction between area and the national park was not a determinant for exposure to *B. anthracis* in kudus and zebras. The goodness-of-fit test showed that the data fitted well the final regression models (χ^2<0.001, *p*=1.0).

Comparison of the frequency of anti-PA antibodies between endemic area and non-endemic area in each national park, separately for each host species, showed that animals in endemic areas were more frequently exposed to the pathogen than those in non-endemic areas, although the effect was only significant for zebra in Etosha National Park, i.e. 19/20 (95%) in the endemic area Vs 14/20 (70%) in non-endemic area (p=0.046) (Figure 4.2 and Appendix 1).

Comparing Steenkamp et al. (2018) anthrax suitability map for KNP south shows that a higher proportion of positive animals were found around the places marked as suitable (Figure 4.3).

Table 4-3 Multivariable logistic regression analysis for the association between exposure to *Bacillus anthracis* in wild animals in two national parks in southern Africa, and predictor variables

Variable	Category	No. of animals sampled	No. of positive animals (%)	Odds ratio	95% CI of OR	*p*-value
Kudu[a]						
National park	ENP	40	26 (40.0)			
	KNP	37	36 (97.3)	19.4	2.4, 156.8	0.005[a]
Zebra						
National park	KNP	40	25 (62.5)			
	ENP	40	33 (82.5)	3.1	1.1, 9.2	0.041[a]
Endemic status	Non-endemic	40	24 (60.0)			
	Endemic	40	34 (85.0)	4.1	1.4, 12.4	0.013[a]
Impala	KNP	20	8 (40.0)	NA	NA	NA
Wildebeest	ENP	20	7 (35.0)	NA	NA	NA

[a] The area within a national park was not a determinant for exposure to B. anthracis in Kudus, and therefore eliminated from the final logistic regression
[b] Significant difference in exposure to B. anthracis between categories of a variable.
KNP, Kruger National Park in South Africa; ENP, Etosha National Park in Namibia
NA, non-applicable

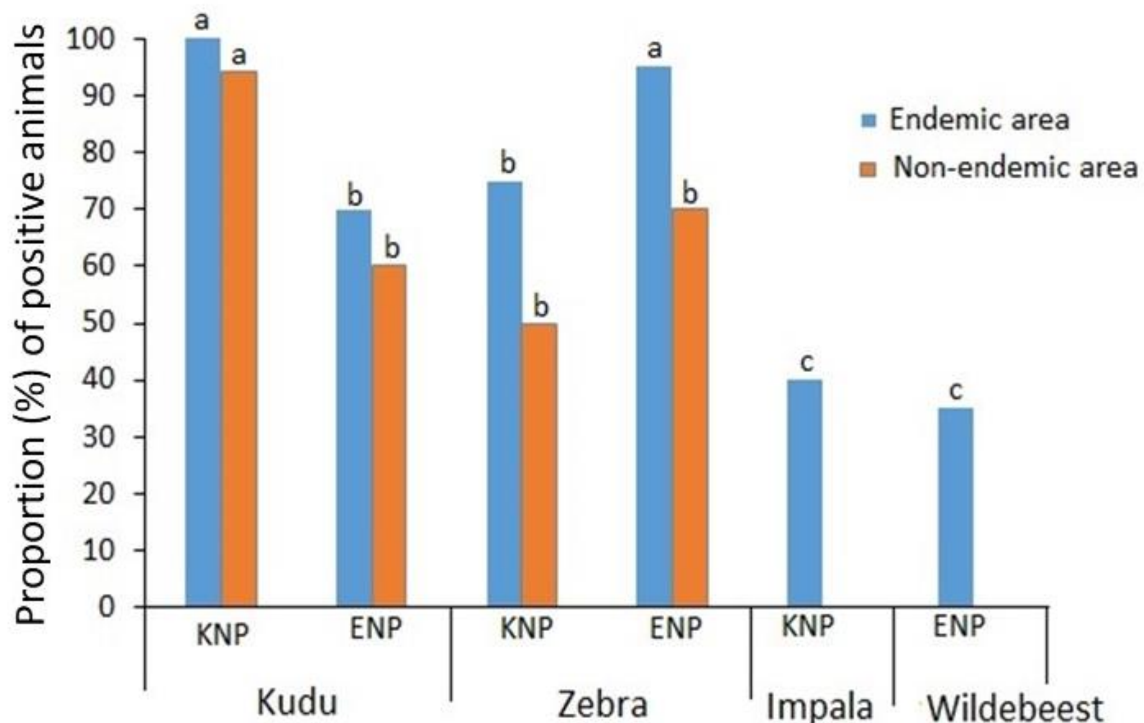

Figure 4-2 Bar graph showing the proportion of wild animals that were seropositive for anti-PA antibodies, as determined using enzyme-linked immunosorbent assay (ELISA). The four wild animal species (kudu, zebra, Impala and wildebeest) were sampled in Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia. Similar letters above the graphs indicate lack of statistically significant difference in the proportion of seropositive animals between endemic and non-endemic areas for the corresponding national park and animal species, as determined using the chi-square test, at a significance level of 0.05. Apart from kudu whose sample sizes in endemic and non-endemic areas in KNP were 19 and 18 respectively, the sample size of animals in each of the other sub-categories was 20.

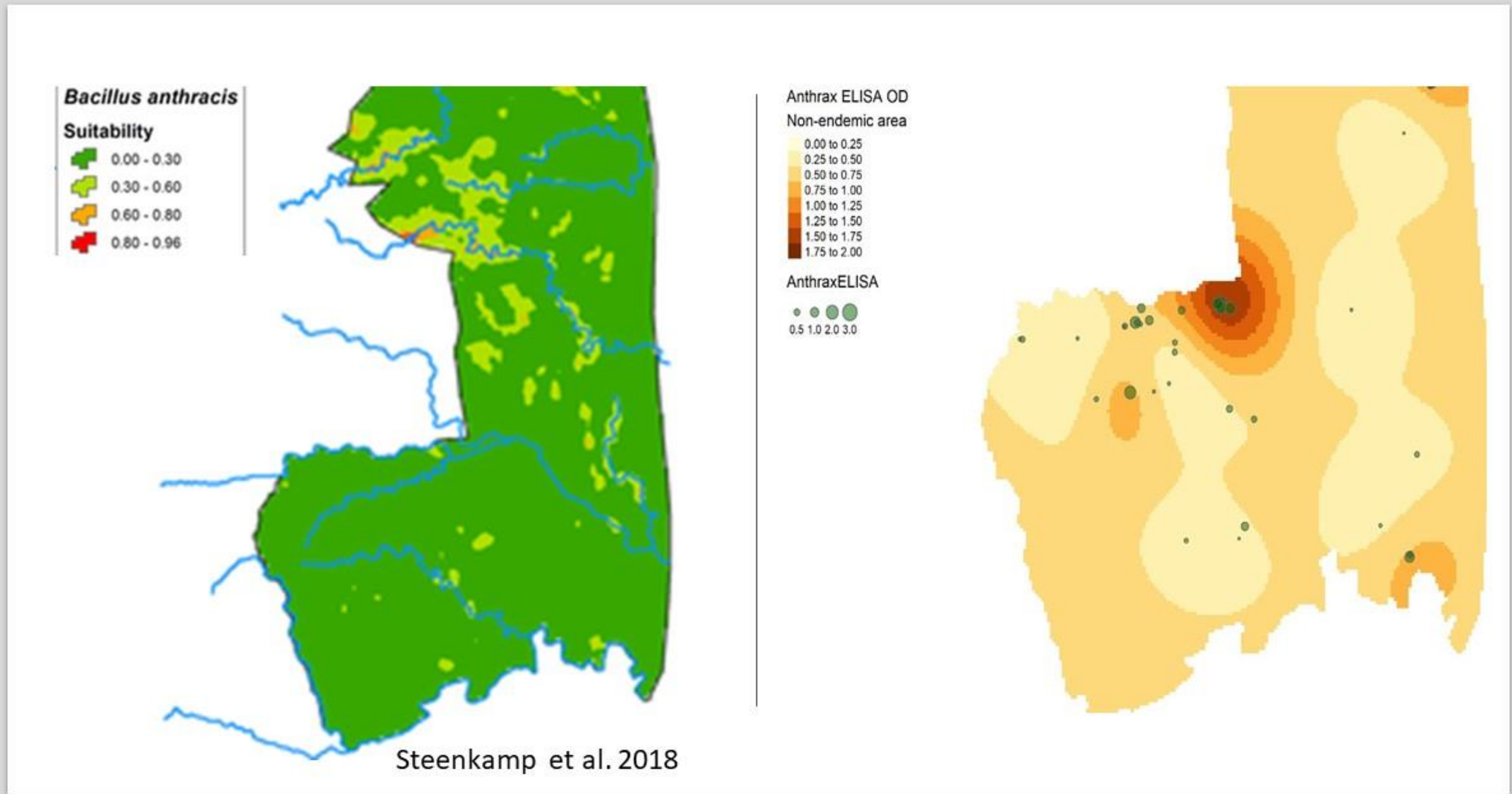

Figure 4-3 Image showing anthrax suitability map on the left of Steenkamp et al (2018) coinciding with areas where animals tested positive for antibodies against Bacillus anthracis protective antigen (PA) antibodies in the southern part of Kruger National.

4.2 Toxin Neutralisation assay

4.2.1 Results of the proportion of animals that neutralized the anthrax lethal toxin

Multivariable logistic regression analysis showed that the predictors of *B. anthracis* toxin neutralisation were host species and national park, and there was a significant interaction between the host species and national park. Kudus were more frequently associated with *B. anthracis* toxin neutralisation than zebras (odds ratios [OR]=16.7, $p<0.001$) (Table 4.4). Similarly, for the national park, wild animals in Kruger showed a significantly higher frequency of toxin neutralisation than those in Etosha (OR=23.7, $p<0.001$). Interaction between the national park and host species showed that kudus in Etosha, kudus in Kruger, and zebras in Kruger, all showed significantly higher odds of *B. anthracis* toxin neutralisation frequency (>45%) than zebras in Etosha (10%, 4/40) ($p<0.001$) (Table 4.4). The Hosmer-Lemeshow (χ^2) goodness-of-fit test showed that the model fitted well the data (Table 4.4). The area in national parks had no significant effect on the frequency of toxin neutralisation in the wild animals, i.e. endemic area =51.9% (41/79) Vs non-endemic (36/78, 46.2%) ($p=0.47$) (data excluded from the final regression model in Table 4.4). All comparisons between the endemic and non-endemic areas in each park for each host species showed no significant difference ($p>0.05$) (Figure 4.4 and Appendix 2). Further analysis of the association of the frequency of toxin neutralisation in kudu, or zebra, between the two national parks (irrespective of endemic status), showed a significantly higher proportion of zebra neutralizing the anthrax lethal toxin in KNP than in ENP (p<0.001) (Figure 4.4 and Appendix 2), while for kudu, although those in ENP showed a higher frequency of neutralisation than in Kruger, the difference was not statistically significant (p=0.15) (Figure 4.4 and Appendix 2).

Only 3 of the 20 impala sampled in Kruger National Park showed toxin neutralisation, while the frequency of toxin neutralisation in wildebeest was 9 out of the 20 animals sampled (Figure 1, Appendix 1).

Table 4-4 Multivariable logistic regression analysis for the association between the frequency of *Bacillus anthracis* toxin neutralisation in wild animals in two national parks in southern Africa, and the identified predictor variables

Variable	Category	No. of animals sampled	No. of positive animals (%)	Odds ratio and 95% CI	*p*-value
Host species	Zebra[a]	80	33 (41.3)		
	Kudu	77	44 (57.1)	16.7 (4.9,56.6)	<0.001[b]
Park	ENP[a]	80	30 (37.5)		
	KNP	77	47 (61.0)	23.7 (6.8, 82.4)	<0.001[b]
Host species.*Park	Zebra_ENP[a]	40	4 (10.0)		
	Kudu_ENP	40	26 (65.0)	16.7 (4.9, 56.6)	<0.001[b]
	Kudu_KNP	37	18 (48.6)	8.5 (2.5, 28.8)	<0.001[b]
	Zebra_KNP	40	29 (72.5)	23.7 (6.8, 82.4)	<0.001[b]

KNP, Kruger National Park in South Africa; ENP, Etosha National Park in Namibia
Laboratory analysis was conducted using toxin neutralisation assay (TNA).
Goodness-of-fit: χ^2=13.69, df=8, p=0.090
[b] Significant difference in the frequency of toxin neutralisation as compared to the reference category [a]

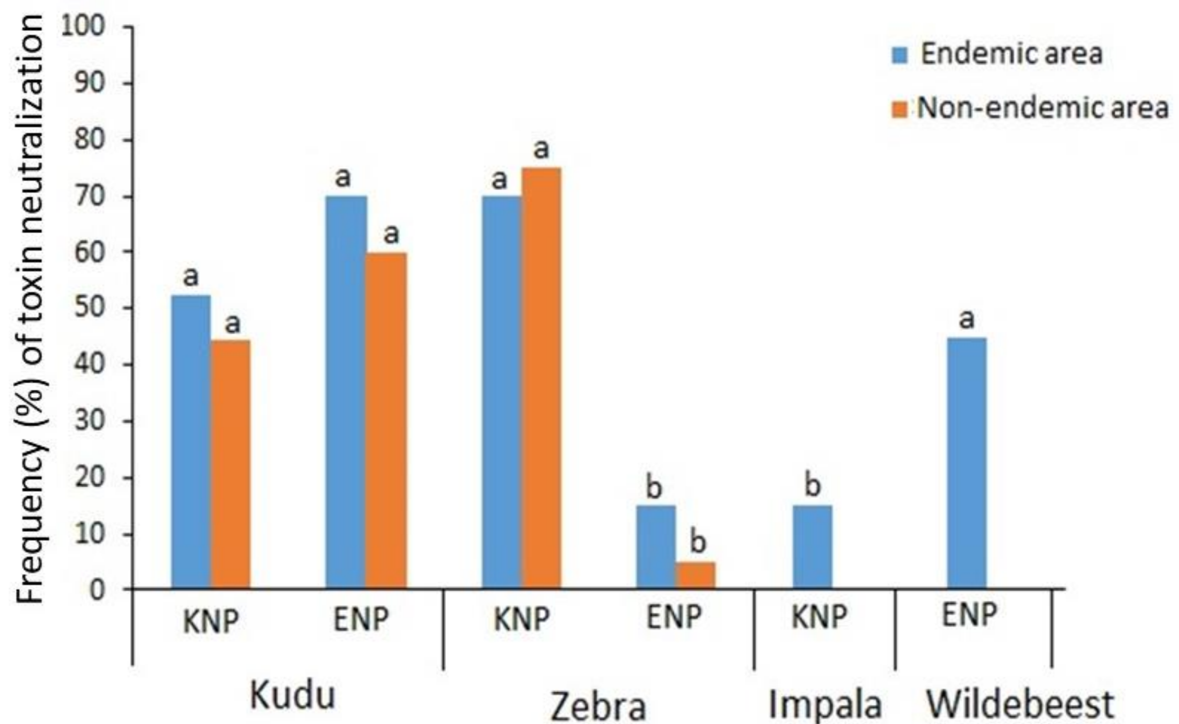

Figure 4-4 Bar graph representing anthrax toxin neutralisation across four wild animal species from Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia. On the Y-axis is the proportion of animals whose antibodies in sera protected 50% of cells in culture from the toxin. The cells in the culture were of the J774A.1 cell line. Similar letters above the graphs mean that there was no significant difference between endemic and non-endemic areas for the corresponding national park and animal species, as determined using the chi-square test, at a significance level of 0.05. Apart from kudu whose sample sizes in endemic and non-endemic areas in KNP were 19 and 18 respectively, all other subcategories each had a sample size of 20 animals.

4.2.2 Results of the analysis of the distribution of the *Bacillus anthracis* neutralisation titres (NT) in the wildlife species

Descriptive analysis for wildebeest and impala data showed that of the 20 impala sampled in KNP, only 3 showed toxin neutralisation (mean titre=3.4 ± 1.9) (Figure 4.3 and Table 4.6). Toxin neutralisation in wildebeest was observed in 14 of the 20 sampled in ENP (17.4 ± 7.4 (Figure 4.5). Details of toxin neutralisation scores for all host species each national park, segregated by area are shown in Figure 4.5 and Table 4.6

A negative binomial regression for toxin neutralisation scores showed that kudus had significantly higher anti-*B. anthracis* toxin neutralisation scores (54.5 ± se 8.35) than Zebras (25.2 ± se 4.35) (p<0.001) (Table 4.5). Kudu and zebra in KNP had significantly higher *B. anthracis* toxin neutralisation scores (44.9 ± 6.74) than those in ENP (34.4 ± se 6.79) (p<0.001), while locality within the national park was not a significant determinant to toxin neutralisation scores amongst wild animals (p=0.17) (Table 4.5). Four interactions between the variables, host species, area and national park were assessed, and only one interaction (host species*national park) was a significant determinant for toxin neutralisation in wild animals (p<0.001) (Table 4.5). Within the host species*national park interaction, kudus in ENP had the highest toxin neutralisation scores (64.0 ± se 11.66), followed by zebras in KNP (45.5 ± se 7.05), and the least was from zebras in ENP (4.8 ± se 2.39).

Kudus in ENP showed higher TNA titres (64.0 ± 11.7) and those os KNP (44.2 ± 11.9se) even though this was not statistically significant (regardless of endemic status) (p=0.12) (Table 4.7) and between endemic and non-endemic areas (regardless of the park) (p=0.45) (Table 4.13). However, for zebra, the TNA titres were significantly higher in KNP (45.5 ± 7.0) than ENP (4.8 ± 2.4) (regardless of endemic status) (p<0.001) (Table 4.7), but did not differ between endemic and non-endemic areas (p=0.71) (Table 4.7). The titres of the two animal species were also compared by national park (regardless of endemic status), showing that kudu in KNP had slightly lower titres than zebra in KNP (44.2 Vs 45.5 respectively; p=0.072), but significantly different titres between the two species in ENP (64.0 Vs 4.8 respectively; p<0.001) (Table 4.7).

Table 4-5 Negative binomial regression analysis for the significance of differences in anti-*Bacillus anthracis* toxin neutralisation scores from Kudu and Zebra

Variable	Category	No. of animals sampled	Mean TNA scores ± se	*p*-value
Host species	Zebra	80	25.2 ± 4.35	
	Kudu	77	54.5 ± 8.35	<0.001
National park	ENP	80	34.4 ± 6.79	
	KNP	77	44.9 ± 6.74	<0.001
Area	Non-endemic	78	35.6 ± 6.33	
	Endemic	79	43.5 ± 7.18	0.17
Host species*national park	Zebra*ENP	40	4.8 ± 2.39	
	Kudu*KNP	37	44.3 ± 11.88	
	Zebra*KNP	40	45.5 ± 7.05	
	Kudu*ENP	40	64.0 ± 11.66	<0.001

Serum samples were collected from Kudus and Zebras in Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia; Negative binomial regression analysis performed considering the factors; national park, area, host species, and interactions: host species*area, host species*national park, national park*area, and national park*area*host species.
[a] Factors; host species, national park and the interaction host species*national park were significant determinants for toxin neutralisation.
Area, endemic and non-endemic; TNA, toxin neutralisation assay; Se, standard error of mean.

Table 4-6 *Bacillus anthracis* toxin neutralisation assay (TNA) titres in wild animal species based on two national parks and the endemic status of wildlife habitat

Animal species	National park	Endemic status	Mean TNA titres ± se	1st, 3rd quartiles	Maximum	p-value
Kudu	ENP	Endemic (20)	59.5 ± 13.8	0,102.9	194.3	0.67
		Non-endemic (20)	68.6 ± 19.1	0,114.4	316.6	
	KNP	Endemic (19)	61.0 ± 21.4	0,83.5	270.9	0.51
		Non-endemic (18)	26.5 ± 8.0	0,44.4	84.3	
Zebra	ENP	Endemic (20)	7.1 ± 4.2	0,0	70.2	0.32
		Non-endemic (20)	2.5 ± 2.4	0,0	47.8	
	KNP	Endemic (20)	47.3 ± 10.7	0,61.6	208.7	0.96
		Non-endemic (20)	43.7 ± 9.4	7.8,56.3	123.6	
Impala	KNP	Endemic (20)	3.4 ± 1.9	0,0	31.7	NA
Wildebeest	ENP	Endemic (20)	17.4 ± 7.4	0,21.8	140.9	NA

KNP, Kruger National Park; ENP, Etosha National Park
TNA, Toxin neutralisation assay
p>0.05, no significant difference in TNA titres between endemic and non-endemic areas in each national park, for each animal species
Number of animals sampled are indicated in parenthesis

Table 4-7 Comparison of *Bacillus anthracis* TNA titres between two national parks in southern Africa, based on wild animal species

	National park	Mean TNA titres ± se	p-value
Kudu	KNP (n=37)	44.2 ± 11.9	0.12
	ENP (n=40)	64.0 ± 11.7	
Zebra	KNP (n=40)	45.5 ± 7.0	<0.001
	ENP (n=40)	4.8 ± 2.4	

KNP, Kruger National Park; ENP, Etosha National Park
TNA, Toxin neutralisation assay
Se, standard error of mean

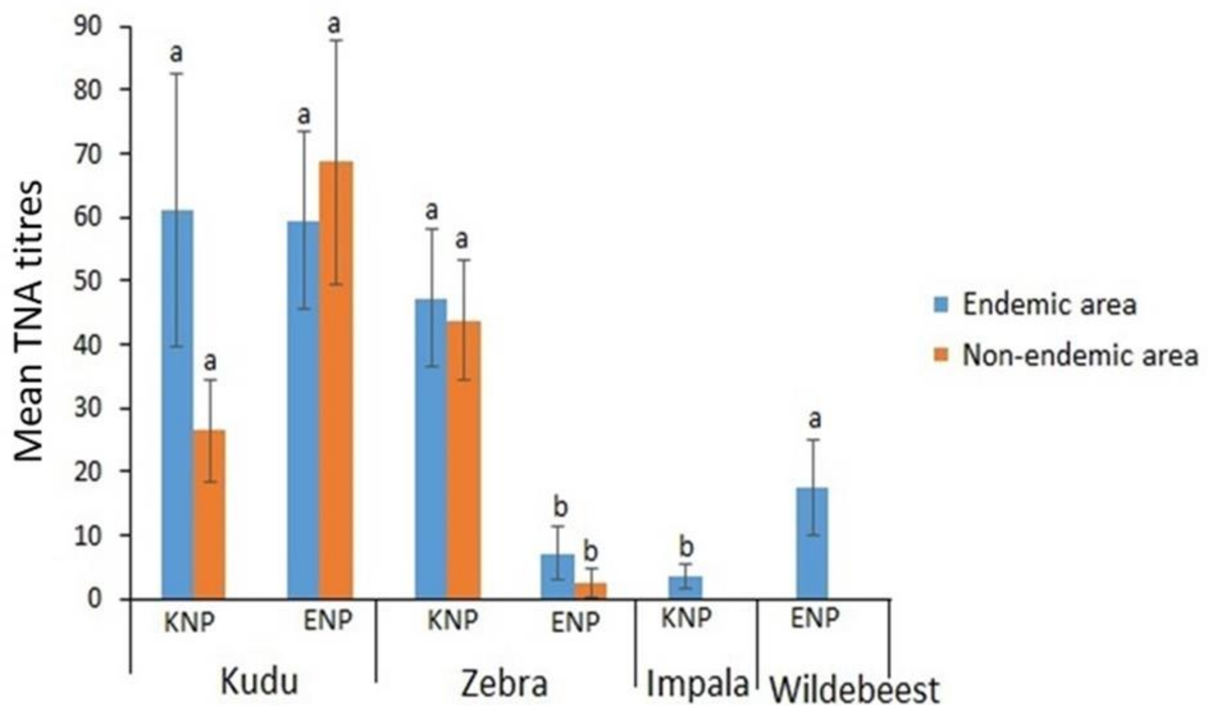

Figure 4-5 Bar chart showing Bacillus anthracis toxin neutralisation assay (TNA) titres among four wild animal species in Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia. The Y-axis represents means ± standard error of the means. Similar letters above graphs imply no significant difference in the mean TNA titres, as determined using the independent samples T-test, at a significance level of 0.05.

Table 4-8 Comparison of results from ELISA and TNA binary outcome in four wild animal species from Kruger and Etosha National Parks in South Africa and Namibia respectively

	Number and percentage of positive samples		
	TNA		
ELISA	Positive	Negative	
Positive	73 (37.1)	62 (31.5)	135 (68.5)
Negative	16 (8.1)	46 (23.4)	62 (31.5)
Total	89 (45.2)	108 (54.8)	n=197

n, number of wildlife samples tested by both ELISA and TNA.
Wild animal species tested: kudu, wildebeest, impala, kudu and zebra

4.2.3 Result of comparison of the two tests and correlation analysis

The comparison between the results from qualitative outcome of ELISA and TNA in four wildlife species is shown in Table 4.8. There was a fair agreement between the two tests (kappa=0.24, 95% CI 0.12, 0.35), and this was statistically significant (p=0.0001), implying that this determined level of agreement was not due to chance.

There was a medium and significant positive relationship between ELISA titres and TNA values from both Pearson correlation (r=0.47, $p<0.001$), and Spearman's rank correlation (rho=0.40, $p<0.001$). The correlation was very obvious in kudu in both parks and zebra in KNP as well as wildebeest in ENP (Figure 4.6)

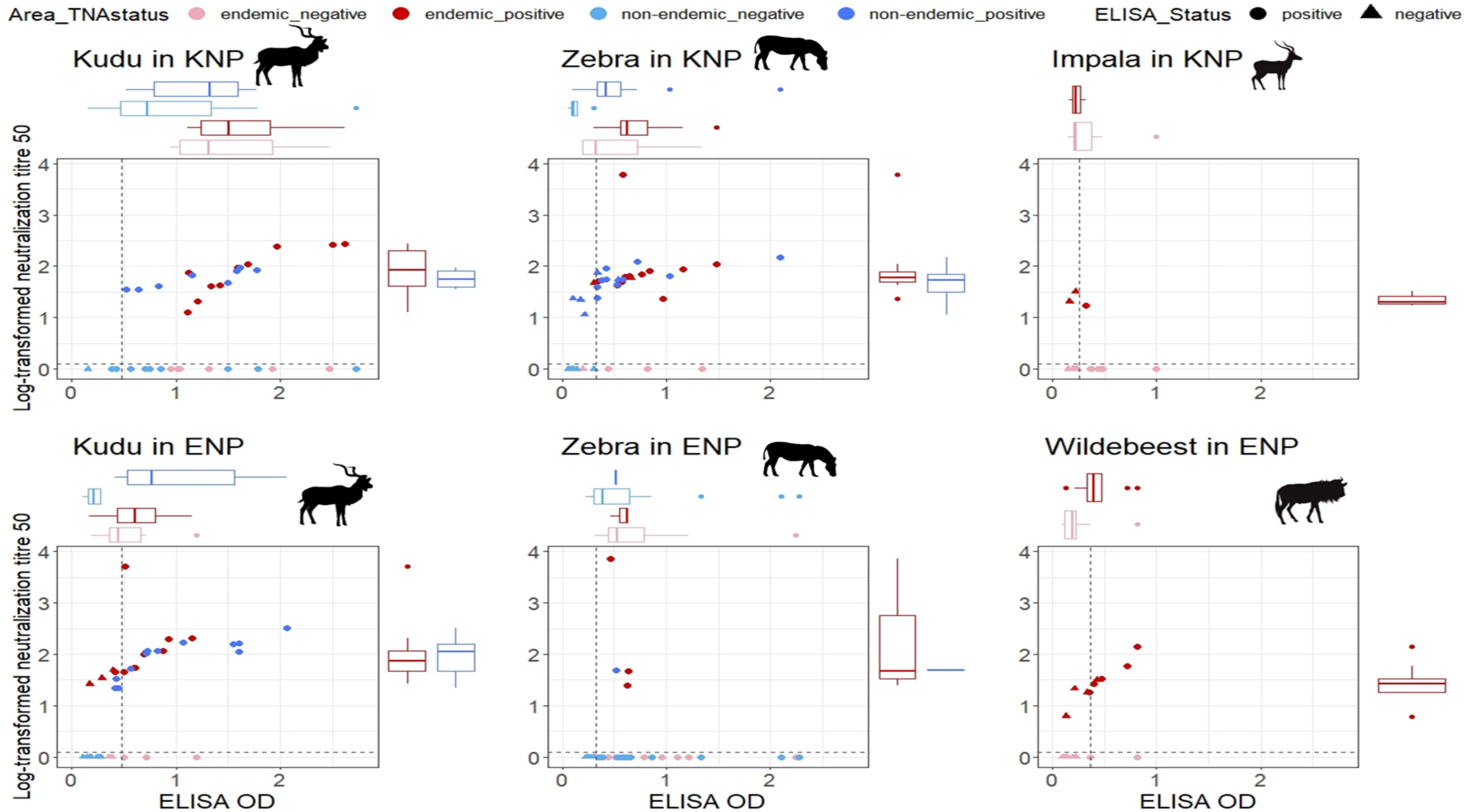

Figure 4-6 scatter plots and box and whiskers representing anthrax toxin neutralization and ELISA ODs across four wild animal species from Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia. The TNA status indicates if there was neutralization or not and the samples below the set cut-off are considered as negatives. TNA negative sera are seen below the dotted horizontal lines in each plot while ELISA negative samples can be seen behind the vertical dotted lines of each plot.

4.3 Analysis of the Reverse Line Blot (RLB) hybridization results

Out of the 196 animal samples analysed using RLB, 180 (91.8%) tested positive for at least one of the tick-borne parasites examined (appendix 2). Of the aforementioned 180 animals, 90.9% (N=77) of kudu, 92.4% (N=79) of Zebra, 95% (N=20) of Wildebeest and 100% (N=20) of Impala showed positive signals with the RLB oligonucleotide probes. Out of the 180 animals that tested positive for any of the haemoparasites, 50% (N=90) were from the KNP while 50% (N=90) were from ENP. There was a statistically significant difference ($P<0.05$) between the Kudu in both parks in that 100% (N=37) of kudu in KNP tested positive for the tick-borne parasites while only 70% (N=40) in ENP tested positive. Of the zebra in ENP, 100% (N=40) tested positive while only 84.6% of zebra in KNP tested positive ($P<0.05$). All the Impala 100% (N=20) in KNP and 85%(N=20) in ENP tested positive. There was also a significant difference ($P<0.05$) between kudu and zebra within each park.

A comprehensive representation of the samples that showed genus-specific signals irrespective of species-specific status can be seen in figure 4.7. Of the 196 animals that were sampled, 107 showed genus-specific signals without any species-specific signals. A total of 109 samples showed *Anaplasma*/*Ehrlichia* genus-specific signals without any *Anaplasma* or *Ehrlichia* species-specific signals. A sum of 82 samples showed *Theileria*/*Babesia* signals without *Theileria* or *Babesia* species-specific signals. While 57 samples were positive for just *Theileria* genus-specific RLB probe without any *Theileria* species-specific signals and 68 samples were positive for *Babesia* genus-specific probes without any species-specific signals (Figure 4.8). Of the 109 samples that were positive for *Anaplasma*/*Ehrlichia* genus-specific signals without any *Anaplasma* or *Ehrlichia* species-specific signals, 42 were from Kudu (12 in KNP, 30 in ENP), 53 from zebra (24 in KNP, 29 in ENP), 1 from Impala in KNP and 13 from Wildebeest in Etosha (Figure 4.8). The 82 *Theileria*/*Babesia* genus-specific signals without *Theileria* or *Babesia* species-specific signals were from Kudu (2 in KNP, 16 in ENP), Zebra (29 in KNP, 30 in ENP) and 13 from Wildebeest in ENP. Out of the 57 *Theileria* genus-specific RLB probe without any *Theileria* species-specific signals, 27 were from Zebra in KNP while 30 were from Zebra in ENP. Of the 68 *Babesia* genus-specific signals, 1 was from Kudu in KNP and 67 from Zebra (32 in KNP, 35 in ENP) as evident in figure 4.8.

Of all the 196 animals sampled, 107 (54.6%) were classified as having “high diversity”, while 89 (45.4%) animals were classified as having “low diversity’. Of the 107 animals that had high diversity, 30.6% were kudu from KNP, 18.5% were zebra from KNP, 27.8% were Zebra from ENP and 18.5% were Impala from KNP and 4.6% from wildebeest in ENP.

The zebra in ENP showed a prevalence of 75% (N=40) of high diversity and 25% of a low diversity while in KNP, 51.3% (N=39) had high diversity while 48.7% had low diversity. 89.2% (N=37) kudu in KNP had high diversity while 10.8% had low diversity. All the kudu in ENP had less ≤3 parasites, thus having low diversity.

The occurrence of various species of tick-borne pathogens in 4 species of wild animals is shown below(Figure 4.6). *Anaplasma centrale*, the cause of mild anaplasmosis in cattle, was found mostly in Impala (15/20) in KNP, and in a few samples from Zebra in KNP (2/39) and wildebeest in ENP (3/20) as seen in figure 4.6. *Ehrlichia ruminantium*, a highly pathogenic species for heartwater in domestic ruminants was detected in only 2 of the 39 zebras sampled in KNP (figure 4.9). *Anaplasma bovis*, *A. platys*, *Theileria* sp. (kudu), and *T. taurotragi* were found only in Kudu in KNP, in proportions of 19%, 28%, 92% and 76% of the 37 sampled kudu, respectively (figure 4.9). *Theileria equi*, one of the causative agents of equine piroplasmosis, was found only in zebra, and this was in both ENP (8/40) and KNP (5/39) (figure 4.4). *Theileria* sp. (*sable*) was in high abundance in kudu in KNP (31/37), but in none of the kudus in ENP. The pathogen was also detected in wildebeest (9/20) in ENP and Impala (11/20) in KNP (figure 4.9). *T. separata* was found only in wildebeests in ENP (6/20).

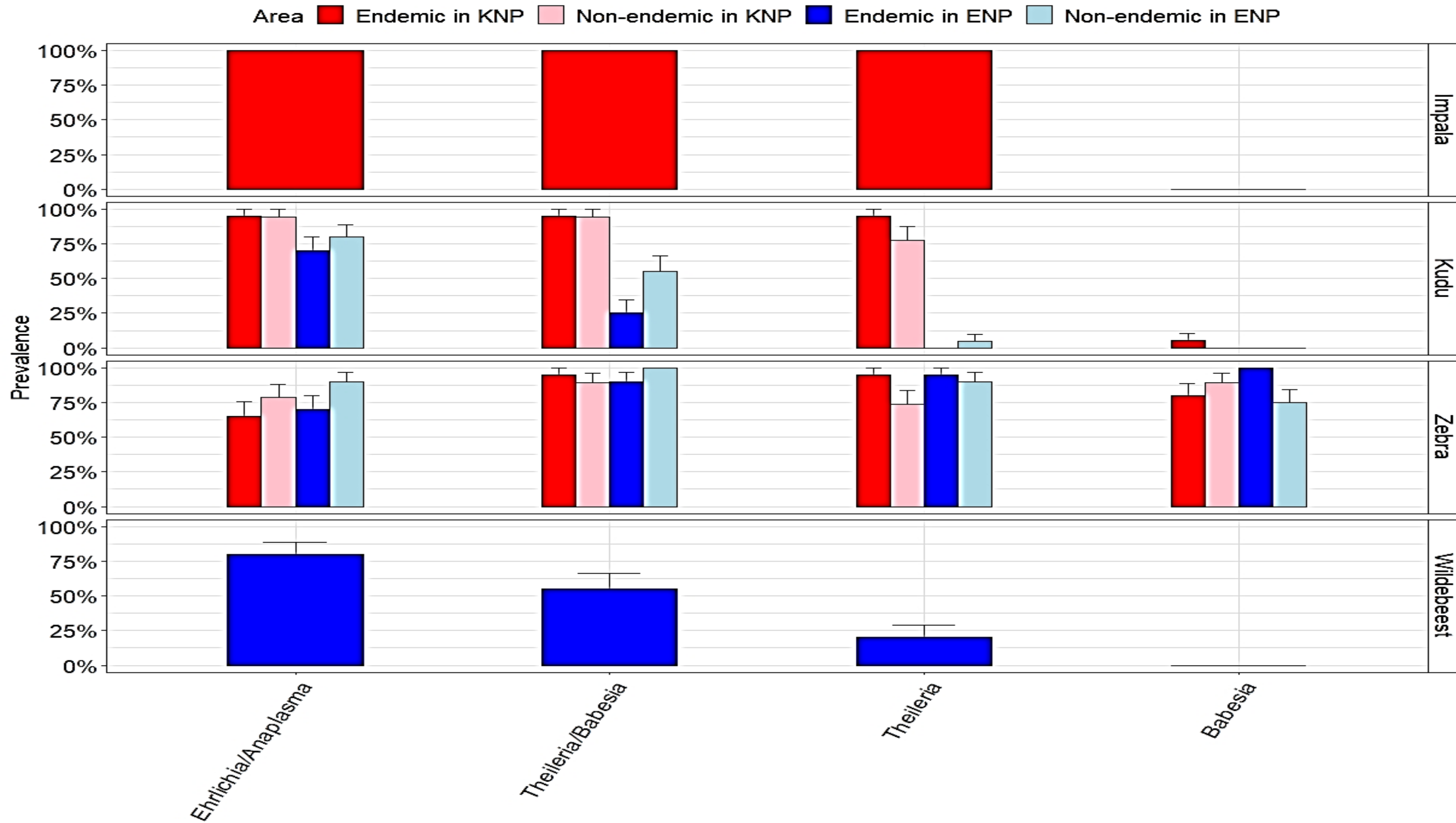

Figure 4-7 Prevalence of different genera of haemoparasites in Kudu, Zebra (Kruger and Etosha National parks), Impala (Kruger National park) and Wildebeest (Etosha National park) using the reverse line blot hybridization.

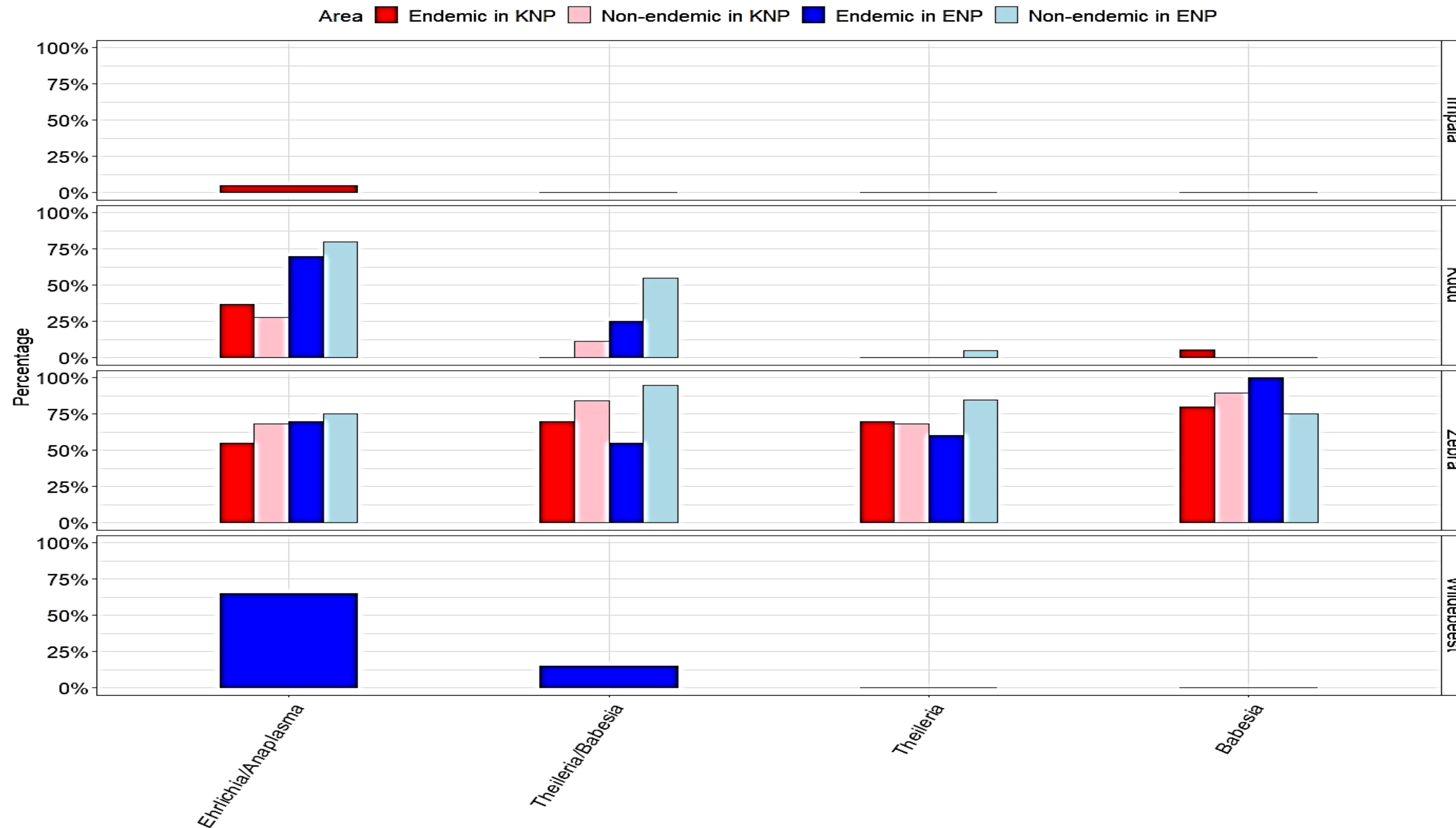

Figure 4-8 Bar charts representing Ehrlichia/Anaplasma, Therileria/Babesia, Theileria and babesia genera-specific signals without corresponding species-specific signals across four wild animal species from Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia. The data represents the percentage of samples that had only genus-specific signals out of the total population sampled in each park and each region (endemic and non-endemic) of the parks.

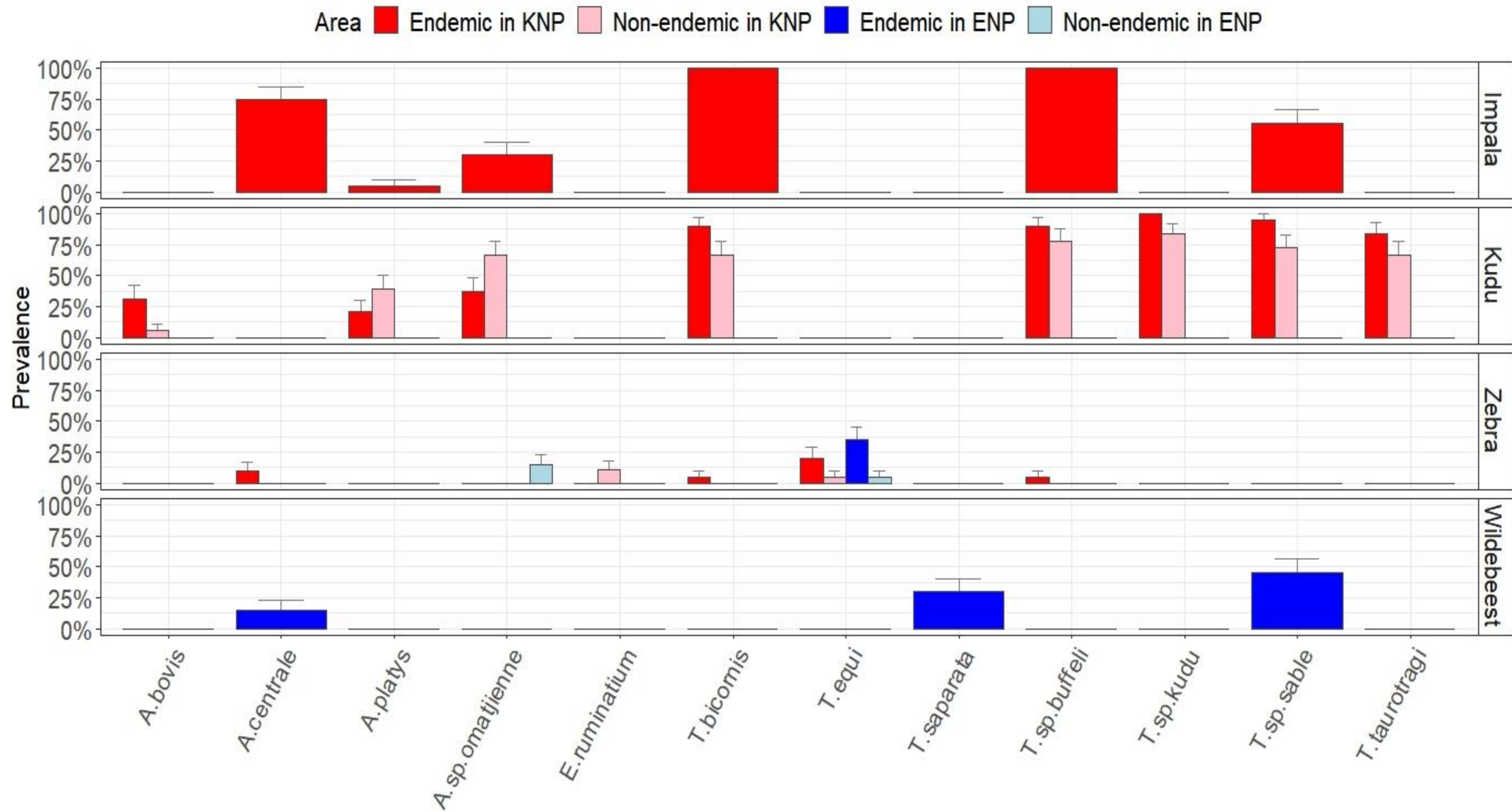

Figure 4-9 Prevalence of different species of haemoparasites in kudu, zebra (Kruger and Etosha National parks), impala(Kruger National park) and wildebeest (Etosha National park) identified using the reverse line blot hybridization for Theileria, Anaplasma and Ehrlichia species.

4.3.1 Results of logistic regression analysis

The final logistic regression analysis between haemoparasite infections and exposure variables amongst zebra and kudu in ENP and KNP is shown in Table 4.9, while details of the initial models that include non-significant associations are shown in Appendix 3. An increase in toxin neutralisation was significantly associated with less likelihood of infection with *Babesia* (OR=0.97, p=0.002), *Theileria* (OR=0.96, p=0.0005), *Ehrlichia/Anaplasma* (OR=0.96, p=0.0004) parasites in zebras (Table 4.9). A similar trend was observed for kudu, regarding anthrax toxin neutralisation, with lower odds of infection with *Theileria* (OR=0.99, p=0.09) *Ehrlichia/Anaplasma* (OR=0.98, p=0.004) parasites (Table 4.9). Only one kudu was positive for *Babesia*, and therefore not included in the analysis.

For the area within a national park; the odds of infection with *Ehrlichia* parasites amongst zebras was 3.4 times higher in anthrax non-endemic areas (32/39) than in endemic areas (25/40), and the association was statistically significant (p=0.048) (Table 4.9). The analysis also revealed higher odds of infection with *Anaplasma* parasites amongst zebras in non-endemic (30/39) than endemic (25/40), although not significant (p=0.17). On the other hand, the area within a national park did not affect infection with *Babesia* and *Theileria* parasites amongst zebras (Appendix 3). Kudus in non-endemic areas were associated with higher odds of infection with *Theileria* (OR=4.4, p=0.04) and *Ehrlichia* (OR=2.5, p=0.097) parasites than in endemic areas (Table 4.9). The odds of infection of kudus with *Theileria* parasites were higher in KNP than ENP (OR=76.6, p=0.0005), but higher for *Ehrlichia* parasites amongst kudus in ENP than those in KNP (OR=7.8, p=0.001). However, the national park had no significant effect on tick-borne haemoparasite infections in zebra (Appendix 3). There was a significant interaction between the national park and area for *Theileria* infection in kudus (p=0.016). The goodness-of-fit test shows that the data was in good fit with the final model (Table 4.9).

Table 4-9 Final logistic regression analysis for the association between infection with tick-borne haemoparasites and the predictors of infection in wild animal populations in two national parks in South Africa and Namibia

	Variable	Category	No. of positive animals (%)	OR	Goodness-of-fit test	p-value
Zebra						
Babesia	Anthrax TN	TN		0.97	X2 =1.99, p=0.98	0.002
Theileria	Anthrax TN	TN		0.96	X2 =1.51, p=0.99	0.0005
Ehrlichia/ Anaplasma	Area	Endemic	25/40 (62.5)			
		Non-endemic	32/39 (82.1)	3.4		0.048
	Anthrax TN	TN		0.96	X2 =8.66, p=0.37	0.0004
Kudu						
Theileria	Park	ENP	16/40 (40.0)			
		KNP	32/37 (86.5)	76.7		0.0005
	Area	Endemic	23/39 (59.0)			
		Non-endemic	25/38 (65.8)	4.4		0.04
	Anthrax TN	TN		0.99		0.09
	Park*Area	Interaction		0.03	X2 =3.68, p=0.88	0.016
Ehrlichia/ Anaplasma	Park	ENP	30/40 (75.0)	7.8		0.001
		KNP	16/37 (43.2)			
	Area	Endemic	20/39 (51.3)			0.097
		Non-endemic	26/38 (68.4)	2.5		
	Anthrax TN	TN		0.98	NA	0.004

The haemoparasites were detected in blood samples using the reverse line blot hybridisation (RLB) assay. A sample was considered positive for a parasite genus if there was a positive signal with the RLB genus-specific probe and/or with the corresponding species-specific probe (s)

KNP, Kruger National Park (South Africa); ENP, Etosha National Park (Namibia)

TN, toxin neutralisation; OR, odds ratio

Logistic regression analysis was done separately for each parasite genus and each host species, including four factors; area, national park, TNA score, interaction between area and national park. Only factors retained in the final logistic regression for each parasite genus analysis are shown in the table.

4.3.2 Results of the correlation analysis

The outcome of the Chi-squared analysis demonstrated a significant difference (P<0.001) between the categories of parasite diversity and neutralisation. A total of 196 animals were analysed and of this number, 89 (45.4%) showed neutralisation while 107 (54.6%) did not. Of the animals that showed neutralisation, 32 (36%) had a high parasite diversity while 57 (64%) had a low diversity. Out of the 107 animals that didn't show neutralisation, 80 (74.7%) had a high parasite diversity while 27 (25.3%) had low diversity (Table 4.10).

A very significant negative correlation was found between parasite diversity and NT50 values for all the animal species together with Spearman's rank correlation (rho= -0.40, $p<0.001$) and for intra-species analysis regardless of parks and also for impala and wildebeest combined. Spearman's rank correlation for zebra (rho= -0.59, $p<0.001$), kudu (rho= -0.40, $p<0.001$), impala and wildebeest combined (rho= -0.436, $p<0.005$) also indicated a significant negative correlation. Of the 77 kudu sampled, 33 had a high diversity and of the 33, 14(42.4%) were able to neutralise the toxin, while 19 (57.6%) did not. Also, 44 (57.6%) of the kudu had a low diversity and of this number, 30 (68.2%) were able to neutralize while 14 (31.8%) could not. Of the 79 zebra tested, animals with high diversity were 50 (63.3%) while those with low diversity were 29 (36.7%) (Table 4.15). Of the 50 animals with high diversity 11 (22%) were able to neutralize the anthrax lethal toxin while 39(78%) could not. And of the animals with low diversity, 23 (79.3%) neutralized the lethal toxin while only 6 (20.7%) could not. A total of 34 zebra showed neutralizing activities. And out of this number, 79.3% had a low diversity of parasites while only 21.7% had a high diversity (Table 4.15). Impala and wildebeest were put together making a total of 40 animals. And of this number of animals, 25 (62.5%) had high diversity while 15 (37.5%) had a low diversity. Of the animals with High diversity 4 (16%) were able to neutralize while 21 (84%) could not. Out of the animals with Low diversity, 7 (46.7%) animals were able to neutralize while 8 (53.3%) could not. A total of 11 animals were able to neutralize the toxin and of this number, 4 (36.4%) had a High diversity while 7(63.6%) had a Low diversity (Table 4.10).

Table 4-10 Parasite diversity by Neutralisation 50 (NT50) cross tabulation

			Neutralisation status		
			Neutralisation (% within diversity)	**No neutralisation** (% within diversity)	**Total (N)**
Parasite Diversity	Overall	High diversity	32 (36%)	80 (74.7%)	112
		Low diversity	57 (64%)	27 (25.3%)	84
	Kudu	High diversity	14 (42.4%)	19 (57.6%)	33
		Low diversity	30 (68.2%)	14 (31.8%)	44
	Zebra	High diversity	11 (22%)	39 (78%)	50
		Low diversity	23 (79.3%)	6 (20.7%)	29
	Wildebeest and Impala	High diversity	4 (16%)	21 (84%)	25
		Low diversity	7 (46.7%)	8 (53.3%)	15

P<0.005

Chapter 5

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

This study reveals a wide presence of anti-PA antibodies in the various host species sampled. Interestingly, even though these parks share similar host species, there were significant differences in the proportions of animals that tested positive for anti-PA antibodies and the titres between the two parks. More so, there was a negative agreement between the TNA NT50 titres and the previous mortality data of the various host species in these parks (Havarua et al., 2014, de-Vos and Bryden, 1996, Turner et al., 2013). This agreement further gives credence to earlier reports of the positive correlation between NT50 titres and protection of animals against anthrax (Weiss et al., 2006, Little et al., 2004, Reuveny et al., 2001, Peachman et al., 2006). We also report a negative correlation between haemoparasite presence/diversity and the ability of the animals to neutralize anthrax lethal toxin.

The anti-PA antibodies reported in this study indicated that the animals in these parks are exposed to varying doses of *B. anthracis* spores and/or repeated exposures in the environment and are able to mount an immune response. These results build on existing evidence that herbivores exposed to sublethal doses of *B. anthracis* in the environment develop antibodies against the pathogen (Cizauskas et al., 2014). Moreover, this claim contradicts previous studies suggesting herbivores in anthrax endemic regions are susceptible and naïve to *B. anthracis* and die following severe and sudden exposure. These assumptions of previous studies were based on a lack of detectable anti-PA tires (Turnbull et al., 1992, Lembo et al., 2011). However, the current study and previous studies each used a different serological method namely indirect anti-PA ELISA, QuickELISA kit (Anthrax-PA kit, Immunetics, Incorporated, USA) (Lembo et al., 2011) and competitive indirect anti-PA ELISA (Turnbull et al., 1992) which could account for the different results. The competitive indirect anti-PA ELISA, unlike the indirect anti-PA ELISA requires a high quantity of antibodies for there to be a 0.2 OD difference between two consecutive dilutions due to the inhibited counterpart and are thus less sensitive than the latter (Cizauskas et al., 2014). The Quick ELISA kit also lacks the sensitivity to detect animals with low antibody titres (Cizauskas et al., 2014). The indirect ELISA used in this study was not without limitations as the conjugate will only allow optimal binding for specific species developed and closely related species (Feir et al., 1993, Smith, 2017). In this study, protein A/G conjugate was used for zebra and wildebeest while protein G was used for kudu and impala. These conjugates were tested against different wildlife species with varying binding ability (Feir et al., 1993, Smith, 2017,

Stöbel et al., 2002) and therefore species-specific conjugates to overcome this limitation need to be developed.

Both kudu and zebra in the two parks tested positive for the anti-PA antibodies. It was also noted that a higher proportion of kudu were positive for ELISA anti-PA antibodies in KNP than ENP (Figure 4.1 and Table 4.1). Anthrax outbreaks in kudu in KNP have been linked to the population of blowflies in the park (Basson et al., 2018, Braack and de Vos, 1987). Hugh-Jones and de Vos (2002) indicated that *Chrysomya spp.* blowflies feeding on anthrax carcasses in KNP deposit *B. anthracis* spores onto the leaves of trees/shrubs near the carcass at the height that kudu feed thereby creating a higher inoculum and exposure to the kudu in KNP. The increase in *B. anthracis* inoculum by the *Chrysomy*a flies on shrubs eaten by kudu browsers might cause the higher mortality rate of ~75% of recorded cases from 1960-1990s in KNP (de-Vos and Bryden, 1996) compared to the ~0.46% in ENP (Havarua et al., 2014, Turner et al., 2013). Although there is no available specific report of the blow-fly transmission pathway in ENP, Nalisa (2013) recorded the presence of *B. anthracis* in flies of the *Muscidae* and *Calliphoridae* families but these may be in significantly lesser number due to the landscape and climate of ENP compared to KNP. This suggests that kudus in ENP can be exposed but to a lesser extent due to a smaller amount of dissemination and inoculum through flies or other vectors depositing the spores on the leaves (Fasanella et al., 2010, Blackburn et al., 2014, Nalisa, 2013). The role of blowflies in the transmission of *B. anthracis* needs further investigation in ENP to allow comparison to KNP.

Watson and Keir (1994), suggested that herbivores can ingest a relatively large dose of spores in the environment that boost their immunity whereas significantly higher dose is required to kill the animals. If this is true, it can be hypothesized that higher spore doses for kudu are present in KNP than ENP, and combined with other factors like blowfly populations, these might cause exposure with larger mortalities in KNP due to higher numbers of spores ingested by kudu. Also, it has been shown in a previous study that an animal host may ingest a high amount of spores that pass through the digestive tract without any invasion or establishment or poor establishment resulting in a sublethal infection (Aloni-Grinstein et al., 2005). This might cause the high anti-PA titers in KNP kudu without mortality. Our study recorded kudu in KNP are 19.4 times more likely to be exposed to the pathogen than their counterparts in ENP, which is reflected by the kudu mortality data of approximately 75% of recorded cases in KNP (de-Vos and Bryden, 1996) while kudu in ENP contributes only 0.46% of recorded cases (Turner et al., 2013). Even though, recent report puts it that impala is now the most impacted host species in KNP and this may further reduce the percentage accorded to kudu in this park (Basson et al., 2018)

In ENP, anthrax affects mainly grazers rather than browsers (Turner et al., 2013). There is a report of kudu mortality in ENP due to anthrax which might indicate infrequent exposure. However, the anti-PA titres indicate that kudu in ENP is exposed to the *B. anthracis* spores in the environment. This suggests that kudus in ENP are exposed, but likely to a lesser dose by browsing/grazing on grasses, shrubs and forbs closer to the ground due to the vegetation in ENP (Owen-Smith, 1979, Owen-Smith and Cooper, 1985, du Plessis and Skinner, 1987, Owen-smith, 1990), but this needs to be further investigated. Furthermore, the low number of kudu cases reported in ENP over the years (Ebedes, 1976a, Havarua et al., 2014) might be underreported as they occur in the inaccessible woodlands regions of the park compared to the central region consisting of plain and open regions (le Roux et al., 1988, Berry and Jenssen, 1997).

Zebra in ENP had significantly higher antibodies titres than zebra in KNP (Figure 4.1, Table 4.1). A high number of zebra (82.5%) in ENP tested positive for anti-PA antibodies in this study. In a previous study conducted in ENP, 24 zebra sampled tested negative for anthrax anti-PA antibodies in ENP (Turnbull et al., 1992). Similarly, none of the 85 zebra tested positive in the Serengeti National Park in Tanzania using the QuickELISA kit (Anthrax-PA kit, Immunetics, Incorporated, USA) (Lembo et al., 2011). The two aforementioned negative results may be connected to the sensitivity of the assays used, as both may not be able to detect antibodies in sera with lower titres (Cizauskas et al., 2014). Cizauskas et al. (2014) suggested the difference between the antibody titres in animals in the Serengeti and ENP could be as a result of the concentration of the spores in the environment. Results of this study show that 82.5% of zebra in ENP tested positive using indirect anti-PA ELISA which was similar to Cizauskas et al. (2014) that demonstrated a 52-87% prevalence of anti-PA antibodies in zebra that were tested in ENP. Zebra in ENP are 3.1 times more likely to encounter this pathogen than zebra in KNP. This is reflected by the 55% of anthrax mortalities in ENP coming from the zebra population (Turner et al., 2013) compared to less than 10% of recorded mortalities in KNP (personal communication with Skukuza State Veterinary Services). The difference between the exposures and titres in the two populations of zebras could be associated with the spore concentration in the soil ingested during grazing (Turner et al., 2014) or interactions between zebra diet and foraging behaviour altering exposure risk (Havarua et al., 2014).

Secondly, the difference may also arise from behavioural- and ecological factors as well as climate extremes such as droughts and flooding (Hampson et al., 2011). Furthermore, the season of anthrax outbreaks between the two parks (de-Vos and Bryden, 1996, Turner et al., 2013) may also contribute towards the difference observed between animal species in the two parks. The results confirm that kudu in KNP and zebra in ENP are the most dominant affected species in each park followed by impala for KNP and wildebeest in ENP (de-Vos and Bryden, 1996, Turner

et al., 2013, Basson et al., 2018). These species are exposed to spores more often and results in antibodies (relatively higher antibody tires) against the pathogen. It can therefore be assumed that kudu in KNP and zebra in ENP with higher antibody titres encounter and ingest the pathogen more often with lethal doses in the environment resulting in the higher mortality rates.
It has been shown that the endemicity of *B. anthracis* in a number of regions is related to calcium concentrations and alkalinity of the soil (Van Ness, 1971, Hugh-Jones and Blackburn, 2009). Thus the spore concentration could be influenced by the soil chemistry as well as composition of the soils in these regions. The animals in the endemic region of KNP had higher antibody titres than animals in the non-endemic region of the park. These animals are 2.8 times more likely to be seropositive for *B. anthracis* anti-PA antibodies than animals in the non-endemic region of the park. This may suggest that the soil composition in KNP could be responsible for the difference between the endemic and non-endemic regions. Also, soil types supporting different strains and dissemination during anthrax outbreaks have been reported (Smith et al., 2000, Blackburn et al., 2007, Garofolo et al., 2010). Ancient A (A.Br005/005) and B clade strains occur in KNP (Smith et al., 2000). Northern KNP, lying between the Limpopo and Luvuvhu rivers has a higher diversity of strains with Pafuri in a lower elevation (with a high diversity of strains consisting of A.Br005/006, A.Br.001/002, A.Br.V770 SNP subclades and B-clade) (Lekota, 2018) surrounded by higher elevations (Figure 3.3). This may explain why there was a significant difference between the two regions in KNP if the non-endemic area is less conducive to spore survival than the endemic area (Smith et al., 2000). Also, with the presence of physical barriers such as rivers in KNP, long-range movement of animals is restricted (Personal communication with Skukuza State Veterinary services). Also, home ranges are much smaller in KNP (Huang Yen-Hua unpublished data of monthly home range size). This may also be responsible for the difference noted as there is restriction to spore distribution.

The result of seropositive zebras and kudu in KNP shows that animals are also exposed in the "non-endemic" (southern KNP) area though in lesser proportions, and cases could be underreported due to surveillance bias in an area considered as non-endemic for anthrax. Steenkamp et al. (2018) identified the region where 'non-endemic' kudu were sampled and tested seropositive as *B. anthracis* spore suitability regions (Figure 4.3). Also, previous anthrax reports from KNP show that outbreaks in 1960 spread from the north downwards to the central part of KNP (Pienaar, 1961). Passive surveillance conducted in KNP from 1988 – 2019 shows that over 50% of samples received are from the northern part and merely 15.8% are from the southern regions located within the "non-endemic" study area, while the remaining percentage is from the central regions of the park (Personal communication with Skukuza State Veterinary services).

In contrast, there was no significant difference in antibody titres in animals in the endemic and non-endemic regions of ENP. The absence of a significant difference in the titers between the animals in the endemic and non-endemic regions of ENP could be a result of the absence of any physical barrier (such as rivers) that would prevent or slow movement between the west and central regions of the park, and thus animals are able to move across regions (Hipondoka et al., 2013, Zidon et al., 2017)Haung Yen-Hua, unpublished data). Secondly, animals in ENP have very large home ranges and animals do move between the western and central parts of the park (Huang Yen-Hua, unpublished data). A study suggested that spores could concentrate more in the waterholes dispersed in the western part of ENP as 26% of waterholes in the western part tested positive for anthrax spores (Lindeque and Turnbull, 1994). However Turner et al. (2016), found that spore concentrations in waterhole sediments are too low for lethal exposures. Also, Cloete (2013) reported that there was no significant difference in spore survival by soil types sampled from different regions of the park. Together, these results suggest that the whole park is a suitable habitat for *B. anthracis* without barriers to restrict herbivore movement or spore distribution. Thus, most of the ENP could be endemic for anthrax but cases in the west could be underreported due to inadequate surveillance over time due to accessibility to this area. More studies should focus on anthrax occurrence in the western part of ENP.

In ENP, a single SNP group (A.Br.Aust94) are reported. Two other SNP groups in Namibia, group 6 (Ancient A; A.Br.005/006) and group 9 (Sterne; A.Br.001/002) were found in Namibia but not in ENP (Beyer et al., 2012). This aligns with the hypothesis of a clonal expansion of an ancestral strain , which was brought into ENP a long time ago with no inflow or outflow of rivers (one catchment area), although with some seasonal rivers feeding into the Etosha salt pan. This may explain why there was no difference between the two regions in ENP as the whole park could be inhabited by a clonal strain.

Within each park, there were also considerable differences between the major host species (kudu and zebra). Kudu in KNP and zebra in ENP had higher seroprevalences than zebra in KNP and kudu in ENP. Thus the same species within a different ecosystem can be affected differently as observed in the literature (Lindeque and Turnbull, 1994). Due to the complexity of the *B. anthracis* life cycle, many factors could be responsible for this disparity, some of which could be host-dependent variations in exposure and susceptibility which could be linked to ecological and behaviour related traits (Hampson et al., 2011). Another driver of this difference between species within the same park could be related to the foraging behaviours of these animals (Havarua et al., 2014). Outbreaks in zebra populations have been shown to occur mostly during the wet season or towards the end of the rainy season , with some cases occurring during droughts or extended

dry period (Muoria et al., 2007, Wafula et al., 2008). Whereas, outbreaks in kudu occur largely during the dry season as seen in KNP and other parks (Pienaar, 1960, Pienaar, 1961, de-Vos, 1990, Turnbull et al., 1991, de-Vos and Bryden, 1996). The grazing versus browsing transmission pathways occur at different timescales, which may have important effects on disease dynamics, pathogen diversity, and host resistance. Browsing-based transmission should occur shortly after host death before rainfall or leaf loss by deciduous trees/shrubs prevents further browser exposure (Ebedes, 1976a). Grazing-based transmission occurs only upon the regeneration of vegetation at a carcass site, and continues for years, with exposure dose decaying over time (Turner et al., 2014, Turner et al., 2016). This could mean that different herbivore species in the same ecosystem could be affected at different times and at different rates, based on differences in their diet.

Toxin Neutralisation

The TNA was performed to measure the immune performance between kudu and zebra in each park and between parks. The TNA determines the protection of mouse macrophages by antibodies in host sera from the cytotoxic effect of the anthrax lethal toxin (Ngundi et al., 2010) and is not species-specific (Omland et al., 2008). The assay quantitates only the functional subunit of the antibodies compared to the total anti-PA antibodies detected by ELISA (Ngundi et al., 2010). This study represents the first to measure the immune performance of these host species against *B. anthracis* lethal toxin.

The two main host species from the two parks demonstrated interesting variation in levels of neutralisation. Lincoln et al (1967) hypothesized that there is a trade-off between infection resistance and toxin susceptibility. Hosts that are more resistant to infection require a higher exposure dose for mortality, yet are highly susceptible to the anthrax toxin (i.e. die with low levels of bacteremia) once an infection is established. Susceptible hosts, that become infected with exposure to a lower dose are in turn more resistant to the effects of the toxin and thus die with high terminal bacteremia.

Kudu in ENP had a higher TNA response than kudu in KNP. Similarly, zebra in KNP had a higher immune response against the toxin than zebras in ENP. This suggests that, at the species level, the more often a species is exposed to *B. anthracis*, the less it can cope with the effects of its toxin. These results agree with the mortality records of these species in the two parks (de-Vos and Bryden, 1996, Turner et al., 2013, Havarua et al., 2014). In view of the hypothesis concerning dose in our study, zebra in ENP as well as kudu in KNP are exposed more often, likely to larger doses, and die more readily compared to kudu in ENP and zebra in KNP. Thus, kudu in ENP and zebra in KNP are more likely to be exposed to trace amounts of the pathogen based on their foraging behaviour and the relative risk of that behaviour on the two landscapes (de-Vos and

Bryden, 1996, Turner et al., 2013, Havarua et al., 2014), yet show greater resistance than kudus in KNP and zebras ENP and respectively.
Since higher percentages of anthrax mortalities for zebra in ENP and kudu in KNP contribute to the total mortalities recorded in the respective parks, these species are more likely to be exposed to the spores at higher concentrations and at more frequent intervals. We, therefore, hypothesize that animals that are exposed to higher doses of the anthrax antigen are less able to produce antibodies with high affinity (Eisen and Siskind, 1964). This further means that the more antigens encountered from frequent and large exposures the less time or resources are available to mature the high-affinity antibodies that are required to bind and neutralize the toxin. The affinity maturation process allows for high-affinity antibodies to be produced and this process takes place in the germinal centres which are effectually activated structures found in the secondary lymphoid tissues following stimulation by the presence of an antigen (Shlomchik and Weisel, 2012). Production of viable antibodies through affinity maturation which is a mutation-selection process is very important in the stimulation of a very effective immune response (Eisen and Siskind, 1964, Wang et al., 2015). When the concentration of the antigen is high or ingested more frequently, this leads to very low competition among the B-cells and the germinal centres become occupied with antibodies that have a very low affinity (Eisen and Siskind, 1964, Goidl et al., 1968, Wang et al., 2015). Dumas et al. (2020) also suggested that a higher immune response is derived from severe disease caused by exposure to a high amount of antigen over longer periods of time. Thus, we assume that the low neutralisation in zebra from ENP could be due to exposure to high doses of antigen in the environment leading to less competition among the antibodies as such leading to lower affinity and hence lower neutralisation.

A previous study showed that animals that were immunized with antigens of spore origin conferred protection by the production of antibodies that reduced spore germination (Oscherwitz et al., 2015). This sublethal passive natural “vaccination” may have induced anti-spore antibodies and reduced germination in zebra in KNP and kudu in ENP, but this will need further investigation. From the above-mentioned reasons, variability in the kinetics of the antibody affinity maturation process and anti-spore activities in the animals sampled may add to the diversity of the neutralizing ability observed (Ngundi et al., 2010).

Within the same park, it was seen that zebra in KNP and kudu in ENP had higher proportions of animals that neutralized the lethal toxin and demonstrated higher titres than kudu in KNP and zebra in ENP, respectively. The better neutralisation could be connected to their ability to be better protected from the effect of the lethal toxin (Weiss et al., 2006, Peachman et al., 2006). This ability to be protected may be caused by their ability to develop neutralizing titres of high

affinity (Ngundi et al., 2010). As earlier discussed, a relationship has been established between antigen dose, "immunization" (exposure) interval and development of antibodies with high affinity (Eisen and Siskind, 1964, Goidl et al., 1968). This relationship may play a role in animals with higher neutralisation that may have moderate doses and at longer intervals. Also, Verma et al. (2009) suggested that factors such as the characteristics of the antibodies (such as the species of origin, subclasses and isotype) being examined in the test could largely affect the measure to which Fc-gamma receptor-reliant neutralisation can influence the extent of neutralisation. As such, we suspect that species idiosyncrasies could have also played a role in the differences observed.

Another hypothesis to the reason zebra in ENP and other animals that showed anti-PA titres without neutralizing titres might be due to cross-reaction with closely related antigens to *B. anthracis* PA (Dumas et al., 2020), which needs further investigation. Cross-reactivity will reflect on the sensitivity of the technique (PA-ELISA). *Bacillus cereus* biovar *anthracis* and atypical *B. cereus* have been reported to cause anthrax-like infections in humans and animals (Marston et al., 2016, Antonation et al., 2016, Baldwin, 2020). Furthermore, members of *B. cereus sensu stricto* have been reported to be closely related to *B. anthracis* (van Tongeren et al., 2014). Future study will investigate *B. cereus* isolates with similar *B. anthracis* pag gene. Since TNA quantitates only the neutralizing antibodies in a serum, it may account for the ELISA positive samples that were negative for TNA (Ngundi et al., 2010). Kudu in ENP (Havarua et al., 2014) and zebra in ENP (personal communication with Skukuza Veterinary Services) are considered as less susceptible (not major hosts) hosts species in these parks. We suggest that their ability to mount neutralizing immune responses against the toxin could be to an extent responsible for their protection (Reuveny et al., 2001, Weiss et al., 2006, Peachman et al., 2006).

Some studies have demonstrated a correlation between anti-PA titres and the neutralizing titres (Parreiras et al., 2009, Ndumnego et al., 2013). Ndumnego et al. (2013) quantified the anti-PA IgG titres and reported a high correlation with neutralizing antibodies in vaccinated goats. Parreiras et al. (2009) compared anti-PA ELISA and TNA in mice vaccinated with PA. Furthermore, other laboratory studies reported that lethal toxin neutralizing antibodies in rabbits (Weiss et al., 2006, Little et al., 2004), guinea pigs (Reuveny et al., 2001) and mice (Peachman et al., 2006) correlated with survival rates of these animals. In our study, a significant positive correlation was found between the anti-PA ELISA titres and the toxin neutralizing NT50 titres in animals that naturally acquired the antigen, despite differences in zebra in ENP and kudu in KNP. Not all seropositive animals based on anti-PA ELISA titres showed neutralizing activities, but most animals with neutralizing activities had a high anti-PA titre. Owing to the fact that most studies previously conducted were controlled laboratory studies, there will be a need to further

investigate this correlation in field studies. This further confirms the presence of the *B. anthracis* lethal toxin in the animal sera and the neutralizing titres are a subset of the total antibodies present (Ngundi et al., 2010). The agreement and correlation between anti-PA tires and neutralizing antibodies validate these tests in these wild herbivores.

Haemoparasites co-infection vs Anthrax toxin neutralisation

Tick-transmitted diseases pose serious threats to livestock farming and wildlife populations (Mbati et al., 2002, Nijhof et al., 2005). The wild animals sampled in this study reacted to the 4 genera of haemoparasite tested: *Theileria, Babesia, Anaplasma and Ehrlichia.* Theileriosis is an important disease that is capable of inducing fatal loss in African antelopes and has also been reported in wild equids. *Theileria* are obligate intracellular organisms that live in lymphocytes and erythrocytes of the host (McKeever, 2009). Previous studies have reported lethal Theileriosis in kudu, sable and gray duiker (Nijhof et al., 2005), eland, roan (Steyl et al., 2012) and wildebeest (Dondona et al., 2012). Some of the pathogenic *Theileria* species of the African antelopes include *Theileria* sp.(sable) (Nijhof et al., 2005, Steyl et al., 2012), *Theileria* sp.(gray duiker) (Nijhof et al., 2005), and *Theileria* sp. (kudu) (Nijhof et al., 2005). *Theileria equi, which* is considered highly pathogenic has also been found in zebra (Lampen et al., 2009, Laus et al., 2015). In a study done by Hawkins et al. (2014) on the prevalence of *T. equi* and *Babesia caballi*, they demonstrated that 72% (N=71) of donkeys and 100% (N=16) of Grevy's zebras were positive for *T. equi*. In our study, we found an overall prevalence of 65.3%(N=196) for *Theileria* infections. *Theileria* species were found in kudu, zebra, impala and wildebeest. *Theileria* sp. (sable) was found in kudu, impala and wildebeest, *Theileria* sp. (kudu) was found in only kudu and *T. separata* was only detected in wildebeest. Although zebra were predominantly infected with *Theileria equi*, hybridization to genera-specific *Theileria* sp. oligonucleotide probes was observed. We, therefore, assume that infection with *Theileria* species could increase host susceptibility to other pathogens given that *Theileria* inhabits and destroy white and red cells (McKeever, 2009).Although a limitation to this hypothesis is the fact that the technique employed in the detection of these parasites does not distinguish between sick and asymptomatic carriers and this will require further investigation.

Babesiosis is a very important disease in livestock, wildlife and humans, which is caused by the haemoprotozoan of the genus *Babesia* (Bock et al., 2004, Thomas et al., 1982, Gray, 2006). In this study, most of the zebras reacted to the *Babesia* genus-specific oligonucleotide probe. This agrees with other studies that identified *Babesia* species in equids such as horses, zebra, donkey (Rothschild, 2013, Onyiche et al., 2019, Bhoora, 2010). While other studies have reported babesiosis in kudu, impala, wildebeest, tsessebe (*Damaliscus lunatus*), eland (Carmichael and

Hobday, 1975), roan (Oosthuizen et al., 2009), sable (Oosthuizen et al., 2008) and red deer (*Cervus elaphus*) (Zintl et al., 2011), with the exception of one kudu, the antelopes in this study were negative for babesiosis. The absence of the *Babesia* species in these antelopes in these parks might be partly that the RLB was not sensitive enough to detect these parasites (Bhoora, 2010). Secondly, it could be connected to the preference of the Ixodid ticks in the regions as to the best of our knowledge, no *Babesia* species have been reported in the antelopes in these parks.

Anaplasmosis is known to have a devastating effect on domestic livestock leading to great economic loss with 99% of cattle in South Africa being at risk and especially common in the south of the Sahara and endemic in South Africa (de Waal, 2000). In this study, we detected *Anaplasma* species in kudu, impala and zebra, which corroborated the findings of Ngeranwa et al. (2008) that reported the prevalence of *Anaplasmaanaplasmosis* in these species. AnaplasmaAnaplasmosis has also been reported in buffalo, blue and black wildebeest, waterbuck and eland in provinces in South Africa (Eygelaar et al., 2015, Khumalo et al., 2017), although we did not detect it in the wildebeest sampled in ENP. *Anaplasma* species are obligate intracellular organisms that parasitize the erythrocytes, granulocytes and monocytes of their host (Woldehiwet, 1987, Woldehiwet and Ristic, 1993, Dumler et al., 2001, Aubry and Geale, 2011). Some species of this organism can cause anaemia (Ristic, 1981) and or leucopenia thereby causing immunosuppression (Woldehiwet, 1987, Woldehiwet, 2008).

Ehrlichiosis *Ehrlichia* is a Rickettsial haemoparasite that affects livestock (Van Winkelhoff and Uilenberg, 1981), wildlife and humans (Groen et al., 2002). This disease is caused by the rickettsia of the genus *Ehrlichia* (Dumler et al., 2001) and is extensively distributed across the world and endemic in Africa (Provost and Bezuidenhout, 1987). Interestingly, *Ehrlichia ruminantium* was found only in zebras from KNP and to the best of our knowledge, this is the first report of *E. ruminantium* in this species. *Amblyoma* ticks responsible for the transmission of this pathogen have been reported in KNP and could therefore explain the detection of this parasite in zebra (Horak et al., 2011). King'ori et al. (2019) reported a prevalence of 5.8% (N=66) in elephants in Kenya. Susceptibility of African and non-African wildlife infected either experimentally or naturally with these parasites, have been reported. This includes white tailed-deer (*Odocoileus virginianus*) in the United States of America (Dardiri et al., 1987), and other African wildlife such as; kudu, blue wildebeest, eland and giraffe (*Giraffa camelopardalis*) (Peter et al., 1998), impala (Peter et al., 1999) and donkeys (Halajian et al., 2018).

Haemoparasites of the genera *Theileria, Babesia, Anaplasma* and *Ehrlichia* have been reported to have debilitating effects on the health of domestic animals (Mbati et al., 2002). Most African wildlife are considered to be reservoirs and only suffer deleterious effects under stressful

conditions (Nijhof et al., 2005). But it has also been shown that these animals can suffer fulminating TBDs (Oosthuizen et al., 2009, Steyl et al., 2012).

We noticed in this study that the animals in ENP reacted mostly to the genus-specific probes with very few animals reacting to species-specific probes as compared to the animals in KNP. The kudu in ENP did not react to any species-specific probe while a large proportion of kudu in KNP reacted to species-specific probes. We assume that this could be because the probes used for this assay were largely created from haemoparasites found in South Africa and they may vary from the strains or species found in ENP (Sibeko et al., 2008). It could also be that the parasitaemia was lower in the animals in ENP (Hanafusa et al., 1998, Bhoora, 2010).

In ENP a higher tick burden in zebra (data not shown) than kudu and wildebeest in the same park, possibly contributed to the higher amount of parasites detected or higher parasite diversity (Horak et al., 1983, Nijhof et al., 2003, Dondona et al., 2012, Raboloko et al., 2020). Although tick-burdens were not noted for animals in KNP, we hypothesize that there is a correlation between tick burden and parasite prevalence (Byaruhanga et al., 2015, Byaruhanga et al., 2016, Tembo et al., 2018) which will be investigated in future.

It is hypothesized that zebra in ENP and kudu in KNP are under significantly increased levels of stress imposed by higher tick burdens (Dondona et al., 2012, Raboloko et al., 2020) and therefore haemoparasite infections, than the other species and are thus more likely to succumb to other diseases. We assume that the ability to mount an adequate immune response against other pathogens could be suppressed by the higher parasite burden in these animals (McKeever, 2009). These parasites can infect and destroy red blood cells and/ or the white cells of different wildlife, livestock and humans (Duh et al., 2008). Protozoal infections often lead to some level of immunosuppression of the host. This results in delayed identification of antigens of different variation and ultimately the inability of the immune system to elicit an appropriate response (Seed, 1996). These parasites exert this immunosuppression mechanism by either manufacturing-specific immune suppressors or by the multiplication of suppressor T-cells or macrophages (Erard and Le Gros, 1994). The production of regulatory cytokines prevents the defence system by releasing cytokines of the regulatory type (Erard and Le Gros, 1994). A number of these parasites invade and multiply within host cells such as the red blood cells (Hadley, 1986, Brown, 2012), white blood cells (Duh et al., 2008, Brown, 2012) and macrophages as in the case of some *Theileria* (Dyer and Tait, 1987) and in most cases leading to anaemia (Hooshmand-Rad, 1976) and leukopenia (Dumler et al., 2005).

Most humans and animals alike are often presented with an enormous number of parasites showing a very high prevalence of concomitant infections (Christensen et al., 1987, Lello and Hussell, 2008). These interactions often modulate and/ or alter the immunity of the host and hence increasing susceptibility of the host to one or the other pathogen (Chen et al., 2005) or increase the severity of other pathogenic organisms (Borkow et al., 2001). Co-infection with tick-borne diseases (TBDs) has also been shown to exacerbate diseases and leading to higher fatality (Munson et al., 2008). Thus, we investigated the correlation between the diversity (number of haemoparasites genera) identified and the toxin neutralisation titres of these animals. Animals infected with more haemoparasites showed less toxin neutralisation (lower NT50 titres) and vice versa. This negative relationship was observed across the animals. These findings reflect a correlation but not necessarily a causal effect. Although, we hypothesize that with such a significant negative correlation, there might be some coinfection interplays that are not being accounted for, which will be the subject of future research. As previously mentioned, some studies have demonstrated the effect of coinfections on the host and how it hampers immune response against other pathogens. Munson et al. (2008) showed that coinfection can cause immunosuppression and exacerbate the effect of the other pathogen. Other studies have also shown that in situations of concomitant infections, infectious organisms can compete for niches in the host (Lello et al., 2008), lead to an intensification of the severity of disease pathology (Borkow et al., 2001, Chen et al., 2005, Hughes and Shafran, 2006), increase the susceptibility of the host to other pathogens (Chen et al., 2005) and also lengthen the course of the infection (Pathak et al., 2012).

Within-guild coinfections and interactions have been shown to also have a devastating effect on the host's immunity (McKeever, 2009, Henrichs et al., 2016). Owing to the ability of *Theileria* species to inhabit and destroy the host's white blood cells that are responsible for cellular immunity, this may lead to immunosuppression. This action can allow *Anaplasma* species for example and other pathogens to colonize the host's system (Henrichs et al., 2016). It was also demonstrated by Henrichs et al. (2016) that there is a considerable increase in the odds of infection with *T. parva* in an animal infected with *A. marginale*. It has also been hypothesized that excessive and sustained inflammatory responses to protozoans like *Plasmodium* can cause severe immunopathology (Mabbott, 2018) and this can, in turn, increase the effect of other pathogens in the host. As was noted in this study, there was a negative relationship between the odds of infection with haemoparasites and the ability to neutralize the lethal toxin. An increase in toxin neutralisation was significantly associated with less likelihood of infection with *Babesia, Theileria, Ehrlichia,* and *Anaplasma* parasites in zebras and kudus except for the absence of *Babesia* in kudus. This further confirms that there could be some co-infection interplay that has

not been fully unravelled. Further investigations are required into which combination of parasites and what parasite loads could potentially affect this response.

5.2 Conclusion

The comparison between the endemic and non-endemic regions within parks reveals that animals in endemic regions of both parks put together were more exposed than those in non-endemic regions. Although a narrow look reveals that ENP is entirely endemic as there was almost similar exposures between both regions. This is largely attributed to the fact the entire park is suitable for spore germination and absence of physical barriers makes spore distribution easy.

Between parks where a given species is considered to be a more (ENP: zebra, KNP: kudu) or less (ENP: kudu, KNP: zebra) susceptible host, it was seen that kudu in KNP and zebra in ENP are more likely to be exposed to the antigen by ingesting higher doses or encountering the antigen more regularly than those of ENP and KNP respectively. This is mainly due to the foraging behaviour of the animals as well as the presence of factors and vectors responsible for transmission and dispersal of the anthrax spores.

Varying levels of toxin neutralisation titres were reported with higher neutralizing titres in a higher proportion of zebras in KNP than ENP. This supports the species differences in mortality data in the two parks, such that animals that have higher anthrax mortality showed lesser neutralisation. Sampled kudu in ENP and zebra in KNP are more able to mount a successful immune response against the anthrax toxin than their respective counterparts in the respective parks. The ability to mount successful neutralizing activity could have emanated from the ability of the animals to produce neutralizing antibodies with high affinity. This ability is largely influenced by the dose of the antigen and interval between exposures. This trend was also observed within parks in that zebra in KNP showed better neutralisation than the kudu in the same park. Similarly, kudu in ENP are better able to neutralize the toxin than zebra in the same park.

Even though positive correlation between the antibody titres and the NT50 titres were reported in this study, anti-PA antibodies do not necessarily translate to the presence of neutralizing antibodies which could be influenced by various factors. These factors include the dose of the antigen; antibody properties (species of origin, subclasses and isotype); the interval and frequency of exposure; cross-reactivity with other pathogens and pathogen or immune system burden.

Haemoparasites were detected in the host species in ENP and KNP. Animals with a lower haemoparasite diversity were better able to neutralize the anthrax lethal toxin. So many factors

could be responsible for this effect some of which include the parasite burden, within guild co-infections, co-infections interplay between the haemoparasites and *B. anthracis.*

5.3 Future Recommendations

The finding of this study reveals that animals in both regions of the parks are exposed to anthrax spores in the environment. Further investigation could investigate the detection of *B. anthracis* in the non-endemic regions of these parks as well as the detection of closely related *B. cereus* species in the parks.

Environmental conditions like foraging, rainfall, landscape, biological vectors of the same animal host species should be compared between parks to establish the reason(s) for the variation in the exposure status of the same animal species in the two parks.

Lastly, we noticed a correlational interplay that requires further investigation between a coinfection with haemoparasites and *B. anthracis* to determine which combination of parasites and also what parasite load could potentially affect the neutralizing ability of the host against anthrax toxins.

References

ALONI-GRINSTEIN, R., GAT, O., ALTBOUM, Z., VELAN, B., COHEN, S. & SHAFFERMAN, A. 2005. Oral Spore Vaccine Based on Live Attenuated Nontoxinogenic Bacillus anthracis Expressing Recombinant Mutant Protective Antigen. *Infection and immunity,* 73**,** 4043-53.

ANDERSON, K., EZENWA, V. O. & JOLLES, A. E. 2013. Tick infestation patterns in free ranging African buffalo (Syncercus caffer): Effects of host innate immunity and niche segregation among tick species. *International Journal for Parasitology: Parasites and Wildlife,* 2**,** 1-9.

ANTONATION, K. S., GRÜTZMACHER, K., DUPKE, S., MABON, P., ZIMMERMANN, F., LANKESTER, F., PELLER, T., FEISTNER, A., TODD, A., HERBINGER, I., DE NYS, H. M., MUYEMBE-TAMFUN, J.-J., KARHEMERE, S., WITTIG, R. M., COUACY-HYMANN, E., GRUNOW, R., CALVIGNAC-SPENCER, S., CORBETT, C. R., KLEE, S. R. & LEENDERTZ, F. H. 2016. Bacillus cereus Biovar Anthracis Causing Anthrax in Sub-Saharan Africa-Chromosomal Monophyly and Broad Geographic Distribution. *PLoS neglected tropical diseases,* 10**,** e0004923-e0004923.

AUBRY, P. & GEALE, D. W. 2011. A review of bovine anaplasmosis. *Transbound Emerg Dis,* 58**,** 1-30.

AYDIN, S. 2015. A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides,* 72**,** 4-15.

BAGAMIAN, K. H., ALEXANDER, K. A., HADFIELD, T. L. & BLACKBURN, J. K. 2013. Ante- and postmortem diagnostic techniques for anthrax: rethinking pathogen exposure and the geographic extent of the disease in wildlife. *J Wildl Dis,* 49**,** 786-801.

BAILLIE, L. W., HUWAR, T. B., MOORE, S., MELLADO-SANCHEZ, G., RODRIGUEZ, L., NEESON, B. N., FLICK-SMITH, H. C., JENNER, D. C., ATKINS, H. S., INGRAM, R. J., ALTMANN, D. M., NATARO, J. P. & PASETTI, M. F. 2010. An anthrax subunit vaccine candidate based on protective regions of Bacillus anthracis protective antigen and lethal factor. *Vaccine,* 28**,** 6740-8.

BALDWIN, V. M. 2020. You Can't B. cereus - A Review of Bacillus cereus Strains That Cause Anthrax-Like Disease. *Front Microbiol,* 11**,** 1731.

BARTH, H., AKTORIES, K., POPOFF, M. R. & STILES, B. G. 2004. Binary bacterial toxins: biochemistry, biology, and applications of common Clostridium and Bacillus proteins. *Microbiol Mol Biol Rev,* 68**,** 373-402, table of contents.

BASSON, L., HASSIM, A., DEKKER, A., GILBERT, A., BEYER, W., ROSSOUW, J. & HEERDEN, H. V. 2018. Blowflies as vectors of Bacillus anthracis in the Kruger National Park. *Koedoe: African Protected Area Conservation and Science,* 60**,** 1-6.

BEKKER, C. P., DE VOS, S., TAOUFIK, A., SPARAGANO, O. A. & JONGEJAN, F. 2002. Simultaneous detection of Anaplasma and Ehrlichia species in ruminants and detection of Ehrlichia ruminantium in Amblyomma variegatum ticks by reverse line blot hybridization. *Vet Microbiol,* 89**,** 223-38.

BELLAN, S. E., CIZAUSKAS, C. A., MIYEN, J., EBERSOHN, K., KÜSTERS, M., PRAGER, K. C., VAN VUUREN, M., SABETA, C. & GETZ, W. M. 2012. BLACK-BACKED JACKAL EXPOSURE TO RABIES VIRUS, CANINE DISTEMPER VIRUS, AND BACILLUS ANTHRACIS IN ETOSHA NATIONAL PARK, NAMIBIA. *J Wildl Dis,* 48**,** 371-381.

BELLAN, S. E., TURNBULL, P. C. B., BEYER, W. & GETZ, W. M. 2013. Effects of experimental exclusion of scavengers from carcasses of anthrax-infected herbivores on Bacillus anthracis sporulation, survival, and distribution. *Applied and environmental microbiology,* 79**,** 3756-3761.

BERRY, J. & JENSSEN, H. Aspects of wildebeest Connochaetes taurinus ecology in the Etosha National Park - a synthesis for future management. 1997.

BEYER, W., BELLAN, S., EBERLE, G., GANZ, H. H., GETZ, W. M., HAUMACHER, R., HILSS, K. A., KILIAN, W., LAZAK, J., TURNER, W. C. & TURNBULL, P. C. B. 2012. Distribution and molecular evolution of bacillus anthracis genotypes in Namibia. *PLoS neglected tropical diseases,* 6**,** e1534-e1534.

BEYER, W. & TURNBULL, P. C. B. 2009. Anthrax in animals. *Molecular Aspects of Medicine,* 30**,** 481-489.

BEYER, W., VAN HEERDEN, H., BASSON, L., GILBERT, A., DEKKER, A., ROSSOUW, J. & HASSIM, A. 2018. Blowflies as vectors of Bacillus anthracis in the Kruger National Park. *Koedoe : African Protected Area Conservation and Science,* 60**,** 1-6.

BHOORA, R. 2010. Molecular characterization of Babesia caballi and Theileria equi , the aetiological agents of equine piroplasmosis, in South Africa.

BLACKBURN, J. K., CURTIS, A., HADFIELD, T. L., O'SHEA, B., MITCHELL, M. A. & HUGH-JONES, M. E. 2010. Confirmation of Bacillus anthracis from flesh-eating flies collected during a West Texas anthrax season. *J Wildl Dis,* 46**,** 918-22.

BLACKBURN, J. K., MCNYSET, K. M., CURTIS, A. & HUGH-JONES, M. E. 2007. Modeling the geographic distribution of Bacillus anthracis, the causative agent of anthrax disease, for the contiguous United States using predictive ecological [corrected] niche modeling. *Am J Trop Med Hyg,* 77**,** 1103-10.

BLACKBURN, J. K., VAN ERT, M., MULLINS, J. C., HADFIELD, T. L. & HUGH-JONES, M. E. 2014. The necrophagous fly anthrax transmission pathway: empirical and genetic evidence from wildlife epizootics. *Vector Borne Zoonotic Dis,* 14**,** 576-83.

BOCK, R., JACKSON, L., DE VOS, A. & JORGENSEN, W. 2004. Babesiosis of cattle. *Parasitology,* 129 Suppl**,** S247-69.

BORKOW, G., WEISMAN, Z., LENG, Q., STEIN, M., KALINKOVICH, A., WOLDAY, D. & BENTWICH, Z. 2001. Helminths, human immunodeficiency virus and tuberculosis. *Scand J Infect Dis,* 33**,** 568-71.

BOSMAN, A. M., VENTER, E. H. & PENZHORN, B. L. 2007. Occurrence of Babesia felis and Babesia leo in various wild felid species and domestic cats in Southern Africa, based on reverse line blot analysis. *Veterinary Parasitology,* 144**,** 33-38.

BRAACK, L. E. & DE VOS, V. 1987. Seasonal abundance of carrion-frequenting blow-flies (Diptera: Calliphoridae) in the Kruger National Park. *The Onderstepoort journal of veterinary research,* 54**,** 591-597.

BRAACK, L. E. & DE VOS, V. 1990. Feeding habits and flight range of blow-flies (Chrysomyia spp.) in relation to anthrax transmission in the Kruger National Park, South Africa. *Onderstepoort J Vet Res,* 57**,** 141-2.

BROTHERS, P. S., COLLINS, N. E., OOSTHUIZEN, M. C., BHOORA, R., TROSKIE, M. & PENZHORN, B. L. 2011. Occurrence of blood-borne tick-transmitted parasites in common tsessebe (Damaliscus lunatus) antelope in Northern Cape Province, South Africa. *Vet Parasitol,* 183**,** 160-5.

BROWN, W. C. 2012. Adaptive immunity to Anaplasma pathogens and immune dysregulation: implications for bacterial persistence. *Comp Immunol Microbiol Infect Dis,* 35**,** 241-52.

BUTLER, C. M., NIJHOF, A. M., VAN DER KOLK, J. H., DE HASETH, O. B., TAOUFIK, A., JONGEJAN, F. & HOUWERS, D. J. 2008. Repeated high dose imidocarb dipropionate treatment did not eliminate Babesia caballi from naturally infected horses as determined by PCR-reverse line blot hybridization. *Veterinary Parasitology,* 151**,** 320-322.

BYARUHANGA, C., COLLINS, N. E., KNOBEL, D., CHAISI, M. E., VORSTER, I., STEYN, H. C. & OOSTHUIZEN, M. C. 2016. Molecular investigation of tick-borne haemoparasite infections among transhumant zebu cattle in Karamoja Region, Uganda. *Veterinary Parasitology: Regional Studies and Reports,* 3-4**,** 27-35.

BYARUHANGA, C., COLLINS, N. E., KNOBEL, D., KABASA, W. & OOSTHUIZEN, M. C. 2015. Endemic status of tick-borne infections and tick species diversity among transhumant zebu cattle in Karamoja Region, Uganda: Support for control approaches. *Vet Parasitol Reg Stud Reports,* 1-2**,** 21-30.

CARMICHAEL, I. H. & HOBDAY, E. 1975. Blood parasites of some wild bovidae in Botswana. *Onderstepoort J Vet Res,* 42**,** 55-62.

CHAISI, M. E., BAXTER, J. R., HOVE, P., CHOOPA, C. N., OOSTHUIZEN, M. C., BRAYTON, K. A., KHUMALO, Z. T. H., MUTSHEMBELE, A. M., MTSHALI, M. S. & COLLINS, N. E. 2017. Comparison of three nucleic acid-based tests for detecting Anaplasma marginale and Anaplasma centrale in cattle. *2017,* 84.

CHEN, C. C., LOUIE, S., MCCORMICK, B., WALKER, W. A. & SHI, H. N. 2005. Concurrent infection with an intestinal helminth parasite impairs host resistance to enteric Citrobacter rodentium and enhances Citrobacter-induced colitis in mice. *Infect Immun,* 73**,** 5468-81.

CHIKEREMA, S. M., PFUKENYI, D. M., MATOPE, G. & BHEBHE, E. 2012. Temporal and spatial distribution of cattle anthrax outbreaks in Zimbabwe between 1967 and 2006. *Trop Anim Health Prod,* 44**,** 63-70.

CHRISTENSEN, N. Ø., NANSEN, P., FAGBEMI, B. O. & MONRAD, J. 1987. Heterologous antagonistic and synergistic interactions between helminths and between helminths and protozoans in concurrent experimental infection of mammalian hosts. *Parasitology Research,* 73**,** 387-410.

CIZAUSKAS, C. A., BELLAN, S. E., TURNER, W. C., VANCE, R. E. & GETZ, W. M. 2014. Frequent and seasonally variable sublethal anthrax infections are accompanied by short-lived immunity in an endemic system. *The Journal of animal ecology,* 83**,** 1078-1090.

CIZAUSKAS, C. A., TURNER, W. C., PITTS, N. & GETZ, W. M. 2015. Seasonal patterns of hormones, macroparasites, and microparasites in wild African ungulates: the interplay among stress, reproduction, and disease. *PLoS One,* 10**,** e0120800.

CLAIRE, H. 2020. *Enzyme-linked immunosorbent assay (ELISA)* [Online]. Available: https://www.immunology.org/public-information/bitesized-immunology/experimental-techniques/enzyme-linked-immunosorbent-assay [Accessed 7th August 2020].

CLEGG, S. B., TURNBULL, P. C., FOGGIN, C. M. & LINDEQUE, P. M. 2007. Massive outbreak of anthrax in wildlife in the Malilangwe Wildlife Reserve, Zimbabwe. *Vet Rec,* 160**,** 113-8.

CLOETE, C. C. Influence of site and soil type on the distribution of anthrax infectious sites and the contribution of anthrax to elephant mortality in Ethosha National park, Namibia. 2013.

COHEN, J., COHEN, P., WEST, S., AIKEN, L. 2003. *Applied Multiple Regression/Correlation Analysis for the Behavioral Sciences. New York: Routledge,* New York: Routledge.

CRESSLER, C. E., MC, L. D., ROZINS, C., J, V. D. H. & DAY, T. 2016. The adaptive evolution of virulence: a review of theoretical predictions and empirical tests. *Vet Parasitol,* 143**,** 915-30.

CROMARTIE, W. J., BLOOM, W. L. & WATSON, D. W. 1947. Studies on infection with Bacillus anthracis; a histopathological study of skin lesions produced by B. anthracis in susceptible and resistant animal species. *J Infect Dis,* 80**,** 1-13.

CROWTHER, J. R. 2000. The ELISA guidebook. *Methods Mol Biol,* 149**,** Iii-iv, 1-413.

DARDIRI, A. H., LOGAN, L. L. & MEBUS, C. A. 1987. Susceptibility of white-tailed deer to experimental heartwater infections. *J Wildl Dis,* 23**,** 215-9.

DE-VOS, V. 1990. The ecology of anthrax in the Kruger National Park, South Africa. *Salisbury Medical Bulletin,* 68S:**,** 19-23.

DE-VOS, V. & BRYDEN, H. B. 1996. Anthrax in the Kruger National Park: temporal and spatial patterns of disease occurrence. *Salisbury Medical Bulletin,* 87S**,** 26-30.

DE ECHAIDE, S. T., ECHAIDE, I. E., GAIDO, A. B., MANGOLD, A. J., LUGARESI, C. I., VANZINI, V. R. & GUGLIELMONE, A. A. 1995. Evaluation of an enzyme-linked immunosorbent assay kit to detect Babesia bovis antibodies in cattle. *Preventive Veterinary Medicine,* 24**,** 277-283.

DE WAAL, T. 2000. Anaplasmosis Control and Diagnosis in South Africa. *Annals of the New York Academy of Sciences,* 916**,** 474-483.

DONDONA, A., CAMMA, C., ASCHENBORN, O., JAGO, M., DI DOMENICO, M., BISHI, A., MARCACCI, M., LELLI, R. & CAPORALE, V. 2012. *Detection of haemoparasites in springboks and wildebeests in the Etosha National Park in Namibia*.

DROBNIEWSKI, F. A. 1993. Bacillus cereus and related species. *Clinical microbiology reviews,* 6, 324-338.

DU PLESSIS, M. A. & SKINNER, J. D. 1987. FEEDING PATTERNS OF THE GREATER KUDU, TRAGELAPHUS STREPSICEROS IN AN AGRICULTURAL AREA ON THE SPRINGBOK FLATS, NORTHERN TRANSVAAL. *Transactions of the Royal Society of South Africa,* 46, 199-208.

DUH, D., PUNDA-POLIC, V., TRILAR, T. & AVSIC-ZUPANC, T. 2008. Molecular detection of Theileria sp. in ticks and naturally infected sheep. *Vet Parasitol,* 151, 327-31.

DUMAS, E. K., DEMIRASLAN, H., INGRAM, R. J., SPARKS, R. M., MUNS, E., ZAMORA, A., LARABEE, J., GARMAN, L., BALLARD, J. D., BOONS, G.-J., JAMES, J. A., KAYABAS, U., DOGANAY, M. & FARRIS, A. D. 2020. Toxin-neutralizing antibodies elicited by naturally acquired cutaneous anthrax are elevated following severe disease and appear to target conformational epitopes. *PLOS ONE,* 15, e0230782.

DUMLER, J. S., BARBET, A. F., BEKKER, C. P., DASCH, G. A., PALMER, G. H., RAY, S. C., RIKIHISA, Y. & RURANGIRWA, F. R. 2001. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of Ehrlichia with Anaplasma, Cowdria with Ehrlichia and Ehrlichia with Neorickettsia, descriptions of six new species combinations and designation of Ehrlichia equi and 'HGE agent' as subjective synonyms of Ehrlichia phagocytophila. *Int J Syst Evol Microbiol,* 51, 2145-2165.

DUMLER, J. S., CHOI, K.-S., GARCIA-GARCIA, J. C., BARAT, N. S., SCORPIO, D. G., GARYU, J. W., GRAB, D. J. & BAKKEN, J. S. 2005. Human granulocytic anaplasmosis and Anaplasma phagocytophilum. *Emerging infectious diseases,* 11, 1828-1834.

DYER, M. & TAIT, A. 1987. Control of lymphoproliferation by Theileria annulata. *Parasitology Today,* 3, 309-311.

EASTERDAY, W. R., PONCIANO, J. M., GOMEZ, J. P., VAN ERT, M. N., HADFIELD, T., BAGAMIAN, K., BLACKBURN, J. K., STENSETH, N. C. & TURNER, W. C. 2020. Coalescence modeling of intrainfection Bacillus anthracis populations allows estimation of infection parameters in wild populations. *Proceedings of the National Academy of Sciences,* 117, 4273-4280.

EBEDES, H. 1976a. Anthrax Epizootics in Wildlife in the Etosha National Park, South west Africa. *In:* PAGE, L. A. (ed.) *J Wildl Dis.* Boston, MA: Springer US.

EBEDES, H. 1976b. Anthrax epizootics in Etosha National Park. *Madoqua,* 10, 99-118.

EISEN, H. N. & SISKIND, G. W. 1964. Variations in Affinities of Antibodies during the Immune Response *. *Biochemistry,* 3, 996-1008.

ELIASSON, M., OLSSON, A., PALMCRANTZ, E., WIBERG, K., INGANAS, M., GUSS, B., LINDBERG, M. & UHLEN, M. 1988. Chimeric IgG-binding receptors engineered from staphylococcal protein A and streptococcal protein G. *J Biol Chem,* 263, 4323-7.

ENGVALL, E. 2010. The ELISA, enzyme-linked immunosorbent assay. *Clin Chem,* 56, 319-20.

ENGVALL, E. & PERLMANN, P. 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry,* 8, 871-4.

ERARD, F. & LE GROS, G. 1994. Th2-like CD8 T cells: their role in protection against infectious diseases. *Parasitol Today,* 10, 313-5.

EYGELAAR, D., JORI, F., MOKOPASETSO, M., SIBEKO, K. P., COLLINS, N. E., VORSTER, I., TROSKIE, M. & OOSTHUIZEN, M. C. 2015. Tick-borne haemoparasites in African buffalo (Syncerus caffer) from two wildlife areas in Northern Botswana. *Parasites & vectors,* 8, 26-26.

FASANELLA, A., SCASCIAMACCHIA, S., GAROFOLO, G., GIANGASPERO, A., TARSITANO, E. & ADONE, R. 2010. Evaluation of the house fly Musca domestica as a mechanical vector for an anthrax. *PLoS One,* 5**,** e12219.

FEIR, D., LAU, C. & JUNGE, R. 1993. Protein A and protein G in the diagnosis of diseases in zoo

animals. *Transactions of the Missouri Academy of Science,* 27**,** 9-14.

FRIEDLANDER, A. M. 1986. *Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process*.

GALE, K. R., DIMMOCK, C. M., GARTSIDE, M. & LEATCH, G. 1996. Anaplasma marginale: Detection of carrier cattle by PCR-ELISA. *International Journal for Parasitology,* 26**,** 1103-1109.

GAN, S. D. & PATEL, K. R. 2013. Enzyme immunoassay and enzyme-linked immunosorbent assay. *J Invest Dermatol,* 133**,** e12.

GANZ, H. H., TURNER, W. C., BRODIE, E. L., KUSTERS, M., SHI, Y., SIBANDA, H., TOROK, T. & GETZ, W. M. 2014. Interactions between Bacillus anthracis and plants may promote anthrax transmission. *PLoS Neg Trop Dis,* 8**,** e2903-e2903.

GAROFOLO, G., CIAMMARUCONI, A., FASANELLA, A., SCASCIAMACCHIA, S., ADONE, R., PITTIGLIO, V. & LISTA, F. 2010. SNR analysis: molecular investigation of an anthrax epidemic. *BMC veterinary research,* 6**,** 11-11.

GATES, C., ELKIN, B. & DRAGON, D. 2001. Anthrax. *In:* WILLIAMS, E. S. & BARKER, I. K. (eds.) *Infectious diseases of wild mammals.* 3 ed. Iowa: Iowa State University Press, Ames.

GEORGES, K., LORIA, G., RIILI, S., GRECO, A., CARACAPPA, S., JONGEJAN, F. & SPARAGANO, O. 2001. Detection of haemoparasites in cattle by reverse line blot hybridisation with a note on the distribution of ticks in Sicily. *Veterinary parasitology,* 99**,** 273-86.

GLEISER, C. A., BERDJIS, C. C., HARTMAN, H. A. & GOCHENOUR, W. S. 1963. Pathology of experimental respiratory anthrax in Macaca mulatta. *British journal of experimental pathology,* 44**,** 416-426.

GLOMSKI, I. J., PIRIS-GIMENEZ, A., HUERRE, M., MOCK, M. & GOOSSENS, P. L. 2007. Primary involvement of pharynx and peyer's patch in inhalational and intestinal anthrax. *PLoS pathogens,* 3**,** e76-e76.

GOIDL, E. A., PAUL, W. E., SISKIND, G. W. & BENACERRAF, B. 1968. The effect of antigen dose and time after immunization on the amount and affinity of anti-hapten antibody. *J Immunol,* 100**,** 371-5.

GRAY, J. S. 2006. Identity of the causal agents of human babesiosis in Europe. *Int J Med Microbiol,* 296 Suppl 40**,** 131-6.

GROEN, J., KORAKA, P., NUR, Y., ZUPANC, T., GOESSENS, W., OTT, A. & OSTERHAUS, A. 2002. Serologic Evidence of Ehrlichiosis Among Humans and Wild Animals in The Netherlands. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology,* 21**,** 46-9.

GROOTENHUIS, J. G., YOUNG, A. S., DOLAN, T. T. & STAGG, D. A. 1979. Characteristics of Theileria species (eland) infections in eland and cattle. *Research in Veterinary Science,* 27**,** 59-68.

GUBBELS, J. M., DE VOS, A. P., VAN DER WEIDE, M., VISERAS, J., SCHOULS, L. M., DE VRIES, E. & JONGEJAN, F. 1999. Simultaneous detection of bovine Theileria and Babesia species by reverse line blot hybridization. *J Clin Microbiol,* 37**,** 1782-9.

HADLEY, T. J. 1986. Invasion of erythrocytes by malaria parasites: a cellular and molecular overview. *Annu Rev Microbiol,* 40**,** 451-77.

HALAJIAN, A., PALOMAR, A. M., PORTILLO, A., HEYNE, H., ROMERO, L. & OTEO, J. A. 2018. Detection of zoonotic agents and a new Rickettsia strain in ticks from donkeys from South Africa: Implications for travel medicine. *Travel Med Infect Dis,* 26**,** 43-50.

HAMPSON, K., LEMBO, T., BESSELL, P., AUTY, H., PACKER, C., HALLIDAY, J., BEESLEY, C. A., FYUMAGWA, R., HOARE, R., ERNEST, E., MENTZEL, C., METZGER, K. L., MLENGEYA, T., STAMEY, K., ROBERTS, K., WILKINS, P. P. & CLEAVELAND, S. 2011. Predictability of anthrax infection in the Serengeti, Tanzania. *J Appl Ecol,* 48**,** 1333-1344.

HANAFUSA, Y., CHO, K.-O., KANEMARU, T., WADA, R., SUGIMOTO, C. & ONUMA, M. 1998. Pathogenesis of Babesia caballi infection in experimental horses. *Journal of veterinary medical science,* 60**,** 1127-1132.

HANNA, P. C., ACOSTA, D. & COLLIER, R. J. 1993. On the role of macrophages in anthrax. *Proc Natl Acad Sci U S A,* 90**,** 10198-201.

HANSEN, M. B., NIELSEN, S. E. & BERG, K. 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Methods,* 119**,** 203-210.

HANSON, J. F., TAFT, S. C. & WEISS, A. A. 2006. Neutralizing Antibodies and Persistence of Immunity following Anthrax Vaccination. *Clinical and Vaccine Immunology,* 13**,** 208.

HAVARUA, Z., TURNER, W. C. & MFUNE, J. K. E. 2014. Seasonal variation in foraging behaviour of plains zebra (Equus quagga) may alter contact with the anthrax bacterium (Bacillus anthracis). *Can J Zool,* 92**,** 331-337.

HAWKINS, E., KOCK, R., MCKEEVER, D., GAKUYA, F., MUSYOKI, C., CHEGE, S., MUTINDA, M., KARIUKI, E., DAVIDSON, Z., LOW, B., SKILTON, R., NJAHIRA, M., WAMALWA, M. & MAINA, E. 2014. Prevalence of Theileria equi and Babesia caballi as well as the identification of associated ticks in sympatric Grevy's zebras (Equus grevyi) and donkeys (Equus africanus asinus) in northern Kenya. *Journal of wildlife diseases,* 51.

HAWKINS, E., KOCK, R., MCKEEVER, D., GAKUYA, F., MUSYOKI, C., CHEGE, S. M., MUTINDA, M., KARIUKI, E., DAVIDSON, Z., LOW, B., SKILTON, R. A., NJAHIRA, M. N., WAMALWA, M. & MAINA, E. 2015. Prevalence of Theileria equi and Babesia caballi as well as the identification of associated ticks in sympatric Grevy's zebras (Equus grevyi) and donkeys (Equus africanus asinus) in northern Kenya. *J Wildl Dis,* 51**,** 137-47.

HE, L., FENG, H.-H., ZHANG, Q.-L., ZHANG, W.-J., KHAN, M. K., HU, M., ZHOU, Y.-Q. & ZHAO, J.-L. 2011. DEVELOPMENT AND EVALUATION OF REAL-TIME PCR ASSAY FOR THE DETECTION OF BABESIA ORIENTALIS IN WATER BUFFALO (BUBALUS BUBALIS, LINNAEUS, 1758). *The Journal of Parasitology,* 97**,** 1166-1169.

HENRICHS, B., OOSTHUIZEN, M. C., TROSKIE, M., GORSICH, E., GONDHALEKAR, C., BEECHLER, B. R., EZENWA, V. O. & JOLLES, A. E. 2016. Within guild co-infections influence parasite community membership: a longitudinal study in African Buffalo. *J Anim Ecol,* 85**,** 1025-34.

HERING, D., THOMPSON, W., HEWETSON, J., LITTLE, S., NORRIS, S. & PACE-TEMPLETON, J. 2004. Validation of the anthrax lethal toxin neutralization assay. *Biologicals,* 32**,** 17-27.

HIPONDOKA, M., KEMPF, J. & JOUSSE, H. 2013. Palaeo and Present Ecological Value of the Etosha Pan, Namibia: An Integrative Review. 61**,** 61-2013.

HOOSHMAND-RAD, P. 1976. The pathogenesis of anaemia in Theileria annulata infection. *Res Vet Sci,* 20**,** 324-9.

HOOVER, D. L., FRIEDLANDER, A. M., ROGERS, L. C., YOON, I. K., WARREN, R. L. & CROSS, A. S. 1994. Anthrax edema toxin differentially regulates lipopolysaccharide-induced monocyte production of tumor necrosis factor alpha and interleukin-6 by increasing intracellular cyclic AMP. *Infect Immun,* 62**,** 4432-4439.

HORAK, I. G., BIGGS, H. C., HANSSEN, T. S. & HANSSEN, R. E. 1983. The prevalence of helminth and arthropod parasites of warthog, Phacochoerus aethiopicus, in South West Africa/Namibia. *Onderstepoort J Vet Res,* 50**,** 145-8.

HORAK, I. G., GALLIVAN, G. J., BRAACK, L. E., BOOMKER, J. & DE VOS, V. 2003. Parasites of domestic and wild animals in South Africa. XLI. Arthropod parasites of impalas, Aepyceros melampus, in the Kruger National Park. *Onderstepoort J Vet Res,* 70, 131-63.

HORAK, I. G., GALLIVAN, G. J. & SPICKETT, A. M. 2011. The dynamics of questing ticks collected for 164 consecutive months off the vegetation of two landscape zones in the Kruger National Park (1988–2002). Part I. Total ticks, Amblyomma hebraeum and Rhipicephalus decoloratus. *2011,* 78.

HOUSTON, D. C. & COOPER, J. E. 1975. The digestive tract of the whiteback griffon vulture and its role in disease transmission among wild ungulates. *J Wildl Dis,* 11, 306-13.

HUGH-JONES, M. & BLACKBURN, J. 2009. The ecology of Bacillus anthracis. *Mol Aspects Med,* 30, 356-67.

HUGH-JONES, M. E. & DE VOS, V. 2002. Anthrax and wildlife. *Revue scientifique et technique (International Office of Epizootics),* 21, 359-383.

HUGHES, C. & SHAFRAN, S. 2006. Treatment of Hepatitis C in HIV-Coinfected Patients. *The Annals of pharmacotherapy,* 40, 479-89; quiz 582.

HUNTLEY, B. J. 1982. Southern African savannas. *In:* HUNTLEY, B. J. & WALKER, B. H. (eds.) *Ecology of Tropical Savannas.* Springer-Verlag.

IVINS, B. E., FELLOWS, P. F. & NELSON, G. O. 1994. Efficacy of a standard human anthrax vaccine against Bacillus anthracis spore challenge in guinea-pigs. *Vaccine,* 12, 872-4.

IVINS, B. E. & WELKOS, S. L. 1988. Recent advances in the development of an improved, human anthrax vaccine. *Eur J Epidemiol,* 4, 12-9.

JACOBSON, E. R. 2007. *Infectious diseases and pathology of reptiles : color atlas and text,* Boca Raton, FL, CRC/Taylor & Francis.

JANG, J., CHO, M., CHUN, J. H., CHO, M. H., PARK, J., OH, H. B., YOO, C. K. & RHIE, G. E. 2011. The poly-gamma-D-glutamic acid capsule of Bacillus anthracis enhances lethal toxin activity. *Infect Immun,* 79, 3846-54.

JELACIC, T. M., CHABOT, D. J., BOZUE, J. A., TOBERY, S. A., WEST, M. W., MOODY, K., YANG, D., OPPENHEIM, J. J. & FRIEDLANDER, A. M. 2014. Exposure to Bacillus anthracis Capsule Results in Suppression of Human Monocyte-Derived Dendritic Cells. *Infection and Immunity,* 82, 3405.

JEON, J., LEE, H.-R., CHO, M.-H., PARK, O.-K., PARK, J. & RHIE, G.-E. 2015. The Poly-γ-d-Glutamic Acid Capsule Surrogate of the Bacillus anthracis Capsule Is a Novel Toll-Like Receptor 2 Agonist. *Infection and immunity,* 83.

JERNIGAN, D., RAGHUNATHAN, P., BELL, B., BRECHNER, R., BRESNITZ, E., BUTLER, J., CETRON, M., COHEN, M., DOYLE, T., FISCHER, M., GREENE, C., GRIFFITH, K., GUARNER, J., HADLER, J., HAYSLETT, J., MEYER, R., PETERSEN, L., PHILLIPS, M., PINNER, R. & GERBERDING, J. 2002. Investigation of Bioterrorism-Related Anthrax, United States, 2001: Epidemiologic Findings. *Emerging infectious diseases,* 8, 1019-28.

JOHNSON-WINEGAR, A. 1984. Comparison of enzyme-linked immunosorbent and indirect hemagglutination assays for determining anthrax antibodies. *Journal of clinical microbiology,* 20, 357-61.

JONGEJAN, F. & UILENBERG, G. 2004. The Global Importance of Ticks. *Parasitology,* 129 Suppl, S3-14.

KAMAL, S., RASHID, A., BAKAR, M. & AHAD, M. 2011. Anthrax: An update. *Asian Pacific journal of tropical biomedicine,* 1, 496-501.

KEIM, P., KALIF, A., SCHUPP, J., HILL, K., TRAVIS, S. E., RICHMOND, K., ADAIR, D. M., HUGH-JONES, M., KUSKE, C. R. & JACKSON, P. 1997. Molecular evolution and diversity in Bacillus anthracis as detected by amplified fragment length polymorphism markers. *J. Bacteriol.,* 179, 818-824.

KELLY, P. J., TAGWIRA, M., MATTHEWMAN, L., MASON, P. R. & WRIGHT, E. P. 1993. Reactions of sera from laboratory, domestic and wild animals in Africa with protein A and a recombinant chimeric protein AG. *Comp Immunol Microbiol Infect Dis,* 16**,** 299-305.

KHUMALO, Z. T. H., UNIVERSITY OF PRETORIA. DEPARTMENT OF VETERINARY TROPICAL, D., OOSTHUIZEN, M. C. O. M. C. S., COLLINS, N. E. C. N. E. C.-S. & BRAYTON, K. A. B. K. A. C.-S. 2017. *Anaplasma centrale in South Africa : occurrence, phylogeny and genetic diversity.*

KING'ORI, E., OBANDA, V., CHIYO, P. I., SORIGUER, R. C., MORRONDO, P. & ANGELONE, S. 2019. Molecular identification of Ehrlichia, Anaplasma, Babesia and Theileria in African elephants and their ticks. *PLOS ONE,* 14**,** e0226083.

KLEIN, F., HODGES, D. R., MAHLANDT, B. G., JONES, W. I., HAINES, B. W. & LINCOLN, R. E. 1962. Anthrax toxin: causative agent in the death of rhesus monkeys. *Science,* 138**,** 1331-3.

KOCAN, K., DE LA FUENTE, J., BLOUIN, E. F. & GARCIA-GARCIA, J. C. 2004. Anaplasma marginale (Rickettsiales: Anaplasmataceae): recent advances in defining host-pathogen adaptations of a tick-borne Rickettsia. *Parasitology,* 129 Suppl**,** S285-300.

KOCK, N. D., JONGEJAN, F., KOCK, M. D., KOCK, R. A. & MORKEL, P. 1992. Serological Evidence for Cowdria ruminantium Infection in Free-Ranging Black (Diceros bicornis) and White (Ceratotherium simum) Rhinoceroses in Zimbabwe. *Journal of Zoo and Wildlife Medicine,* 23**,** 409-413.

KOCK, N. D., VAN VLIET, A. H., CHARLTON, K. & JONGEJAN, F. 1995. Detection of Cowdria ruminantium in blood and bone marrow samples from clinically normal, free-ranging Zimbabwean wild ungulates. *Journal of clinical microbiology,* 33**,** 2501-2504.

KRAMSKY, J. A., MANNING, E. J. & COLLINS, M. T. 2003. Protein G Binding to Enriched Serum Immunoglobulin from Nondomestic Hoofstock Species. *J Vet Diagn Invest,* 15**,** 253-261.

KUTTLER, K. L. 1984. ANAPLASMA INFECTIONS IN WILD AND DOMESTIC RUMINANTS: A REVIEW. *Journal of Wildlife Diseases,* 20**,** 12-20.

LAMPEN, F., BHOORA, R., COLLINS, N. E. & PENZHORN, B. L. 2009. Putative clinical piroplasmosis in a Burchell s zebra (Equus quagga burchelli). *Journal of the South African Veterinary Association,* 80**,** 257-260.

LANDIS, J. R. & KOCH, G. G. 1977. The measurement of observer agreement for categorical data. *Biometrics,* 33**,** 159-74.

LAUS, F., SPATERNA, A., FAILLACE, V., VERONESI, F., RAVAGNAN, S., BERIBÉ, F., CERQUETELLA, M., MELIGRANA, M. & TESEI, B. 2015. Clinical investigation on Theileria equi and Babesia caballi infections in Italian donkeys. *BMC Veterinary Research,* 11**,** 100.

LE ROUX, C. J. G., GRUNOW, J. O., BREDENKAMP, G. J., MORRIS, J. W. & SCHEEPERS, J. C. 1988. A classification of the vegetation of the Etosha National Park. *South African Journal of Botany,* 54**,** 1-10.

LEKOTA, K. E. 2018. *Genomic study of Bacillus anthracis and Bacillus species isolated from anthrax outbreaks in South Africa.* Ph.D, University of Pretoria.

LELLO, J. & HUSSELL, T. 2008. Functional group/guild modelling of inter-specific pathogen interactions: a potential tool for predicting the consequences of co-infection. *Parasitology,* 135**,** 825-39.

LELLO, J., NORMAN, R., BOAG, B., HUDSON, P. & FENTON, A. 2008. Pathogen Interactions, Population Cycles, and Phase Shifts. *The American naturalist,* 171**,** 176-82.

LEMBO, T., HAMPSON, K., AUTY, H., BEESLEY, C. A., BESSELL, P., PACKER, C., HALLIDAY, J., FYUMAGWA, R., HOARE, R., ERNEST, E., MENTZEL, C., MLENGEYA, T., STAMEY, K., WILKINS, P. P. & CLEAVELAND, S. 2011. Serologic surveillance of anthrax in the Serengeti ecosystem, Tanzania, 1996-2009. *Emerging infectious diseases,* 17**,** 387-394.

LEPPLA, S. H. 1982. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. *Proc Natl Acad Sci U S A,* 79**,** 3162-6.

LEPPLA, S. H., ROBBINS, J. B., SCHNEERSON, R. & SHILOACH, J. 2002. Development of an improved vaccine for anthrax. *The Journal of clinical investigation,* 110**,** 141-144.

LEWERIN, S. S., ELVANDER, M., WESTERMARK, T., HARTZELL, L. N., NORSTRÖM, A. K., EHRS, S., KNUTSSON, R., ENGLUND, S., ANDERSSON, A.-C., GRANBERG, M., BÄCKMAN, S., WIKSTRÖM, P. & SANDSTEDT, K. 2010. Anthrax outbreak in a Swedish beef cattle herd--1st case in 27 years: Case report. *Acta veterinaria Scandinavica,* 52**,** 7-7.

LINCOLN, R. E. & FISH, D. C. 1970. Anthrax toxin. *In:* MONTIE, T. C., KADIS, S. & AJL, S. J. (eds.) *Microbial toxins: Bacterial protein toxins.* New York: Academic Press, Inc.

LINCOLN, R. E., WALKER, J. S., KLEIN, F., ROSENWALD, A. J. & JONES, W. I., JR. 1967. Value of field data for extrapolation in anthrax. *Fed Proc,* 26**,** 1558-62.

LINDEQUE, P. M. & TURNBULL, P. C. 1994. Ecology and epidemiology of anthrax in the Etosha National Park, Namibia. *Onderstepoort J Vet Res,* 61**,** 71-83.

LINDSTRÖM, P. & WAGER, O. 1978. IgG Autoantibody to Human Serum Albumin Studied by the ELISA-Technique. *Scandinavian Journal of Immunology,* 7**,** 419-425.

LITTLE, S. F. & IVINS, B. E. 1999. Molecular pathogenesis of Bacillus anthracis infection. *Microbes Infect,* 1**,** 131-9.

LITTLE, S. F., IVINS, B. E., FELLOWS, P. F., PITT, M. L., NORRIS, S. L. & ANDREWS, G. P. 2004. Defining a serological correlate of protection in rabbits for a recombinant anthrax vaccine. *Vaccine,* 22**,** 422-30.

MABBOTT, N. A. 2018. The Influence of Parasite Infections on Host Immunity to Co-infection With Other Pathogens. *Frontiers in Immunology,* 9**,** 2579.

MAHLANDT, B. G., KLEIN, F., LINCOLN, R. E., HAINES, B. W., JONES, W. I., JR. & FRIEDMAN, R. H. 1966. Immunologic studies of anthrax. IV. Evaluation of the immunogenicity of three components of anthrax toxin. *J Immunol,* 96**,** 727-33.

MAKINO, S., UCHIDA, I., TERAKADO, N., SASAKAWA, C. & YOSHIKAWA, M. 1989. Molecular characterization and protein analysis of the cap region, which is essential for encapsulation in Bacillus anthracis. *Journal of Bacteriology,* 171**,** 722.

MAKINO, S., WATARAI, M., CHEUN, H. I., SHIRAHATA, T. & UCHIDA, I. 2002. Effect of the lower molecular capsule released from the cell surface of Bacillus anthracis on the pathogenesis of anthrax. *J Infect Dis,* 186**,** 227-33.

MARSTON, C. K., IBRAHIM, H., LEE, P., CHURCHWELL, G., GUMKE, M., STANEK, D., GEE, J. E., BOYER, A. E., GALLEGOS-CANDELA, M., BARR, J. R., LI, H., BOULAY, D., CRONIN, L., QUINN, C. P. & HOFFMASTER, A. R. 2016. Anthrax Toxin-Expressing Bacillus cereus Isolated from an Anthrax-Like Eschar. *PLoS One,* 11**,** e0156987.

MATJILA, P. T., LEISEWITZ, A. L., JONGEJAN, F. & PENZHORN, B. L. 2008. Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa. *Veterinary Parasitology,* 155**,** 152-157.

MATJILA, P. T., PENZHORN, B. L., BEKKER, C. P. J., NIJHOF, A. M. & JONGEJAN, F. 2004. Confirmation of occurrence of Babesia canis vogeli in domestic dogs in South Africa. *Veterinary Parasitology,* 122**,** 119-125.

MBATI, P. A., HLATSHWAYO, M., MTSHALI, M. S., MOGASWANE, K. R., DE WAAL, T. D. & DIPEOLU, O. O. 2002. Ticks and Tick-Borne Diseases of Livestock Belonging to Resource-poor Farmers in the Eastern Free State of South Africa. *Experimental & Applied Acarology,* 28**,** 217-224.

MCKEEVER, D. J. 2009. Bovine immunity - a driver for diversity in Theileria parasites? *Trends Parasitol,* 25**,** 269-76.

MEHLHORN, H. & SCHEIN, E. 1985. The Piroplasms: Life Cycle and Sexual Stages**Supported by DFG. *In:* BAKER, J. R. & MULLER, R. (eds.) *Advances in Parasitology.* Academic Press.

MIDEO, N., ALIZON, S. & DAY, T. 2008. Linking within- and between-host dynamics in the evolutionary epidemiology of infectious diseases. *Trends in Ecology & Evolution,* 23**,** 511-517.

MINETT, F. C. & DHANDA, M. R. 1941. Multiplication of B. anthracis and Cl. chauvoei in soil and water. *Indian J. Vet. Sci. Ani. Husb,* 11**,** 308-321.

MOAYERI, M., LEPPLA, S., VRENTAS, C., POMERANTSEV, A. & LIU, S. 2015. Anthrax Pathogenesis. *Annual review of microbiology,* 69.

MOAYERI, M. & LEPPLA, S. H. 2004. The roles of anthrax toxin in pathogenesis. *Curr Opin Microbiol,* 7**,** 19-24.

MOAYERI, M. & LEPPLA, S. H. 2009. Cellular and systemic effects of anthrax lethal toxin and edema toxin. *Molecular aspects of medicine,* 30**,** 439-455.

MULLINS, J. C., GAROFOLO, G., VAN ERT, M., FASANELLA, A., LUKHNOVA, L., HUGH-JONES, M. E. & BLACKBURN, J. K. 2013. Ecological niche modeling of Bacillus anthracis on three continents: evidence for genetic-ecological divergence? *PLoS One,* 8**,** e72451.

MUNSON, L., TERIO, K. A., KOCK, R., MLENGEYA, T., ROELKE, M. E., DUBOVI, E., SUMMERS, B., SINCLAIR, A. R. E. & PACKER, C. 2008. Climate Extremes Promote Fatal Co-Infections during Canine Distemper Epidemics in African Lions. *PLOS ONE,* 3**,** e2545.

MUORIA, P., MURUTHI, P., WAITITU, K., HASSAN, B., MIJELE, D. & OGUGE, N. 2007. Anthrax outbreak among Grevy's zebra (Equus grevyi) in Samburu, Kenya. *African Journal of Ecology,* 45**,** 483-489.

NAGORE, D., GARCíA-SANMARTíN, J., GARCíA-PÉREZ, A. L., JUSTE, R. A. & HURTADO, A. 2004. Identification, genetic diversity and prevalence of Theileria and Babesia species in a sheep population from Northern Spain. *International Journal for Parasitology,* 34**,** 1059-1067.

NALISA, M. Investigation of biting and non-biting flies as vectors for Bacillus Anthracis in Etosha National Park, Namibia. 2013.

NDUMNEGO, O. C., CRAFFORD, J., BEYER, W. & VAN HEERDEN, H. 2013. Quantitative anti-PA IgG ELISA; assessment and comparability with the anthrax toxin neutralization assay in goats. *BMC veterinary research,* 9**,** 265.

NDUMNEGO, O. C., KOEHLER, S. M., CRAFFORD, J. E., BEYER, W. & VAN HEERDEN, H. 2018. Immunogenicity of anthrax recombinant peptides and killed spores in goats and protective efficacy of immune sera in A/J mouse model. *Sci Rep,* 8.

NEITZ, W. O. 1935a. The Blesbuck (Damaliscus albifrom) and the Black Wildebeest (Conochaetes Gnu) as Carriers of Heartwater. *Onderstepoort J. vet. Sci,* 5**,** 35-40.

NEITZ, W. O. 1935b. Bovine anaplasmosis: The transmission of Anaplasma marginale to a black wildebeest (Connochaetes gnou). *Onderstepoort Journal of Veterinary Science and Ani-mal Industry,* 5**,** 9–11.

NEWMAN, Z. L., PRINTZ, M. P., LIU, S., CROWN, D., BREEN, L., MILLER-RANDOLPH, S., FLODMAN, P., LEPPLA, S. H. & MOAYERI, M. 2010. Susceptibility to Anthrax Lethal Toxin-Induced Rat Death Is Controlled by a Single Chromosome 10 Locus That Includes rNlrp1. *PLOS Pathogens,* 6**,** e1000906.

NGERANWA, J. J., SHOMPOLE, S. P., VENTER, E. H., WAMBUGU, A., CRAFFORD, J. E. & PENZHORN, B. L. 2008. Detection of Anaplasma antibodies in wildlife and domestic species in wildlife-livestock interface areas of Kenya by major surface protein 5 competitive inhibition enzyme-linked immunosorbent assay. *Onderstepoort J Vet Res,* 75**,** 199-205.

NGUNDI, M. M., MEADE, B. D., LIN, T.-L., TANG, W.-J. & BURNS, D. L. 2010. Comparison of Three Anthrax Toxin Neutralization Assays. *Clinical and Vaccine Immunology,* 17**,** 895.

NIEBERLE, K. & COHRS, P. 1967. *Textbook of the special pathological anatomy of domestic animals,* Oxford, Pergamon Press.

NIJHOF, A., PENZHORN, B., LYNEN, G., MOLLEL, J., MORKEL, P., BEKKER, C. & JONGEJAN, F. 2003. Babesia bicornis sp. nov. and Theileria bicornis sp. nov.: Tick-Borne Parasites Associated with Mortality in the Black Rhinoceros (Diceros bicornis). *Journal of clinical microbiology,* 41, 2249-54.

NIJHOF, A. M., PILLAY, V., STEYL, J., PROZESKY, L., STOLTSZ, W. H., LAWRENCE, J. A., PENZHORN, B. L. & JONGEJAN, F. 2005. Molecular Characterization of Theileria Species Associated with Mortality in Four Species of African Antelopes. *Journal of Clinical Microbiology,* 43, 5907.

NORVAL, R. A. I., PERRY, B. D. & YOUNG, A. 1992. *The epidemiology of theileriosis in Africa*, ILRI (aka ILCA and ILRAD).

NSOH, A. E., KENU, E., FORSON, E. K., AFARI, E., SACKEY, S., NYARKO, K. M. & YEBUAH, N. 2016. Mapping as a tool for predicting the risk of anthrax outbreaks in Northern Region of Ghana. *Pan Afr Med J,* 25, 14.

OKINAKA, R. T. 1999. Sequence and organization of pXO1, the large Bacillus anthracis plasmid harboring the anthrax toxin genes. *J. Bacteriol.,* 181, 6509.

OMLAND, K. S., BRYS, A., LANSKY, D., CLEMENT, K. & LYNN, F. 2008. Interlaboratory Comparison of Results of an Anthrax Lethal Toxin Neutralization Assay for Assessment of Functional Antibodies in Multiple Species. *Clinical and Vaccine Immunology,* 15, 946.

ONYICHE, T. E., SUGANUMA, K., IGARASHI, I., YOKOYAMA, N., XUAN, X. & THEKISOE, O. 2019. A Review on Equine Piroplasmosis: Epidemiology, Vector Ecology, Risk Factors, Host Immunity, Diagnosis and Control. *International journal of environmental research and public health,* 16, 1736.

OOSTHUIZEN, M. C., ALLSOPP, B. A., TROSKIE, M., COLLINS, N. E. & PENZHORN, B. L. 2009. Identification of novel Babesia and Theileria species in South African giraffe (Giraffa camelopardalis, Linnaeus, 1758) and roan antelope (Hippotragus equinus, Desmarest 1804). *Vet Parasitol,* 163, 39-46.

OOSTHUIZEN, M. C., ZWEYGARTH, E., COLLINS, N. E., TROSKIE, M. & PENZHORN, B. L. 2008. Identification of a Novel Babesia sp. from a Sable Antelope (Hippotragus niger Harris, 1838). *Journal of Clinical Microbiology,* 46, 2247.

OSCHERWITZ, J., QUINN, C. P. & CEASE, K. B. 2015. Anthrax vaccine recipients lack antibody against the loop neutralizing determinant: A protective neutralizing epitope from Bacillus anthracis protective antigen. *Vaccine,* 33, 2342-2346.

OURA, C. A. L., BISHOP, R. P., WAMPANDE, E. M., LUBEGA, G. W. & TAIT, A. 2004. Application of a reverse line blot assay to the study of haemoparasites in cattle in Uganda. *International Journal for Parasitology,* 34, 603-613.

OWEN-SMITH, N. 1979. Assessing the foraging effeciency of a large herbivore, the kudu. *South African Journal of Wildlife Research,* 9, 102-110.

OWEN-SMITH, N. 1990. Demography of a Large Herbivore, the Greater Kudu Tragelaphus strepsiceros, in Relation to Rainfall. *Journal of Animal Ecology,* 59, 893-913.

OWEN-SMITH, O. & COOPER, S. M. 1985. Comparative consumption of vegetation components by kudus, impalas and goats in relation to their commercial potential as browsers in savanna regions. *South African Journal of Science,* 81, 72.

PANNIFER, A. D., WONG, T. Y., SCHWARZENBACHER, R., RENATUS, M., PETOSA, C., BIENKOWSKA, J., LACY, D. B., COLLIER, R. J., PARK, S., LEPPLA, S. H., HANNA, P. & LIDDINGTON, R. C. 2001. Crystal structure of the anthrax lethal factor. *Nature,* 414, 229-33.

PAROLA, P. & RAOULT, D. 2001. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. *Clin Infect Dis,* 32, 897-928.

PARREIRAS, P. M., SIROTA, L. A., WAGNER, L. D., MENZIES, S. L. & ARCINIEGA, J. L. 2009. Comparability of ELISA and toxin neutralization to measure immunogenicity of Protective Antigen in mice, as part of a potency test for anthrax vaccines. *Vaccine,* 27, 4537-42.

PATHAK, A. K., PELENSKY, C., BOAG, B. & CATTADORI, I. M. 2012. Immuno-epidemiology of chronic bacterial and helminth co-infections: observations from the field and evidence from the laboratory. *Int J Parasitol,* 42**,** 647-55.

PAYMENT, P. & DESCOTEAUX, J. P. 1978. Enzyme-linked immunosorbent assay for the detection of antibodies to pneumonia virus of mice in rat sera. *Lab Anim Sci,* 28**,** 676-9.

PEACHMAN, K. K., RAO, M., ALVING, C. R., BURGE, R., LEPPLA, S. H., RAO, V. B. & MATYAS, G. R. 2006. Correlation between lethal toxin-neutralizing antibody titers and protection from intranasal challenge with Bacillus anthracis Ames strain spores in mice after transcutaneous immunization with recombinant anthrax protective antigen. *Infection and immunity,* 74**,** 794-797.

PETER, T. F., ANDERSON, E. C., BURRIDGE, M. J. & MAHAN, S. M. 1998. Demonstration of a carrier state for Cowdria ruminantium in wild ruminants from Africa. *J Wildl Dis,* 34**,** 567-75.

PETER, T. F., ANDERSON, E. C., BURRIDGE, M. J., PERRY, B. D. & MAHAN, S. M. 1999. Susceptibility and carrier status of impala, sable, and tsessebe for Cowdria ruminantium infection (heartwater). *J Parasitol,* 85**,** 468-72.

PFITZER, S., OOSTHUIZEN, M. C., BOSMAN, A. M., VORSTER, I. & PENZHORN, B. L. 2011. Tick-borne blood parasites in nyala (Tragelaphus angasii, Gray 1849) from KwaZulu-Natal, South Africa. *Vet Parasitol,* 176**,** 126-31.

PIENAAR, U. D. 1967. Epidemiology of anthrax in wild animals and the control of anthrax epizootics in the Kruger National Park, South Africa. *Federation proceedings,* 26**,** 1496-502.

PIENAAR, U. D. V. 1960. 'N uitbraak van miltsiekte onder wild in die Nasionale Krugerwildtuin 28.9.50 tot 20.11.59. *Koedoe,* 3**,** 238-251.

PIENAAR, U. D. V. 1961. A second outbreak of anthrax amongst game animals in the Kruger National Park. 5th June to 11th October. *Koedoe,* 4**,** 4-17.

PILO, P. & FREY, J. 2011. Bacillus anthracis: Molecular taxonomy, population genetics, phylogeny and patho-evolution. *Infection, Genetics and Evolution,* 11**,** 1218-1224.

PROVOST, A. & BEZUIDENHOUT, J. 1987. The historical background and global importance of heartwater. *Onderstepoort J. Vet. Res,* 54**,** 165-169.

QUESNEL-HELLMANN, A., CLERET, A., VIDAL, D. R. & TOURNIER, J. N. 2006. Evidence for adjuvanticity of anthrax edema toxin. *Vaccine,* 24**,** 699-702.

R CORE TEAM. 2017. *R:A language and environment for statistical computing. R Foundation for Statistical Computing.* [Online]. Vienna, Austria. Available: http://www.R-project.org/ [Accessed].

RABOLOKO, O. O., RAMABU, S. S., GUERRINI, L. & JORI, F. 2020. Seroprevalence of Selected Tick Borne Pathogens and Diversity and Abundance of Ixodid Ticks (Acari: Ixodidae) at the Wildlife-Livestock Interface in Northern Botswana. *Frontiers in Veterinary Science,* 7.

RADNEDGE, L., AGRON, P. G., HILL, K. K., JACKSON, P. J., TICKNOR, L. O., KEIM, P. & ANDERSEN, G. L. 2003. Genome differences that distinguish Bacillus anthracis from Bacillus cereus and Bacillus thuringiensis. *Applied and environmental microbiology,* 69**,** 2755-2764.

RASKO, D. A., ALTHERR, M. R., HAN, C. S. & RAVEL, J. 2005. Genomics of the Bacillus cereus group of organisms. *FEMS Microbiology Reviews,* 29**,** 303-329.

RENNARD, S. I., BERG, R., MARTIN, G. R., FOIDART, J. M. & ROBEY, P. G. 1980. Enzyme-linked immunoassay (ELISA) for connective tissue components. *Anal Biochem,* 104**,** 205-14.

RESTIF, O. & GRAHAM, A. L. 2015. Within-host dynamics of infection: from ecological insights to evolutionary predictions. *Philos Trans R Soc Lond B Biol Sci,* 370.

REUVENY, S., WHITE, M. D., ADAR, Y. Y., KAFRI, Y., ALTBOUM, Z., GOZES, Y., KOBILER, D., SHAFFERMAN, A. & VELAN, B. 2001. Search for correlates of protective immunity conferred by anthrax vaccine. *Infection and immunity,* 69**,** 2888-2893.

RIJKS, J. 1999. *A serological study of bison (Bison bison) in an area of northern Canada experiencing sporadic and epizootic anthrax.*. MSc Thesis, University of London.

RINGERTZ, S. H., HØIBY, E. A., JENSENIUS, M., MAEHLEN, J., CAUGANT, D. A., MYKLEBUST, A. & FOSSUM, K. 2000. Injectional anthrax in a heroin skin-popper. *Lancet,* 356**,** 1574-5.

RISTIC, M. 1981. Anaplasmosis. *In:* RISTIC M., M. W. I. M. (ed.) *Diseases of Cattle in the Tropics. Current Topics.* Dordrecht: Springer.

ROTHSCHILD, C. M. 2013. Equine Piroplasmosis. *Journal of Equine Veterinary Science,* 33**,** 497-508.

SAILE, E. & KOEHLER, T. M. 2006. Bacillus anthracis multiplication, persistence, and genetic exchange in the rhizosphere of grass plants. *Applied and environmental microbiology,* 72**,** 3168-3174.

SCHNITTGER, L., YIN, H., QI, B., GUBBELS, M. J., BEYER, D., NIEMANN, S., JONGEJAN, F. & AHMED, J. S. 2004. Simultaneous detection and differentiation of Theileria and Babesia parasites infecting small ruminants by reverse line blotting. *Parasitol Res,* 92**,** 189-96.

SCHOULS, L. M., VAN DE POL, I., RIJPKEMA, S. G. T. & SCHOT, C. S. 1999. Detection and Identification ofEhrlichia, Borrelia burgdorferi Sensu Lato, andBartonella Species in Dutch Ixodes ricinusTicks. *Journal of Clinical Microbiology,* 37**,** 2215.

SCORPIO, A., CHABOT, D. J., DAY, W. A., O'BRIEN D, K., VIETRI, N. J., ITOH, Y., MOHAMADZADEH, M. & FRIEDLANDER, A. M. 2007. Poly-gamma-glutamate capsule-degrading enzyme treatment enhances phagocytosis and killing of encapsulated Bacillus anthracis. *Antimicrob Agents Chemother,* 51**,** 215-22.

SEED, J. R. 1996. Protozoa: Pathogenesis and Defenses. *In:* IN: BARON S (ed.) *Medical Microbiology.* 4 ed. Galveston (TX) University of Texas Medical Branch at Galveston.

SHLOMCHIK, M. J. & WEISEL, F. 2012. Germinal center selection and the development of memory B and plasma cells. *Immunol Rev,* 247**,** 52-63.

SIBEKO, K. P., OOSTHUIZEN, M. C., COLLINS, N. E., GEYSEN, D., RAMBRITCH, N. E., LATIF, A. A., GROENEVELD, H. T., POTGIETER, F. T. & COETZER, J. A. 2008. Development and evaluation of a real-time polymerase chain reaction test for the detection of Theileria parva infections in Cape buffalo (Syncerus caffer) and cattle. *Vet Parasitol,* 155**,** 37-48.

SIRIGIREDDY, K. R. & GANTA, R. R. 2005. Multiplex detection of Ehrlichia and Anaplasma species pathogens in peripheral blood by real-time reverse transcriptase-polymerase chain reaction. *J Mol Diagn,* 7**,** 308-16.

SMITH, E. R. & PARKER, D. M. 2010. Tick communities at the expanding wildlife / cattle interface in the Eastern Cape Province, South Africa : implications for Corridor disease. *2010,* 81**,** 4.

SMITH, H., KEPPIE, J. & STANLEY, J. L. 1955. The chemical basis of the virulence of Bacillus anthracis. V. The specific toxin produced by B. Anthracis in vivo. *Br J Exp Pathol,* 36**,** 460-472.

SMITH, K. L., DEVOS, V., BRYDEN, H., PRICE, L. B., HUGH-JONES, M. E. & KEIM, P. 2000. Bacillus anthracis diversity in Kruger National Park. *J Clin Microbiol,* 38**,** 3780-4.

SMITH, S. A. 2017. *Evaluation of anti-bovine, anti-equine and recombinant protein A/G horseradish peroxidase conjugates for cross reactivity to wildlife serum antibodies using ELISA.* Master of Science (Tropical Animal Health), University of Pretoria.

SOULSBY, E. J. L. & MÖNNIG, H. O. 1982. *Helminths, arthropods and protozoa of domesticated animals,* London, Baillière Tindall.

SREEKUMAR, C., ANANDAN, R., BALASUNDARAM, S. & RAJAVELU, G. 1996. Morphology and staining characteristics of Ehrlichia bovis. *Comp Immunol Microbiol Infect Dis,* 19**,** 79-83.

STEENKAMP, P. J., VAN HEERDEN, H. & VAN SCHALKWYK, O. L. 2018. Ecological suitability modeling for anthrax in the Kruger National Park, South Africa. *PloS one,* 13**,** e0191704.

STERNE, M. 1959. Anthrax. *In:* STABLEFORTH, A. W. & GALLOWAY, I. A. (eds.) *Infectious diseases of animals diseases due to bacteria* New York: Academic Press.

STEYL, J. C. A., PROZESKY, L., STOLTSZ, W. H. & LAWRENCE, J. A. 2012. Theileriosis (Cytauxzoonosis) in Roan antelope (Hippotragus equinus): Field exposure to infection and identification of potential vectors. *Onderstepoort Journal of Veterinary Research,* 79**,** 01-08.

STÖBEL, K., SCHÖNBERG, A. & STAAK, C. 2002. A new non-species dependent ELISA for detection of antibodies to Borrelia burgdorferi s. l. in zoo animals. *Int J Med Microbiol: Supplement 33,* 291**,** 88-99.

STOLTSZ, H., BYARUHANGA, C., TROSKIE, M., MAKGABO, M., OOSTHUIZEN, M. C., COLLINS, N. E. & NEVES, L. 2020. Improved detection of Babesia bigemina from various geographical areas in Africa using quantitative PCR and reverse line blot hybridisation. *Ticks Tick Borne Dis***,** 101415.

TAFT, S. C. & WEISS, A. A. 2008. Neutralizing Activity of Vaccine-Induced Antibodies to Two Bacillus anthracis Toxin Components, Lethal Factor and Edema Factor. *Clinical and Vaccine Immunology,* 15**,** 71.

TEMBO, S., COLLINS, N. E., SIBEKO-MATJILA, K. P., TROSKIE, M., VORSTER, I., BYARUHANGA, C. & OOSTHUIZEN, M. C. 2018. Occurrence of tick-borne haemoparasites in cattle in the Mungwi District, Northern Province, Zambia. *Ticks and Tick-borne Diseases,* 9**,** 707-717.

TERRA, J. K., COTE, C. K., FRANCE, B., JENKINS, A. L., BOZUE, J. A., WELKOS, S. L., LEVINE, S. M. & BRADLEY, K. A. 2010. Cutting edge: resistance to Bacillus anthracis infection mediated by a lethal toxin sensitive allele of Nalp1b/Nlrp1b. *J Immunol,* 184**,** 17-20.

THERMOFISHER SCIENTIFIC 2019. *Overview of ELISA - ZA* [Online]. Available: https://www.thermofisher.com/za/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html [Accessed].

THOMAS, S. E., WILSON, D. E. & MASON, T. E. 1982. Babesia, Theileria and Anaplasma spp. infecting sable antelope, Hippotragus niger (Harris, 1838), in southern Africa. *Onderstepoort J Vet Res,* 49**,** 163-6.

TURK, B. E. 2007. Manipulation of host signalling pathways by anthrax toxins. *Biochem J,* 402**,** 405-17.

TURNBULL, P. C., BELL, R. H., SAIGAWA, K., MUNYENYEMBE, F. E., MULENGA, C. K. & MAKALA, L. H. 1991. Anthrax in wildlife in the Luangwa Valley, Zambia. *The Veterinary record,* 128**,** 399-403.

TURNBULL, P. C., DOGANAY, M., LINDEQUE, P. M., AYGEN, B. & MCLAUGHLIN, J. 1992. Serology and anthrax in humans, livestock and Etosha National Park wildlife. *Epidemiol Infect,* 108**,** 299-313.

TURNBULL, P. C., LINDEQUE, P. M., LE ROUX, J., BENNETT, A. M. & PARKS, S. R. 1998. Airborne movement of anthrax spores from carcass sites in the Etosha National Park, Namibia. *J Appl Microbiol,* 84**,** 667-76.

TURNBULL, P. C. B. 2008. Anthrax in humans and animals. *W.H.O.*

TURNBULL, P. C. B. C. J. A. L. P. M. J. F. H. O. J. B. & SNOEYENBOS, G. H. 1989. Further progress in understanding anthrax in the Etosha National Park. *Madoqua,* 16**,** 93-104.

TURNER, W. C., IMOLOGHOME, P., HAVARUA, Z., KAAYA, G. P., MFUNE, J. K. E., MPOFU, I. D. T. & GETZ, W. M. 2013. Soil ingestion, nutrition and the seasonality of anthrax in herbivores of Etosha National Park. *Ecosphere,* 4**,** art13.

TURNER, W. C., KAUSRUD, K. L., BEYER, W., EASTERDAY, W. R., BARANDONGO, Z. R., BLASCHKE, E., CLOETE, C. C., LAZAK, J., VAN ERT, M. N., GANZ, H. H., TURNBULL, P. C. B., STENSETH, N. C. & GETZ, W. M. 2016. Lethal exposure: An integrated approach to pathogen transmission via environmental reservoirs. *Scientific Reports,* 6**,** 27311.

TURNER, W. C., KAUSRUD, K. L., KRISHNAPPA, Y. S., CROMSIGT, J. P., GANZ, H. H., MAPAURE, I., CLOETE, C. C., HAVARUA, Z., KÜSTERS, M., GETZ, W. M. & STENSETH, N. C. 2014. Fatal attraction: vegetation responses to nutrient inputs attract herbivores to infectious anthrax carcass sites. *Proc Biol Sci,* 281.

UILENBERG, G. 1995. International collaborative research: significance of tick-borne hemoparasitic diseases to world animal health. *Vet Parasitol,* 57**,** 19-41.

VAN NESS, G. & STEIN, C. D. 1956. Soils of the United States favorable for anthrax. *J Am Vet Med Assoc,* 128**,** 7-12.

VAN NESS, G. B. 1971. Ecology of anthrax. *Science,* 172**,** 1303-7.

VAN TONGEREN, S. P., ROEST, H. I. J., DEGENER, J. E. & HARMSEN, H. J. M. 2014. Bacillus anthracis-like bacteria and other B. cereus group members in a microbial community within the International Space Station: a challenge for rapid and easy molecular detection of virulent B. anthracis. *PloS one,* 9**,** e98871-e98871.

VAN WEEMEN, B. K. & SCHUURS, A. H. 1971. Immunoassay using antigen-enzyme conjugates. *FEBS Lett,* 15**,** 232-236.

VAN WINKELHOFF, A. J. & UILENBERG, G. 1981. Heartwater: Cross-immunity studies with strains ofCowdria ruminantium isolated in west and South Africa. *Tropical Animal Health and Production,* 13**,** 160-164.

VERMA, A., NGUNDI, M. M., MEADE, B. D., DE PASCALIS, R., ELKINS, K. L. & BURNS, D. L. 2009. Analysis of the Fc gamma receptor-dependent component of neutralization measured by anthrax toxin neutralization assays. *Clin Vaccine Immunol,* 16**,** 1405-12.

VERMA, J., SAXENA, S. & GOSIPATALA, S. 2013. ELISA –Based Identification and Detection of Microbes. *Analyzing Microbes- Manual of Molecular Biology TechniquesSpringer Protocols*.

VILAS-BÔAS, G. T., PERUCA, A. P. & ARANTES, O. M. 2007. Biology and taxonomy of Bacillus cereus, Bacillus anthracis, and Bacillus thuringiensis. *Canadian journal of microbiology,* 53**,** 673-87.

VOLLER, A., BIDWELL, D., HULDT, G. & ENGVALL, E. 1974. A microplate method of enzyme-linked immunosorbent assay and its application to malaria. *Bull World Health Organ,* 51**,** 209-11.

W.H.O. 2008. Anthrax in humans and animals. *In:* ORGANIZATION, W. H. (ed.) *World Health Organization.* 4 ed. World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland: WHO Press,.

WAFULA, M. M., PATRICK, A. & CHARLES, T. 2008. Managing the 2004/05 anthrax outbreak in Queen Elizabeth and Lake Mburo National Parks, Uganda. *African Journal of Ecology,* 46**,** 24-31.

WALKER, A., BOUATTOUR, A., CAMICAS, J. L., ESTRADA-PEÑA, A., HORAK, I., LATIF, A., PEGRAM, R. G. & PRESTON, P. M. 2003. Ticks of Domestic Animals in Africa: a guide to identification of species.

WALSH, M. G., DE SMALEN, A. W. & MOR, S. M. 2018. Climatic influence on anthrax suitability in warming northern latitudes. *Sci Rep,* 8**,** 9269-9269.

WANG, S., MATA-FINK, J., KRIEGSMAN, B., HANSON, M., IRVINE, D. J., EISEN, H. N., BURTON, D. R., WITTRUP, K. D., KARDAR, M. & CHAKRABORTY, A. K. 2015. Manipulating the selection forces during affinity maturation to generate cross-reactive HIV antibodies. *Cell,* 160**,** 785-797.

WATSON, A. & KEIR, D. 1994. Information on which to base assessments of risk from environments contaminated with anthrax spores. *Epidemiol Infect,* 113**,** 479-90.

WEISS, S., KOBILER, D., LEVY, H., MARCUS, H., PASS, A., ROTHSCHILD, N. & ALTBOUM, Z. 2006. Immunological correlates for protection against intranasal challenge of Bacillus anthracis spores conferred by a protective antigen-based vaccine in rabbits. *Infection and immunity,* 74**,** 394-398.

WELKOS, S., LITTLE, S., FRIEDLANDER, A., FRITZ, D. & FELLOWS, P. 2001. The role of antibodies to Bacillus anthracis and anthrax toxin components in inhibiting the early stages of infection by anthrax spores. *Microbiology,* 147**,** 1677-1685.

WELKOS, S. L., TROTTER, R. W., BECKER, D. M. & NELSON, G. O. 1989. Resistance to the Sterne strain of B. anthracis: phagocytic cell responses of resistant and susceptible mice. *Microbial Pathogenesis,* 7**,** 15-35.

WOLDEHIWET, Z. 1987. The effects of tick-borne fever on some functions of polymorphonuclear cells of sheep. *Journal of comparative pathology,* 97**,** 481-485.

WOLDEHIWET, Z. 2008. Immune evasion and immunosuppression by Anaplasma phagocytophilum, the causative agent of tick-borne fever of ruminants and human granulocytic anaplasmosis. *Vet J,* 175**,** 37-44.

WOLDEHIWET, Z. & RISTIC, M. 1993. *Rickettsial and chlamydial diseases of domestic animals*, Pergamon press.

WORLD HEALTH ORGANIZATION 2015. *Anthrax in humans and animals* [Online]. Available: https://www.who.int/csr/resources/publications/AnthraxGuidelines2008/en/ [Accessed].

WORTHINGTON, R. W. & BIGALKE, R. D. 2001. A review of the infectious diseases of African wild ruminants. *Onderstepoort J Vet Res,* 68**,** 291-323.

ZIDON, R., GARTI, S., GETZ, W. & SALTZ, D. 2017. Zebra migration strategies and anthrax in Etosha National Park, Namibia. *Ecosphere,* 8.

ZINTL, A., FINNERTY, E. J., MURPHY, T. M., DE WAAL, T. & GRAY, J. S. 2011. Babesias of red deer (Cervus elaphus) in Ireland. *Vet Res,* 42**,** 7.

APPENDIX

Appendix 1. Comparison of exposure of wild animals to *Bacillus anthracis* between endemic and non-endemic areas in two national parks in southern Africa

Animal species	National Park	Endemic status	No. of animals sampled	No. of positive animals (%)	*p*-value
Kudu	KNP	Endemic	19	19 (100)	0.49[a]
		Non-endemic	18	17 (94.4)	
	ENP	Endemic	20	14 (70)	0.51[a]
		Non-endemic	20	12 (60)	
Zebra	KNP	Endemic	20	15 (75)	0.10[a]
		Non-endemic	20	10 (50)	
	ENP	Endemic	20	19 (95)	0.046
		Non-endemic	20	14 (70)	

KNP, Kruger National Park in South Africa; ENP, Etosha National Park in Namibia

[a] No significant difference in Bacillus anthracis exposure between endemic and non-endemic areas within a national park

Appendix 2

Animal Species	No. of Animals	National Park	Anthrax Endemic Status	Number of parasite genus/ Species identified																
				E/A catch all	***A.centrale***	***E.ruminatium***	***A.bovis***	***A.platys***	***A.sp.omajienne***	***T/B catch all***	***T catch all***	***B1 catch all***	***B2 catch all***	***T.sp.kudu***	***T.sp.sable***	***T.bicornis***	***T.sp.buffeli***	***T.taurotragi***	***T.equi***	***T.saparata***
Kudu	20	ENP	Endemic	14	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0
	20		Non-Endemic	16	0	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0
Kudu	19	KNP	Endemic	18	0	0	6	4	7	18	18	0	1	19	18	17	17	16	0	0
	18		Non-Endemic	17	0	0	1	7	12	17	15	0	0	15	13	12	14	12	0	0
Zebra	20	ENP	Endemic	14	0	0	0	0	0	18	19	20	0	0	0	0	0	0	7	0
	20		Non-Endemic	18	0	0	0	0	3	20	18	15	0	0	0	0	0	0	1	0
Zebra	20	KNP	Endemic	12	2	0	0	0	0	19	19	16	0	0	0	0	0	0	4	0
	19		Non-Endemic	15	0	2	0	0	0	17	14	17	0	0	0	0	0	0	1	0
Wildebeest	20	ENP	Endemic	16	3	0	0	0	0	11	4	0	0	0	9	0	0	0	0	6
Impala	20	KNP	Endemic	20	15	0	0	0	6	20	20	0	0	0	11	20	20	0	0	0

Appendix 3. Proportion of wild animals positive for tick-borne haemoparasites, as determined by the reverse line blot hybridisation assay, and an initial logistic regression analysis for predictor variables

	Variable	Category	No. of animals sampled	No. of positive animals (%)	*p*-value
Zebra					
Babesia	National park	ENP	40	35 (87.5)	0.34
		KNP	39	30 (76.9)	
	Area	Endemic	40	34 (85.0)	0.44
		Non-endemic	39	31 (79.5)	
	Anthrax TNA score	Toxin neutralisation			0.005
Theileria	National park	ENP	40	37 (92.5)	0.39
		KNP	39	30 (76.9)	
	Area	Endemic	40	35 (87.5)	0.73
		Non endemic	39	32 (82.1)	
	Anthrax TNA score	Toxin neutralisation			0.003
Ehrlichia/ Anaplasma	National park	ENP	40	32 (80.0)	0.43
		KNP	39	25 (64.1)	
	Area	Endemic	40	25 (62.5)	0.14
		Non endemic	39	32 (82.1)	
	Anthrax TNA score	Toxin neutralisation			0.0016
Kudu					
Theileria	National park	ENP	40	16 (40.0)	0.0005
		KNP	37	32 (86.5)	
	Area	Endemic	39	23 (59.0)	0.04
		Non-endemic	38	25 (65.8)	
	Anthrax TNA score	Toxin neutralisation			0.09
	Park*Area				0.016
Ehrlichia/Anaplasma	National park	ENP	40	30 (75.0)	0.012
		KNP	37	16 (43.2)	
	Area	Endemic	39	20 (51.3)	0.25
		Non-endemic	38	26 (68.4)	
	Anthrax TNA score	Toxin neutralisation			0.005

Blood samples were collected from wild animals in Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia. Only one kudu was positive for *Babesia* parasites, and therefore not included in the analysis.

Appendix 4: Animal Ethics Approval

Faculty of Veterinary Science
Animal Ethics Committee

03 September 2019

Approval Certificate
New Application

AEC Reference No.: REC041-19

Title: Transmission and evolution of a persistent pathogen: anthrax infection dynamics comparing two natural systems

Researcher: Dr SO Ochai
Student's Supervisor: Prof H van Heerden Dear
Dr SO Ochai,
The New Application as supported by documents received between 2019-04-08 and 2019-08-26 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2019-08-26.

Please note the following about your ethics approval: The use of species is approved:

Species and Samples	Number
Zebra (Equus burchelli)	40
Kudu (Tragelaphus strepsiceros)	40
Impala (Aepyceros melampus)	40
Blood samples (60 ml per animal)	120 total
Hair samples (Tuft)	120 total
Tissue collection (thin slice ear) 3mm	120 total
Animals/carcasses that succumb to anthrax during the surveillance period	Opportunistic collections (to be reported on)

1. Ethics Approval is valid for 1 year and needs to be renewed annually by 2020-09-03.
2. Please remember to use your protocol number (REC041-19) on any documents or correspondence with the AEC regarding your research.
3. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research. Yours sincerely

H. Lutermann

Dr. Heike Lutermann

DEPUTY CHAIRMAN: UP-Animal Ethics Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8483
Fax +27 12 529 8321
Email aec@up.ac.za
www.up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

Appendix 5: Animal Ethics Approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

Research Ethics Committee

Project Title	**Immunological study of the Anthrax infection dynamics in Kruger and Etosha National Parks (KNP and ENP)**
Project Number	**REC106-19**
Researcher / Principal Investigator	**Dr SO Ochai**

DIissertation / Thesis submitted for	**Masters**

Supervisor	**Prof H van Heerden**

APPROVED	Date: **2019-08-28**
CHAIRMAN: UP Research Ethics Committee	Signature: