EFFECTS OF ELEPHANT CARCASSES ON VEGETATION COVER, HERBIVORE BEHAVIOUR, AND POTENTIAL ANTHRAX TRANSMISSION IN CENTRAL ETOSHA NATIONAL PARK.

A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENT

FOR THE DEGREE OF

MASTER OF SCIENCE (BIOLOGY)

OF

THE UNIVERSITY OF NAMIBIA

BY

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201308011

SEPTEMBER 2021

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ABSTRACT

Anthrax is primarily a disease of herbivores caused by a soil-borne, spore-forming and Gram-positive bacterium, *Bacillus anthracis*. This study investigated the role large-bodied animal carcasses may play in anthrax transmission dynamics in an African savanna. Specifically, I examined how African elephant (*Loxodonta africana*) carcasses affected vegetation cover, soil pathogen concentrations, and herbivore behaviour at carcass sites over time in central Etosha National Park, Namibia. Carcass site soil and vegetation sampling was conducted three times over a growing season (January, April and July), while motion-triggered camera traps monitored activity at elephant carcass sites over a three-year period in central Etosha National Park. Elephant carcasses were placed into age classes for soil and vegetation sampling based on the time of death as follows: recent (0-2 years old), old (2-5 years old), and very old (>5 years old). This study i) measured the area of soil disturbance around the elephant carcass, ii) measured for vegetation cover at elephant carcass, iii) determined the concentration of *B. anthracis* spores in soils at zebra and elephant carcass sites, and iv) monitored animals activity at elephant carcass sites using motion sensing video camera. The area of soil disturbance at elephant carcass sites, while substantial in the year of death (up to 31.55m^2), showed a sharp decline after the first year (to less than 1m^2). Vegetation cover was generally highest near the centre of carcass sites and declined with increasing distance away, except for some of the younger sites (<2 years old) with more soil disturbance at the closest sampling distance. Among site ages, the 2-5 years old carcass sites had higher vegetation cover than younger or older sites. There was seasonal variation in vegetation cover that varied with site age, with cover at older carcass sites dropping off considerably in the dry season. Although *B. anthracis* spore concentrations showed a declining trend with site age for elephant and zebra carcass sites, these trends were not statistically significant. Over 35 trap months, the video cameras recorded a total of 31,068 videos of which 12,728 were mammals and the rest by wind, rain and birds. Video recordings at elephant carcass sites showed that 21 animal

species (ungulate herbivores, carnivores and small mammals) visited these sites. Behaviours displayed by mammals and vultures were recorded, and ranked according to risk of anthrax exposure in descending order as; foraging, bone contact with mouth, smelling, kicking/touching, resting, investigating and walking by. The behaviours displayed, and which were most common, varied among animal species. These findings were compared to previous work on carcass sites from plains zebra (*Equus quagga*) carcass sites. Both species had significant effects on soil and vegetation at carcass sites, although vegetation responses at elephant carcass sites appear to occur on a longer time lag, in the 2-5 year window, as compared to vegetation at zebra carcass sites peaking the year after death. Anthrax cases of both species showed persistence of *B. anthracis* spores over time at sites, with no significant difference in spore concentrations between the two species. The biggest difference between the two carcass types was in which species visit these sites and how they interacted with potentially infectious material at the carcass sites. Elephant carcasses have the potential to expose a different suite of host species to anthrax, than observed at plains zebra carcass sites. While zebra are the main host species in Etosha, and most cases in the system are detected in grazing herbivores, large-bodied, wide-ranging animals like elephants that use different habitats than grazing hosts could serve as a bridging host, bringing anthrax into different habitats, and exposing a wider variety of host species.

Key words: Anthrax transmission, *Bacillus anthracis*, Behavioural activities, Camera-trapping, Elephant carcasses, Etosha National Park, Herbivorous mammals, *Loxodonta africana*, Soil Disturbance, Vegetation cover

DEDICATION

I dedicate this paper to the Almighty God for the supremacy and to the redeemer of my soul and to my family, friends, and supervisors and to the memory of my grandmother.

ACKNOWLEDGMENTS

I would like to thank the Almighty God for giving me the courage, strength and passion to carry out this project. This study could not have been completed without the input of many people. Firstly, I would like to extend my appreciation to my two supervisors. My sincerest gratitude goes to Prof. John Mfune who has been a source of motivation since my undergraduate years. Thank you for your time, tireless effort and encouragement, and thank you for caring. I deeply appreciate the input of Prof. Wendy Turner; you have been invaluable to this study. Thank you for taking me as your student, and for introducing me to the incredible world of anthrax. I am also very grateful for everything I have learned throughout this study in the laboratory and in the field. I am also indebted to Yen-Hua Huang and Zoe Barandongo for their insightfulness and contribution to the completion of this thesis.

I thank the Ministry of Environment, Forestry and Tourism for granting me permission to do research in the magnificent Etosha National Park. I appreciate the help of Chief Warden Amunime Namandje, Conservation Scientist Claudine Cloete, Warden Gabriel Shatumbu, Martina Küsters, Ranger Johannes Kapner, assistant Rangers Seth Guim and Sox Gariseb, and staff of the APO, NAMPOL and NSF for assisting me throughout my field work. I also appreciate the assistance and advice of the Chief Conservation Scientist, Mr. Werner Kilian, and the rest of the Etosha Ecological Institute staff. Thank you for allowing me the use of equipment, office, and laboratory space. Lastly I would like to give thanks to my family and friends for their never ending support and encouragement.

DECLARATION

This is a thesis submitted in fulfilment of the requirement for the Degree of Master of Science (Biology) of the University of Namibia. I, Hendrina Joel, declare that this thesis has been composed solely by me and that it has not been submitted, in whole or in part, in any previous application for a Master's degree. Except where states otherwise by reference or acknowledgement, the work presented is entirely my own. I grant the University of Namibia to produce this Master's thesis in whole or in part in any manner or format, for any person or institution requiring it for research studies, providing that the University of Namibia shall waive this right if the whole thesis has been or is being published in any manner.

Date: 25 September 2021

Hendrina Joel

LIST OF ABBREVIATIONS

°C	Degree Celsius
CFU	Colony Forming Unit
EDTA	Ethylendiaminetetraacetic acid
EEI	Etosha Ecological Institute
ENP	Etosha National Park
GPS	Global positioning system
LD	Lethal dose
MEFT	Ministry of Environment, Forestry and Tourism
MID	Minimum infectious dose
O	Old
PA	Protective antigen
PET	Polymixin-ethylendiaminetetraacetic acid (EDTA)-thallous acetate
PGPB	Plant Growth- Promoting Bacteria
R	Recent
RCF	Relative centrifugal force
Spg	Spores per gram
VO	Very old
WHO	World Health Organization

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CHAPTER 1: INTRODUCTION

1.1 Orientation of the study

Anthrax is primarily a disease of herbivorous mammals, caused by a soil-borne, spore-forming and Gram-positive bacterium know as *Bacillus anthracis* (WHO, 2008). *Bacillus anthracis* forms spores in the soil and vegetative cells inside a living host (Coker *et al*., 2003). This bacterium was believed to be the only obligate pathogen of vertebrates in the genus *Bacillus* (Turnbull, 1996), before the discovery of *Bacillus cereus biovar anthracis* (Klee *et al.,* 2010). Anthrax can manifest in three forms: pulmonary anthrax (through inhalation), gastrointestinal anthrax (through ingestion) and cutaneous anthrax (through the skin) (WHO, 2008). The disease varies in its seasonality, incidence and type of species affected from one area to another (Hugh-Jones and Blackburn, 2009). Approximately a global total of 63.8 million poor herdsmen and livestock holders, 1.83 billion people and 1.1 billion livestock (including 320 million sheep, 294.9 million pigs, 268.1 million cattle, 211.2 million goats and 0.6 million buffalo) live within vulnerable regions of anthrax exposure (Carlson *et al.,* 2019). It affects wildlife, livestock and humans worldwide of which herbivorous mammals are most at risk of contracting it because they are likely to come into contact with soil when feeding (Turner *et al.*, 2013). Carnivores may also become infected. Human, livestock, wildlife and the environment are the important aspect of epidemiological patterns of anthrax (Mwakapeje *et al*., 2018). Though humans are observed to be relatively resistant to anthrax, outbreaks and epidemics do occur in humans. In previous studies, the most notable was the epidemic in Zimbabwe which began in 1979 until 1984-1985, of which more than 10 000 persons were affected with anthrax (Kobuch *et al*., 1990). Humans become infected with anthrax most commonly through contact with infected animals or their products. Human infections can be treated with penicillin, chloramphenicol, streptomycin, tetracycline and erythromycin (Mwakapeje *et al*., 2018).

Outbreaks of animal anthrax can have severe economic consequences. This is especially true in developing countries, which contain large numbers of poor shepherds and farmers. Anthrax is a major

problem in poor communities as many of these people lack the means to control the disease. Moreover, poor farmers have small herds, so each animal is used for several purposes, such as transportation, ploughing, producing manure for fertilizer, provides clothing and food. Thus to a poor shepherd or farmer, the death of an animal from anthrax is a huge loss. For instance in Bangladesh (areas with food insecurity) people are more likely to slaughter and consume sick/dying animals (Islam *et al*., 2013). Since religion, cannot allow people to consume meat of the animal that died on its own from the infection (Islam *et al*., 2013). This, actions can lead to high anthrax infections in humans. Studies done in Bangladesh investigated 14 outbreaks of anthrax which included 140 animal and 273 human cases in 14 anthrax-affected villages (Islam *et al*., 2013). The disease causes a great loss through mortality, reducing animal products and complete condemnation of carcasses as well as closure of affected abattoirs (Misgie *et al.,* 2015). Although in developing countries vaccination of susceptible animals in affected areas has reduced the prevalence of the disease on a national basis, heavy losses may still occur in individual herds (Misgie *et al.,* 2015). Anthrax outbreaks cause substantial economic losses from livestock and wildlife losses, the cost of laboratory reagents, and carcass disposal (burial or incineration) (Mwakapeje *et al*., 2018). In cases of extreme food insecurity, poor populations may left with no choice but to eat anthrax-infected meat despite understanding the risks (Carlson *et al.*, 2019) thus leading to more infections and deaths. In addition, livestock keepers may continue to sell contaminated meat to recover their financial losses; hence leading to more anthrax cases in urban settings (Carlson *et al.,* 2019). Education campaign is needed, even though it can be logistically challenging in inaccessible rural areas it may be more cost-effective than mass vaccination (Carlson *et al.*, 2019). In addition, responses to these outbreaks require joint collaborative efforts of the ministries responsible for human health, livestock and wildlife services (Mwakapeje *et al*., 2018). Therefore investment in the control of this disease is inevitable (Mwakapeje *et al*., 2018).

Anthrax is endemic in central and northern Namibia, with anthrax cases recorded in wildlife, livestock and humans (Ebedes, 1976; Beyer *et al.,* 2012). Anthrax in Etosha National Park (ENP), Namibia, first documented in 1964 (Ebedes, 1976) , has been the subject of long-term mortality surveillance and

research into the epidemiology of anthrax. In ENP anthrax occurs annually and affects host species such as plains zebra (*Equus quagga*), springbok (*Antidorcas marsupialis*), blue wildebeest (*Connochaetes taurinus*), and African elephant (*Loxodonta africana*) (Turner *et al.*, 2016). The Etosha Ecological Institute (EEI) initiated mortality surveillance in 1976 as a result of anthrax in ENP. Etosha National Park recorded total anthrax mortality of 2182 individuals of all host species combined, from the years 1975-2020 with the highest peak in annual cases observed in 2010 claiming 177 individuals of which 133 were plains zebra (Etosha Ecological Institute, 2020). These numbers include animals who were confirmed positive for anthrax from blood smears or bacterial culture. Anthrax seasonality in the park differ in space and time between plains ungulates (i.e., zebra, wildebeest, springbok, gemsbok) and elephant populations, and correlates with rainfall (Lindeque and Turnbull, 1994; Beyer *et al.,* 2012; Turner *et al.,* 2013) . The peak in anthrax mortality in plains ungulates is observed toward the end of the wet season, whilst the peak in anthrax mortality in elephants is observed towards the end of the dry season in ENP (Lindeque and Turnbull, 1994)

Table 1. Anthrax total cases for the five most common host species in Etosha National Park from 1975- 2020; (Etosha Ecological Institute, 2020).

Species	Total cases-for 44 years	Average mortality per year
Zebra (*Equus quagga*)	1082	24
Blue-wildebeest (*Connochaetes taurinus*)	350	8
African elephant *(Loxodonta Africana)*	341	8
Springbok *(Antidorcas marsupialis)*	331	7
Gemsbok *(Oryx gazelle)*	20	1

This study investigates the effects of large-bodied animal carcasses, specifically, elephant carcasses, in anthrax epidemiology in ENP. Anthrax host species were categorized into three groups based on their body size: small-bodied ungulates (30-65kg) such as springbok and impala, medium-bodied ungulates (120-385kg) such as plains zebra and wildebeest and large-bodied mammals (600-7000kg) such as African elephant, black rhino (*Diceros bicornis*) and giraffe (*Giraffa camelopardalis*) (Carruthers, 2006). Anthrax transmission by medium-sized animal carcasses has been studied (Turner *et al.*, 2016; 2014). Turner *et al.* (2014) revealed that nutrients from medium-sized carcasses, such as from adult zebras, enrich the soil and contribute to higher biomass, more notorious vegetation at carcass sites in the wet season following death. These sites alter the environment and attract herbivore species to *B. anthracis* aggregations (Turner *et al.*, 2016; 2014). The carcass site of an infected animal remains the area with the highest *B. anthracis* spore contamination, therefore, contributing to future infections (Dragon *et al.*, 2005), with the highest counts of *B. anthracis* spores of about 1 000 000 to 1 000 000 000 spores per gram (spg) (Bellan *et al*., 2013). Animals such as zebra, springbok and wildebeest are attracted to anthrax carcass sites for foraging. Zebras have the strongest attraction; they are up to 4 times more likely to graze at sites where zebras died of anthrax than at grassland control sites, thus increasing their risk of exposure to *B. anthracis* (Turner *et al.*, 2014).

Anthrax is one of the most fatal diseases impacting elephants, and anthrax cases in elephants have been reported throughout their range according to a World Organisation for Animal Health (OIE). Elephants are potential reservoirs of infection for other wild and domestic animals (Yasothal, 2013). They show external signs like bleeding from natural orifices, excessive bloating and rapid decomposition of the carcasses when suspected to have died of anthrax (Aggarwal, 2020). Yet little is known about the contribution of large-bodied animal carcasses, such as elephant carcasses to anthrax transmission in ENP. Elephants also use different habitats than the plains herbivores in ENP (Huang *et al*., 2021), raising the question of how they are infected, and how this species contributes to the epidemiology of this multi-host disease.

This study aimed to investigate the effect of large-bodied animal carcasses (African elephants) on soil, vegetation response, pathogen concentration and behaviour of herbivores at carcass sites and to evaluate how carcasses of different body sizes may contribute differently to anthrax transmission in ENP. Studying how large animal carcasses affect soils and vegetation response may help with understanding the magnitude and duration of these effects and their impact on host contact with the anthrax bacterium at these sites. The concentration of *B. anthracis* in the soil is expected to decrease over time, thus knowing when and how animals contact these sites as they age will contribute to a better understanding of the ecology of anthrax in ENP. Understanding more about the epidemiology of this disease will contribute to its management and control efforts in the region, if wildlife managers or livestock farmers surrounding the park seek to reduce its impact on animal populations. Studying behaviours displayed by animals at these sites will help to identify the species at risk, the types of behaviours they conduct at these sites, and the different routes of exposure through which herbivore species might acquire anthrax from elephant carcass sites.

(a) (b)

Figure 1. Animal carcasses in Etosha National Park a) An African elephant carcass, and b) A fresh plains zebra carcass: (Images: Dr. W. C. Turner).

1.2 Statement of Problem

The possibility of animals contracting anthrax depends upon their foraging behaviour when at an infectious site (Havarua *et al*., 2014). Turner *et al.* (2014) reported that nutrients from medium-sized carcasses boost vegetation growth, hence attracting some but not all ungulate herbivores for foraging at sites of pathogen aggregation. In the process, these species contract anthrax via their increased contact with vegetation, and perhaps soils, at the infectious site. Elephants are important species because of their contribution to the ecosystem and tourism in Etosha National Park or else where. Beside their importance they are also subjected to anthrax and die every year just like other host species. Although elephants die of anthrax every year in ENP, their influence on vegetation response, and anthrax transmission, has not been studied. The effect of large-bodied animal carcasses on the environment (soil and vegetation) and their spore concentration are hypothesized to last longer than that of medium or small-bodied animals. Knowledge of the influence of large-bodied carcasses will contribute to an understanding of species differences in anthrax transmission and in turn influence practices on the management of anthrax. The purpose of the study was to investigate the effects of large-bodied carcasses (African elephant) on vegetation response, pathogen concentrations, and behaviour of herbivores at elephant carcass sites, and to evaluate how carcasses of different body sizes may contribute differently to anthrax transmission in ENP.

1.3 Objectives

The objectives of the study were to:

a) Determine the area of physical disturbance on soil caused by large-sized animal carcasses over time.
b) Determine the effect of decomposition of large-sized animal carcasses on vegetation cover around the carcass site over time.

c) Determine the concentrations of *Bacillus anthracis* spores present in the soil at large-sized and medium-sized animal anthrax carcass sites over time.

d) Describe anthrax transmission risk behaviours displayed by animals at large-sized animal carcass sites and relate these to potential pathogen exposure.

1.4 Hypotheses

a) H_o: There is no significant difference in the area of physical disturbance on soil caused by large-sized animal carcasses over time.

b) H_o: There is no significant difference in the effect of decomposition of large animal carcasses on vegetation cover at large-sized animal carcass sites over time.

c) H_o: There is no significant difference in concentrations of *Bacillus anthracis* spores present in soils at large-sized and medium-sized carcass sites, matched by carcass age.

d) H_o: There is no significant difference in the types of anthrax transmission risk behaviours displayed by different herbivore species at large animal carcass sites.

1.5 Significance of the study

The results of the study will improve our understanding on the risk of contact at anthrax carcass sites and how these sites contribute to the exposure of different host species to *B. anthracis* and anthrax outbreak. Evaluating the magnitude and duration of soil and vegetation disturbance at large-bodied carcass sites will inform how herbivores may respond to these carcass sites in ENP. In addition, the concentrations and persistence of *B. anthracis* spores at these carcass sites will inform the potential for *B. anthracis* transmission from these sites to susceptible host species. Furthermore, the specific behaviours of herbivores at carcass sites will affect the potential for these species contracting anthrax in ENP, and the specific routes of transmission most likely to occur. In general, this study provides

important insights into variation in transmission risk for this multi-host pathogen that can inform epidemic control efforts in managed livestock and wildlife populations.

1.6 Limitations of the study

Motion- sensing video camera traps were used to record animal activities at selected carcass sites. One of the challenges was that the sample size used was controlled by availability of the carcass sites with the requirement to use in this study. In addition, the camera traps were pushed over or destroyed by animals such as rhino and elephant while visiting or passing by the carcass sites, creating gaps in the time series. Some of the cameras were also carried away from sites by animals such as hyenas or elephants, and important data lost due to such behaviours. Another limitation was access to potential study sites: during the rainy season, some roads were closed for driving to prevent vehicles from getting stuck in the mud which would delay visitations to the site for data downloads/battery replacement until conditions improved. Therefore, only carcass sites deemed to be accessible year-round were chosen (in terms of road access, fuel costs, and thickness or vegetation around the area) and visited as often as possible to prevent losing important data for this study.

1.7 Delimitation of the study

The study was carried out in Okaukuejo and western Halali management areas in central ENP. The selection of study sites within this area was based on accessibility, selecting adult animal elephant carcasses of either sex, and adult zebra carcasses for spore persistence sampling. In this study juveniles and sub-adults were not considered, given their smaller body sizes. The study was confined to approximately 40 carcass sites; with data collected from July 2017 to June 2020 for camera data and from January 2020 to July 2020 for data on the area of soil disturbance, vegetation cover and *B. anthracis* spore concentration.

CHAPTER 2: LITERATURE REVIEW

2.1 History of Anthrax

The term “anthrax,” derives from the Greek “anthrakites”, meaning coal-like, which refers to the typical black lesions seen in cutaneous anthrax (Sternbach, 2003). Anthrax was documented since biblical times as a disease of herbivores and was the first disease attributed to a specific microorganism (Schwartz, 2009). It is a common infectious disease caused by the Gram-positive spore-forming *Bacillus anthracis*, a bacterium to which all warm-blooded animals are susceptible (Sternbach, 2003). This disease was extensively studied by several researchers, including Robert Koch and Louis Pasteur in the 1870s. In addition, in 1876, Robert Koch was the first to trace and study the complete life cycle of *B. anthracis* using suspended culture methods and discovered that *Bacillus* could form spores that could survive for long periods in unfavourable conditions or environments (Sternbach, 2003). According to Sternbach (2003), during the first half of the 20th century, anthrax cases reported worldwide were about 20,000-100,000 annually, reduced to approximately 2000 cases per year annually during the second half of the century. Currently, approximately 63.8 million herdsmen and livestock holders and 1.1 billion livestock animals are estimated to be at risk (Carlson *et al*., 2018). The rate of anthrax transmission was subsequently reduced between the years 1940-1960 through the introduced Sterne´s livestock vaccine, in combination with antibiotics and different control program (Beyer & Turnbull, 2009). According to, although national vaccination programs have resulted in a global reduction of anthrax, the disease is still common in some part of the world such as Mediterranean countries, some parts of North America, certain countries of central and South America and Central Asia, and several sub-Saharan African countries (WHO, 2008). Anthrax outbreaks also continue to occur elsewhere in the world. In recent years a total outbreak in livestock of 214 with 2911 losses (mainly cattle) was reported in Zimbabwe between 2000- 2018, while 10 outbreaks with 3171 deaths were noted in wildlife (Mukara *et al*., 2020). In addition, 99 outbreaks were recorded in humans involving 903 individual cases with 16 fatalities due to the consumption of infected meat between the years 2010 and 2018 (Mukarati *et al*., 2020). Another outbreak was reported in Uganda in 2004,

whereas many as 200 hippos died from a deadly anthrax outbreak (Gibbens, 2017). Furthermore, in Lake Nakuru National Park, Kenya, there was a loss of 10 buffalo due to an anthrax outbreak (Bett and Gachochi, 2019). Other outbreaks in this system were reported in 2014, 2015, 2017 of which in 2015 out of 766 wild animals, 745 were buffalos that died from this zoonotic disease (Bett and Gachochi, 2019). Therefore once the bacterium is present in the soil, anthrax outbreaks continue to be recorded in the system.

2.2 The Life Cycle of *B. anthracis*

Bacillus anthracis, the organism that causes anthrax, is a large, rod-shaped bacterium. The bacterium forms both vegetative and spore morphologies which alternate based on the availability of nutrients (WHO, 2008). Within the host body, *B. anthracis* occurs as active vegetative cells, but forms spores when exposed to air (presence of oxygen) and conditions unfavourable for vegetative cells growth (Ebedes, 1976; WHO, 2008). Anthrax spores are hardy, thick-walled, oval bodies with an average diameter of about 1 to 3 microns. Spores are resistant to drying, heat, ultraviolet light, gamma radiation, and many disinfectants (Sternbach, 2003), and is why they can survive in the environment for many years after being released. There are three different forms of illnesses depending on the route of infection in animals, namely; cutaneous (skin), pulmonary (inhalation), and gastrointestinal (ingestion) (Schwartz, 2009; Figure 2).

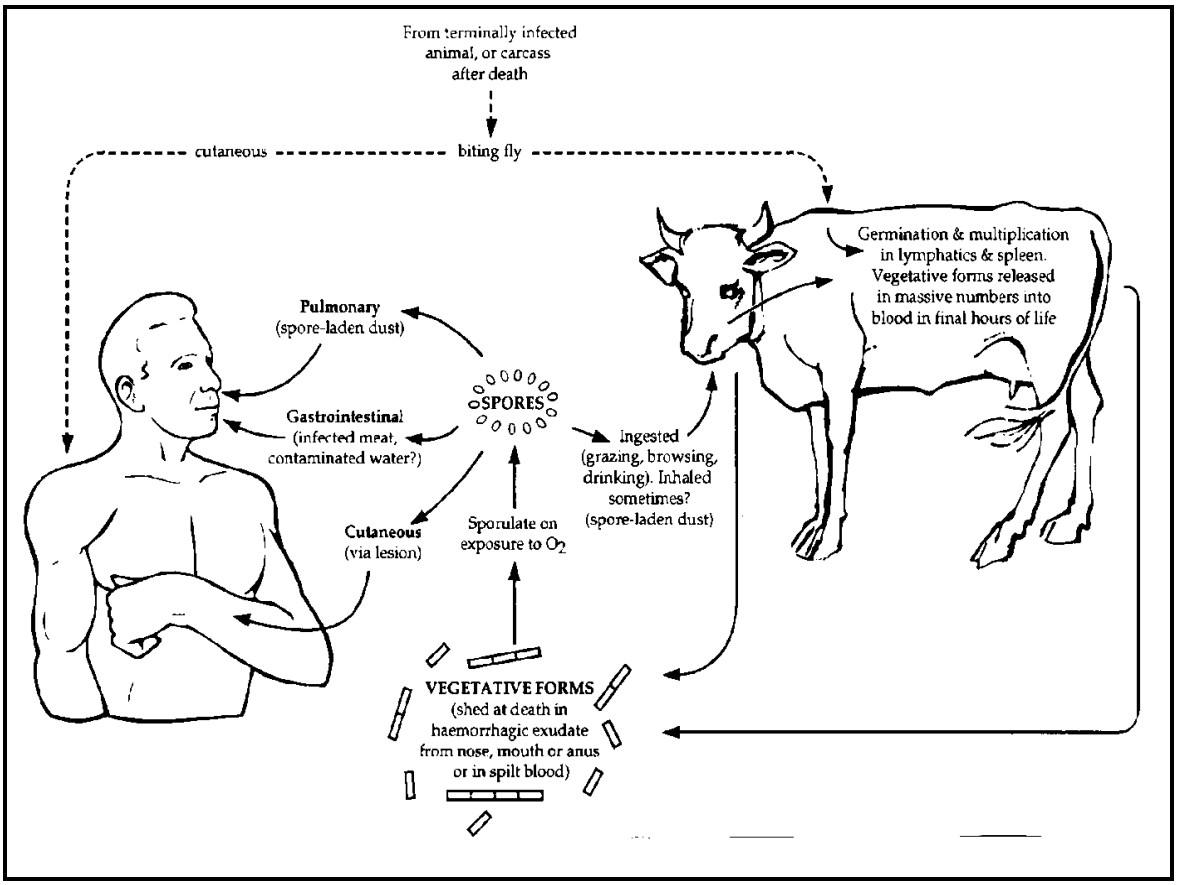


Figure 2. The infectious life cycle of anthrax (Source: World Health Organisation, 2008)

Many anthrax outbreak cases are reported in herbivorous animals, such as cattle, zebra, deer, bison, kudu (WHO, 2008), and hippopotamuses (Gibbens, 2017) that consume *B. anthracis* spores while grazing, browsing, or consuming soil. Health officials in the Kunene and Zambezi regions reported suspected anthrax cases in humans, sheep, goats, and wildlife (hippo) of Namibia (Shikongo, 2019). After hosts die of anthrax, scavenging releases not only the pathogen but also blood and body fluids (WHO, 2008; Valseth *et al*., 2017). These fluids provide nutrients that enhance vegetation growth and attract herbivores to graze at these carcass sites (Turner *et al.*, 2014; Marulanda, 2007). The *B. anthracis* spores also promote the germination of grass seeds (Ganz *et al*., 2014). In addition, some *Bacillus* spp., help plants survive drought conditions through effects on osmoregulation and antioxidant activity (Vardharajul *et al*., 2011). The positive effects of *B. anthracis* on the environment

attract grazing hosts to carcass sites, increasing the rate of anthrax transmission and the cycle begins again.

Figure 3. *Enneapogon desvauxii* grasses growing at a plains zebra carcass site that died from anthrax one year previously in Etosha National Park, Namibia (Source: Dr. W.C. Turner).

2.3 Ecology of *B. anthracis*

Anthrax is a seasonal disease and is commonly characterized by hot and dry weather conditions which are stressful to animals due to food shortage (Hugh-Jones and Blackburn, 2009). Turner *et al.* (2013) noted that most of the anthrax cases in ENP are observed in the wet season in plain ungulates (January-April), although elephants have anthrax mortality peaks in the dry season (November). Turner *et al.* (2013) further stated that the seasonality of anthrax outbreaks varies among locations, making it difficult to develop a single consistent description of its disease ecology. Such dry conditions can reduce an animal's innate resistance to infection, making them more susceptible to infections (Hugh-Jones and Blackburn, 2009). However, drought conditions can also lead to a reduction in anthrax cases, if droughts alter habitat selection, pushing animals into less preferred habitats that have lower anthrax risk (Huang *et al*., 2021). A severe drought in ENP in 2019 was associated with low anthrax mortality, while periods of high rainfall and more forage availability were

associated with larger anthrax outbreaks (Huang *et al*., 2021). In contrast a study carried out in the Zambezi floodplain of western Zambia reports that the seasonality of anthrax is more reflective of human activities on the floodplain rather than climatic effects (Munang'andu *et al*., 2012). This conclusion is supported by the fact that the chances of anthrax outbreaks taking place during the dry months when humans were more dependent on the floodplain were higher than during the periods of flooding (Munang'andu *et al*., 2012). Thus, differences in the ecology of particular ecosystems can make it difficult to determine common causes of anthrax outbreaks among ecosystems.

Bacillus anthracis is a soil-borne, rod-shaped, spore-forming, and Gram-positive microbe (Turnbull, 1996). Within hosts bodies, *B. anthracis* occurs as active vegetative cells (Ebedes, 1976). When conditions inside the dead host become unfavourable for vegetative cell growth and upon exposure to oxygen, *B. anthracis* forms spores that are released into the environment (Lindeque and Turnbull, 1994). The ability of *B. anthracis* to form viable spores allows it to inhabit a diverse variety of environments, including the soil, water and vegetation (Fritze, 2004; Nicholson, 2002). The sites where spores are released have the potential to infect new hosts (Lindeque, 1991). Even though *B. anthracis* spores decline overtime at carcass sites (Lindeque & Turnbull, 1994; Turner *et al.*, 2014), contamination levels at these sites can remain undiminished for many years after an animal death despite exposure to wind, rain and sunlight (Lindeque & Turnbull, 1994; Turnbull *et al.*, 1998). High concentrations of spores are found in soils with low alkaline pH, abundant organic matter and higher calcium content (Hugh-Jones and Blackburn, 2009). Hence, the rate at which a spore pool decays likely depends heavily on local environmental conditions. In addition, features of the outermost spore layer (exosporium) also have been shown to affect the ability of *B. anthracis* spores to bind to different soil types (Williams *et al*., 2013). Therefore, contamination level is believed to be different depending on terminal cell counts in the host's blood, size of the carcass, the scavenging characteristics weather conditions after death, and local soil properties.

2.4 Herbivore susceptibility to *B. anthracis*

The susceptibility of an individual to *B. anthracis* depends on various factors. In general, herbivores are more susceptible to anthrax than carnivores. Among individuals of a susceptible species, individual susceptibility depends on several interactive factors such as age, sex, reproductive status, exposure dose, exposure timing, host nutrition, and other potential stressors. In every epidemic some individuals become sick and some may die, whereas others recover from illness and still others show no signs or symptoms of disease (Casadevall and Pirofski, 2018).

There are various attributes of host susceptibility in general, with application to anthrax. Firstly, immunity; a properly functioning immune system is essential for reducing susceptibility to many infectious diseases including anthrax (Casadevall and Pirofski, 2018). In addition, host immunity can differ from individual to individual by the history of an individual's previous interactions with microbes as well as the fact that immune responses of different individuals may vary in intensity and with time (Casadevall and Pirofski, 2018). Secondly, host sex can be a major determinant of susceptibility to infectious disease (Casadevall and Pirofski, 2018). The mechanisms responsible for sex-related differences in susceptibility include anatomical, hormonal and immune differences (Casadevall and Pirofski, 2018). Thirdly, is the age of the host's species individuals, many infectious diseases are associated with high mortality at the extremes of age (Casadevall and Pirofski, 2018). In addition, according to (Baxter and Griffin, 2016) adults are generally more vulnerable to anthrax susceptibility than young or sub-adults. Fourthly, is the attribute of the environment which includes all conditions that may influence host-microbe interaction, including vitally important entities such as nutrition, climate, crowding and presence of environmental toxins (Casadevall and Pirofski, 2018). Fifth factor is nutritional stress, proper nutrition is critical for homeostasis, and nutritional deficits are likely to have protean effects on physiology, including immune function (Casadevall and Pirofski, 2018). A sixth factor is the *B. anthracis* strain, and individual properties such as coinfections, genetics, pregnancy/lactation and the route of infection (Welkos *et al*., 2011). The anthrax attacks that occur in 2001 caused a fatality of 5 of the 11 individuals infected by the aerosolized spores, assumed to be due to the differences in host susceptibility (Yadav *et al*., 2011). Furthermore, various stressors such as;

competition, heat, parasitism, reproductive activities might lead to host susceptibility to *B. anthracis* (Dragon and Rennie, 1995; Hugh-Jones and de Vos, 2002; WHO, 2008).

The required amount of spores to cause an infection depends on the route of entry into the host species (WHO, 2008). Ingested lethal doses (LD_{50} the dose required to kill half of the exposed population) for herbivores (bovid or equids) are estimated to be 10^7 to 10^8 spores administered as a single dose, however, quantities of spores per gram measured in the environment (in contaminated soil, water or scavenger feces) are many orders of magnitude lower (WHO, 2008; Turner *et al.*, 2016). According to WHO (2008), in guinea pigs, ingestion requires spores exceeding 10^8 to cause an infection while for inhalation the LD_{50} is about 1.6-4.0 $x10^4$ spores. The same applies in sheep in the 1940s, British biological warfare established that the aerosol MID (minimum infectious dose) for sheep was 3.5 $x10^4$ spores and that the dose needed to ensure lethal infections via the oral route in sheep and horses and cattle was 5 x 10^8 spores (WHO, 2008). Therefore, more spores are required for ingestion than inhalation routes to cause a lethal infection (WHO, 2008). Previous work in ENP demonstrated that ungulates such as zebras ingest significantly more soil in the wet season, which might increase exposure to *B. anthracis* at that time (Turner *et al*., 2013). WHO (2008) stated that even though MID can be experimentally established, a MID of *B. anthracis* in a controlled laboratory setting is difficult to relate to exposures that herbivores are likely to encounter in their natural environment. MID estimates of *B. anthracis,* therefore, are largely unknown for wildlife (Hugh-Jones and de Vos, 2002).

Research into host susceptibility in ENP suggests that micro-parasites (such as bacteria, viruses, fungi, protozoa) and macro-parasites (helminths, arthropods) play an important role in both the external and internal ecosystem of their host (Cizauskas *et al*., 2014b; Kamath *et al.*, 2014). According to Cizauskas *et al.* (2014b) factors such as gastrointestinal (GI) parasites can influence host susceptibility to anthrax in the wet season by manipulating the host internal ecosystem. In addition, Cizauskas *et al.* (2014b) suggested that the anti-parasite Th2 immune responses may make hosts less capable of mounting an effective Th1-type immune responses against anthrax infections. Hence this increase in

susceptibility, combined with evidence of increased *B. anthracis* exposure during the wet season, could be a driver of anthrax outbreaks in ENP (Cizauskas *et al*., 2014a; Turner *et al*., 2014).

2.5 Anthrax in Namibia

Anthrax was documented in Namibia starting in pre-colonial times, and sporadic anthrax outbreaks were recorded across the country since the 1870s (Ebedes, 1976). In Namibia, anthrax is considered an endemic disease with infrequent and irregular outbreaks occurring annually throughout the country in livestock and wildlife (Beyer *et al*., 2012). According to Berry (1993), disease epidemics in wild animals and domestic animals, namely anthrax, rabies and rinderpest viruses, are indeed known to have occurred in Namibia during the colonial period. Anthrax has been recorded in both livestock and wildlife, mostly in communal farming communities, commercial farmlands and national parks. Between the periods of 1920-1971, approximately 1541 anthrax cases were confirmed on White-owned farms in the country, even though livestock were regularly vaccinated against the disease (Ebedes, 1976). Based on genotypes of the pathogen, the areas surrounding ENP have wildlife anthrax with shared genetic strains with those found inside ENP. Even though a national park may be fenced off, animals still manage to occasionally break through the fence to surrounding areas (Beyer *et al*., 2012), an indication that the fence is permeable. Further away from the greater ENP area, the genetic strains are quite different (Beyer *et al*., 2012). The similarity of genetic strains in and around ENP is not necessarily due to spillover from ENP, since anthrax in the region predates the fencing of ENP. Likely animals can be exposed on either side of ENP's fences, and the strains in that region are just similar.

Mortality due to anthrax is a regular occurrence in domestic and wild animals in the Zambezi region. In 2004, the Zambezi region reported a major outbreak, where 152 buffalo, 42 cattle, 15 elephants, and two zebras died of the disease (Cloete, 2013). Shigwedha (2004) also reported that anthrax mortality occurs regularly in domestic and wild animals and occasionally in humans. During 2004 in the Zambezi, 11 wildlife animals were killed by anthrax that caused the disease to spread to livestock, of

which 9 cattle were reported dead (Shigwedha 2004). Shigwedha (2004) further stated that during the outbreaks protective measures were taken by the Ministry of Agriculture, Water and Forestry to vaccine all cattle. A commercial farm in the Omaheke region in 2012 was placed under quarantine after it was suspected that three farmworkers had died of anthrax after consumption of meat from a cow that died of unknown causes (Kisting, 2012). The quarantine was later lifted, and the farm owner suffered great financial losses due to the quarantine (Kisting, 2012). Currently, the socio-economic impact of anthrax in Namibia is still unknown. But it shows that the quarantine of commercial livestock and game farms will have negative financial implications on such businesses, as well as communal farmers, should an outbreak occur in these areas. This is because communal farmers rely heavily on their livestock for their livelihoods (WHO, 2005). Major outbreaks continue to occur in recent years in Namibia. In 2019, the Kunene region reported outbreaks that caused the deaths of 68 out of 1 670 small stocks (sheep and goats) and 104 suspected cases of human anthrax at Otjitanga village after the community consumed and had close contact with anthrax-contaminated meat (Shikongo, 2019). The same year, very few cases of anthrax outbreak has been reported in ENP, an indication that causes of outbreak are likely to vary from region to region. In addition, at the Liambezi Lake in the Zambezi, 39 fatalities of the 110 hippos were reported due to anthrax. Therefore, the agriculture and health ministries have cautioned communities against touching or getting into contact with any animal carcasses that die of unknown causes in the affected areas. Farmers were also advised to ensure that their livestock is vaccinated against the disease each year (Shikongo, 2019).

2.6 Anthrax in Etosha National Park

Anthrax was first diagnosed in ENP in 1964, although there was evidence to suggest that it had been present in the region long before this time (Ebedes, 1976). Ebedes (1976) originally proposed that the increased incidence of anthrax in ENP in the late 1960s was due to the increase in the number of gravel pits, which held seasonal water and hence delayed normal migration of animals from the area. He hypothesized that gravel pits provided ideal conditions for *B. anthracis* concentration and

multiplication, exposing animals when drinking at these sites. However, further studies did not find support that spores concentrate at gravel pits or other water sources (Turnbull *et al.*, 1989; Lindeque and Turnbull, 1994; Turner *et al.*, 2016), though their effects on animal movements have not been studied. Early veterinary efforts to control anthrax in ENP, such as burial or incineration of infected carcasses, were unsuccessful in eradicating the disease (Ebedes, 1976).

Experimental studies have showed that spore persistence does not vary among soil types in ENP (Cloete, 2013), suggesting the entire park is suitable for *B. anthracis* spore persistence. Furthermore, Turner *et al.* (2014) found that nutrients from carcasses alter the environment and attract grazing herbivores to pathogen aggregations, thus increasing the transmission rate. Havarua *et al.* (2014) highlighted that host foraging behaviour could be a fundamental component of anthrax epidemiology. This study revealed that the diet of zebra shifts from short grasses during the wet season to more tall grasses during the dry season, thus suggesting greater potential to ingest more soil in the wet season (Havarua *et al.*, 2014). They concluded that the foraging behaviour of zebras increases their potential exposure to *B. anthracis* spores found in the soil and vegetation, and this points to possible ways in which other herbivores may contract anthrax in ENP. This idea is further supported by recent work looking at zebra habitat selection in different seasons and rainfall years (Huang *et al*., 2021). In general herbivorous mammals are more at risk of contracting anthrax than other mammals because they are likely to come into contact with it when feeding (ingestion route) (Nicholson, 2002; Turner *et al.*, 2016). In addition, their low antibody titre against anthrax have been observed, which are considerably lower than those in carnivores, and indicate the exposure to low, sub-lethal doses of the pathogen (Cizauskas *et al.*, 2014a; Bellan *et al*., 2013). Carnivores are more resistant to anthrax, producing strong antibody responses to protect them from lethal infections as they frequently come into contact with infected carcasses (Bellan *et al*., 2012). But, this does not mean they cannot get a lethal anthrax infection; carnivores do succumb to anthrax on occasion (WHO, 2008).

Anthrax occurs annually in ENP and affects host species such as plains zebra, springbok, blue wildebeest, and African elephant (Berry, 1993). In plains ungulates (zebra, blue wildebeest, and

springbok) the highest mortality peak of anthrax is observed toward the end of the wet season in ENP (March–April) (Lindeque *et al*., 2013) with anthrax cases concentrated in open habitats frequented by these species (Huang *et al*., 2021). In elephants, anthrax mortalities peak during the dry season and are more widely distributed, occurring in the central Okaukuejo area, the northeast (Namutoni area) and the west of the park (Otjovasandu area) (Ebedes, 1976; Lindeque and Turnbull, 1994). In addition, both male and female elephants are widely distributed throughout the park, especially during the wet season when anthrax outbreaks are more pronounced in plains zebra. It is their wide distribution that is presumed to be responsible for the increased incidence of anthrax in elephants in the western part of ENP (Lindeque, 1991). Research into anthrax seasonality in ENP suggests that a combination of habitat selection, foraging behaviour, increased exposure, and coinfections with gastrointestinal parasites may all contribute to the wet season timing of anthrax in ENP (Turner *et al.*, 2013; Cizauskas *et al*., 2014a; Cizauskas, *et al*., 2014b; Havarua *et al*.,2014; Huang *et al.*, 2021).

Etosha National Park recorded a total of 2182 anthrax mortalities from 1975-2020 (Etosha Ecological Institute mortality surveillance records, 2020; Figure 4). This includes 14 species with recorded anthrax mortalities, 5 host species detailed in Table 1 and 58 individuals of nine species (Etosha Ecological Institute, 2020). The highest peak was in 2010 (177) individuals, followed by a downward trend in the anthrax mortality for the remaining 9 years, starting 2011 (Figure 4). This recent downward trend is likely to be due to a period of lower than average rainfall (Huang *et al.*, 2021) and reduced surveillance effort when a rise of rhino poaching in ENP diverted resources from monitoring to wildlife protection (personal communication for Kilian).

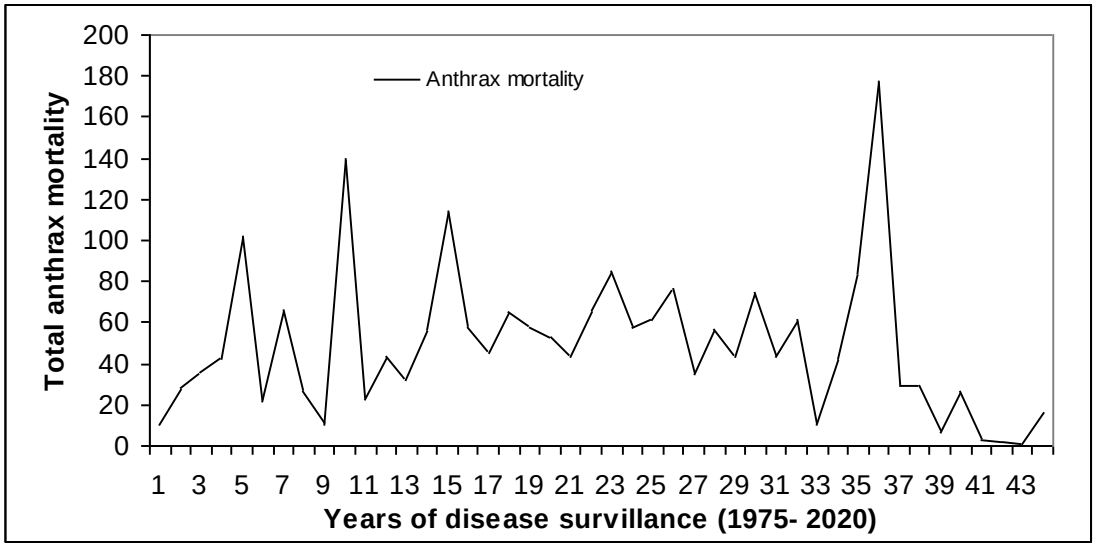


Figure 4. Anthrax mortalities recorded annually in Etosha National Park from 1975-2020 (Etosha Ecological Institute, 2020).

2.7 Anthrax transmission and animal behaviour in medium-sized species

Inhalational exposure requires lethal doses around 10^4-10^5 spores, and doses of about 10^8 (100 million) spores (100% lethal) for oral exposure (WHO, 2008). There is little evidence to support inhalational exposure for species in ENP (Barandongo *et al.,* 2018), or ingestion from drinking contaminated water (Turner *et al.,* 2016). In plains zebras (considered here a medium-sized mammal), anthrax transmission occurs through ingestion of spores while foraging (Turner *et al.*, 2014). Anthrax carcasses create nutrient hotspots that can persist for several years, altering soil fertility and vegetation growth (Turner *et al.*, 2014). At zebra carcass sites host foraging responses changed from avoidance to attraction over time, and ultimately to no preference, and these behavioural responses varied among herbivore species visiting these sites (Turner *et al.*, 2014). According to Turner *et al.* (2014), herbivore species such as zebra, springbok, wildebeest, and gemsbok visit zebra anthrax carcasses, but gemsboks rarely forage there, unlike the other species. Zebra show significantly more attractive than other species to carcass sites for foraging at carcass sites. Elephants did not visit zebra carcass sites (Turner *et al.*, 2014). Choices made by these herbivores to visit carcass sites, and the time spent foraging at

these sites, might have a strong influence on rates of pathogen transmission to susceptible hosts (Turner *et al.*, 2014).

In conclusion anthrax transmission in medium-sized animals is relatively well studied. Herbivores tend to have different responses to a medium-sized carcass site, which range from attraction, avoidance due to deterrence at the sites to no preference hence, they just walk by. These hosts also have to face a trade-off between nutrient acquisition and exposure to environmental pathogens.

2.8 Anthrax in elephant

Anthrax affects both African elephants and Asian elephants (*Elaphus maximus*). When an elephant is suspected to have died due to anthrax, it exhibits external signs like bleeding from natural orifices, bloating and rapid decomposition of the carcass Aggarwal (2020). The presence of anthrax in elephants in hot climates area is considered probable reservoir of infection for another wild as well as domestic animals (Yasothal, 2013). In 2019, Zimbabwe National Parks and Wildlife Management Authority (ZimParks) confirmed a fatality of 11 elephants due to anthrax at Pandamasue Forest (NewZiana, 2020). The outbreak was another leading cause of elephant mortality after the 2019 drought claimed close to 200 individuals (NewZiana, 2020). Drought was thought to lead to the exposure of anthrax spores to the soil surface, thus increasing the rate of exposure (Moyana, 2019). In addition, according to a Sunday News report in 2019, anthrax was suspected in the deaths of several wildlife animals in Zambezi Valley such as; eight elephants, two buffaloes, jackal, impala, and hyenas (Moyana, 2019). In 2006, a forest guard in Kerala, India reported the death of a wild elephant from Chedleth Forest Range to the local veterinary surgeon, showing signs of suspected anthrax such as discharge of blood from the eyes, trunk and also from a deep cut wound in the middle of the trunk (Priya *et al*., 2009). Laboratory results provided evidence that the animal indeed died from anthrax (Priya *et al*., 2009). In addition, control measures like precautionary disposal of a carcass, public awareness programs and vaccination of animals were successfully done by the veterinary officials and the forest officials were also informed (Priya *et al*., 2009). Another anthrax case was reported in

northern Tanzania's district of Ngorongoro where eight elephants had been reported dead, showing signs of anthrax such as bleeding trunks (nose bleeding), strange coloured stools and sudden drunk-like dizziness while moving (IANS, 2018).

The Indian Ministry of Environment came up with a standard operating procedure (SOP) to deal with suspected or confirmed elephant anthrax cases (Aggarwal, 2020). The SOP called for the complete burning of anthrax suspect carcasses and complete sanitization of the contaminated area to prevent its further spread (Aggarwal, 2020). In the past anthrax, control was conducted in ENP from 1968-1981, through methods of burning or burying of carcasses, disinfections of watering points, or closing of water points to restrict animals movements (Turner *et al*., 2013). Control efforts ceased after a large elephant outbreak in 1981 which overwhelmed carcass disposal efforts (Lindeque, 1991). Since then anthrax in Etosha National Park has been considered as part of the natural ecosystem (Turner *et al*., 2013), this is what makes ENP the ‘’perfect natural laboratory” to study anthrax in wildlife. However, limited management still occurs, through movement of carcasses found proximate to roads, water, or high tourist-use areas. Carcasses are moved away from waterholes to prevent contamination of water sources (Claudine Cloete, personal communication), which could also reduce exposure through drinking.

2.8.1 Animal behaviour at elephant carcass sites

Almost all animal types visit elephant carcass sites either by choice or chance. Animals such as hyena, jackal and lion play an important scavenging role on large mammal carrion (Coe, 1978). However, this can also presume that these animals are present in reasonable numbers where the carcass is located (Coe, 1978). Vultures and Marabou stork also visit large animal carcass sites (Coe, 1978). In addition to their scavenging role, vultures also play an important primary role in opening carcasses for vertebrate scavengers and *Diptera* (Coe, 1978). These vertebrate scavengers play an important role in the moving and chewing of bones in earlier and later stages of carcass decay and the dispersal of

nutrients (Coe, 1978). Yet no studies have assessed herbivore responses to large animal carcass sites and what role these may have in anthrax transmission (Turner *et al.*, 2014).

Elephants are recognized for strong behavioural reactions directed towards dead conspecific not restricted to the mother-offspring relationship (Bere, 1966; Sikes, 1971; Douglas-Hamilton *et al.*, 2006; McComb *et al.*, 2006). Observations show that elephants exhibit a generalized interest in their dead, even after the carcass has long decayed and even if there was no strong bond between them (Payne, 2003; Douglas-Hamilton *et al.*, 2006; McComb *et al.*, 2006). Their findings also show elephants spend significantly more time exploring elephant remains than inanimate objects or the remains of other large herbivores. At carcass sites, elephants display common behaviours such as approaching, touching and investigating the carcass and all these behaviours were observed at carcasses in varying stages of decay. The infrequently observed behaviours included mounting and vocalizing which were displayed when they encounter fresh carcasses (Goldenberg and Wittemyer, 2019).

2.8.2 The effects of elephant on the environment and anthrax transmission

The decomposition of animal carcasses contributes to nutrient recycling in ecosystems, including by delivering nutrients to the soil. The inputs of nutrients from a carcass can affect soil chemistry (Macdonald *et al.*, 2014) and plant diversity (Bump *et al.*, 2009; Barton *et al.*, 2013) and these dramatic changes can occur even if the carcass is disturbed or scavenged by vertebrates (Parmenter and MacMahon, 2009). Several studies have examined the effects of carcasses on soil and vegetation a few years after the death of animals to understand the longer-term effects of this particular aspect of carcass decomposition. The effects of carcass decomposition on soils and vegetation will be quite different from what might be expected in an unchanged or intact landscape. For instance, fewer exotic weed species might be expected in less disturbed landscapes. Such would result in less competitive exclusion of native species from carcass patches, reducing the likelihood of creating a new stable state and perhaps stimulating plant community heterogeneity in other ways (Barton *et al.*, 2016).

Coe (1978) carried out a study of elephant carcasses in the Tsavo National Park (east), after a drought caused severe elephant mortality. Low rainfall in 1970 and 1971 led to a serious drought in Tsavo (East) and, as a result, wiped out 5900 elephant population over a period of about 18 months (Corfield, 1973). Which was a very large elephant population mortality recorded. Scavengers played an important role in the environment, as they contribute not only to the breaking down of bones many months after the animals died but also to the dispersal of bones and nutrients (Coe, 1978). Elephants transported individual bones up to 100m from a carcass site (Coe, 1978). Coe (1978) discovered that although a small percentage of the bones (< 15 percent) can be moved by scavengers away from the elephant carcass site, most of the skeleton remains on or close to the death site. Elements such as calcium are present in the bones of large herbivores (Coe, 1978), which may explain why animals such as gemsbok and giraffe chew on them (Hutson *et al.,* 2013). Therefore, the immobilization of calcium in large animals such as elephants may be an important factor in the cycling of nutrients, especially where the mammals have a long life span (Coe, 1978). Coe, (1978) suggested that if we add skeletal material that can be expected to be found decomposing on the ground, assuming that the skeletons remain of elephants take a minimum of 20 years to decay completely, these indicate that an additional 0·7 g m^2 will be unavailable for plant growth. Hence, during a period of severe drought, the input of nutrients from elephant carcasses played an important role in nutrients recycling in the environment (Coe, 1978), which can boost vegetation growth at the carcass site over time. In addition, the future transmission *of B. anthracis* events at carcass sites may be indirectly facilitated by the recruitment of plant-beneficial bacteria (Valseth *et al*., 2017). However, arid soil microbial diversity soil communities can also be affected by the deposition of animal carcasses and their decomposition not only contributed by scavengers and arthropods but also other factors such as precipitation changes (Valseth *et al*., 2017). Thus, carcasses could play an indirect role in the future transmission of *B. anthracis* by stimulating the growth of taxa known to have Plant Growth-Promoting Bacteria (PGPB) (Valseth *et al*., 2017).

CHAPTER 3: METHODOLOGY

3.1 Study Area

The study was carried out in ENP, which is a 22,915 km^2 protected area in northern Namibia with a geographical location between 18°30'-19°30'S and 14°15'-17°10'E (Ministry of Environment and Tourism., 2011; Figure 3). Nearly 1/5 of the area of ENP is salt pans (4,760 km^2) (Hipondoka *et al.*,2006) surrounded by grassland habitats that attract grazing herbivores. The vegetation in ENP is classified as arid savannah of which mopane (*Colophospermum mopane*) tree veld covers much of the park, with an extensive sweet grass veld (the Okaukuejo plains) around the Etosha pan (Le Roux *et al*., 1988). According to Auer, (1997) boreholes and artesian or contact springs supply the only perennial water in the park. There are three seasons recognized in semi-arid ENP, the cool dry season (May-August), the hot dry season (September-December) and the hot wet season (January-April). Rainfall in ENP occurs mostly from November to April, with January and February being the wettest months of all (Engert, 1997).

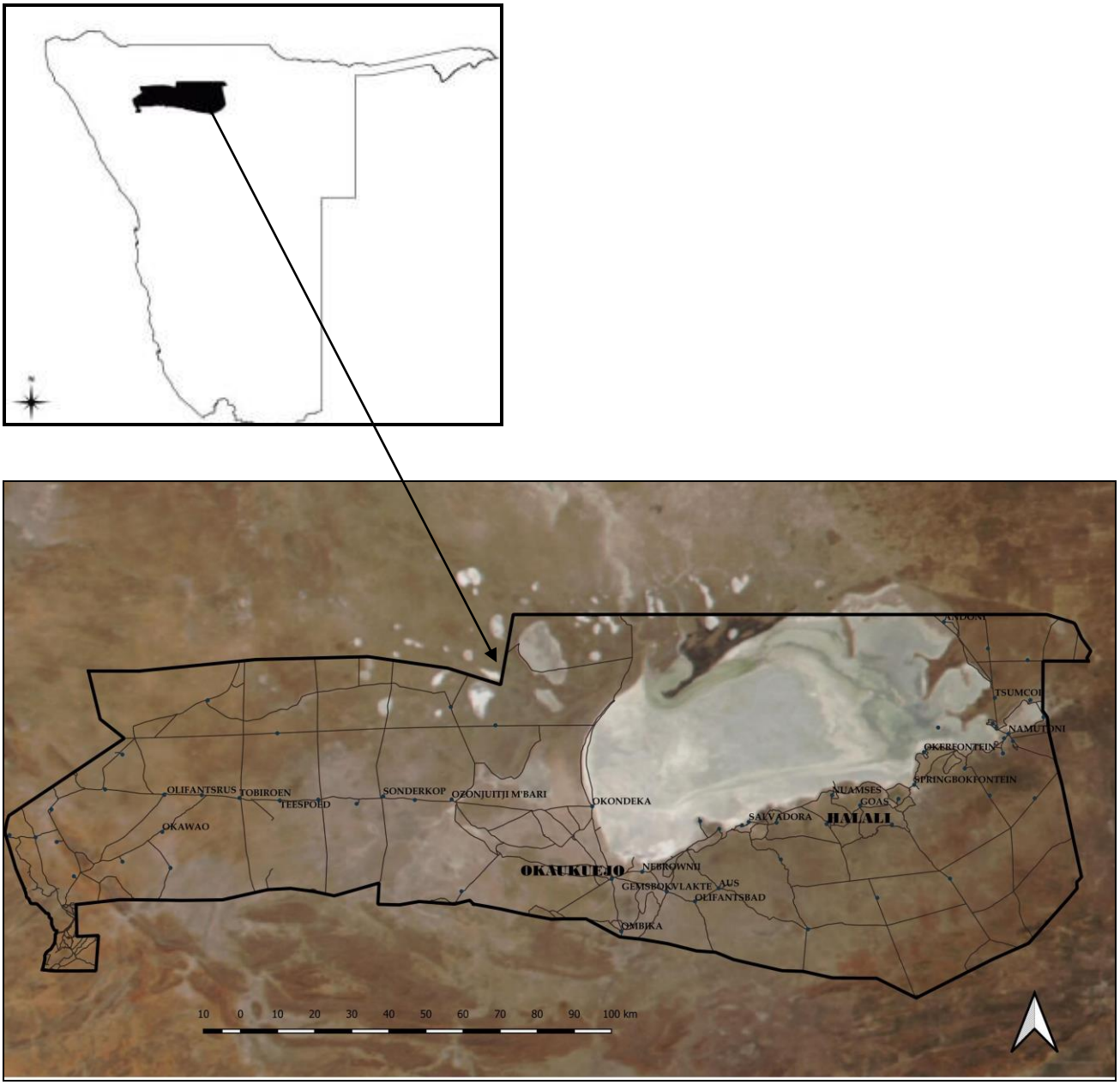


Figure 5**.** A map of Etosha National Park in north-central Namibia, with the inset map showing the tourist camps in the study area (bold black), waterholes (blue circles), salt pans (white) and road network for central, eastern and western Etosha. (Credit: Gabriel Shatumbu).

3.2 Research Design

This study investigated carcasses from large-bodied animals, primarily from elephants, and how these carcasses i) affect the area of bare soil around the carcass over time, ii) affect vegetation cover over time, iii) differ in *B. anthracis* spore concentrations in the soil compared to medium-bodied carcasses,

and iv) how other animals interact with these sites over time. Once carcass sites were chosen for sampling (criteria detailed below in 3.3) some were assigned to the behavioural study (fresh carcasses) and the rest were used for sampling for soil, vegetation cover and spore concentrations. Quantitative measurements were conducted to determine the area of bare soil and the change in vegetation cover at these sites over time since death. Behavioural activities of animals that visit elephant carcass sites were recorded using Browning motion-sensing video camera traps (USA). Soil samples for *B. anthracis* in soils were collected from confirmed anthrax positive carcass sites of different carcass ages and body sizes (African elephant versus plains zebra). Spore concentrations were quantified using bacterial culture in serial dilution following protocols as per (WHO, 2008).

Forty carcass sites were sampled in this study. Fifteen African elephant carcass sites of different ages were sampled for vegetation cover, disturbed soil and concentration of *B anthracis* spores. In addition, fifteen plains zebra carcass sites of different ages were also sampled for the concentration of *B. anthracis spores* (only) to compare with the above-mentioned 15 elephant carcass sites (Figure 6). Furthermore, an additional 10 African elephant carcass sites were used in this study for camera traps for behavioural data (Figure 7). Camera traps were set up immediately or few days after death to capture activities of both carnivores and herbivores, except for (LA02) which was set up a year after death.

3.3 Selection of sampling sites

Detection of potential carcass sites was facilitated by the Etosha Ecological Institute mortality surveillance efforts, which produced mortality records with dates, species, GPS locations and suspected causes of death for all recorded mortalities. During this study, fresh carcasses were detected with the help of GPS tagged scavengers (vultures, lions and hyenas) from other research and monitoring projects looking for scavenger foraging sites ongoing in the study area.

The selection of carcass sites for this study depended on various factors. Firstly, the carcass sites needed to be accessible throughout the year and within range of the study area. Secondly, the carcasses for the two chosen species had to be anthrax positive to enable the determination and comparison of the concentration of *B. anthracis* spores. Thirdly the carcass must be of an adult animal. From among the possible carcasses, these were further divided into groups according to carcass age, selecting five carcass sites per carcass age. Ages were classified as follows: animals that died within a period of 0-2 years from 2019 were categorized as recent carcasses (R), those 2-5 years old carcasses were categorized as old carcasses (O) while those that died >5 years ago were categorized as very old carcasses (VO). The disturbed soil area (i.e. the area of bare soil, denuded of vegetation) of each carcass site was measured to determine the effect and duration of large-sized carcasses on the local soil and vegetation (environment). Soil taken from the selected anthrax-positive carcass sites was cultured to determine the number of colonies of *B. anthracis* present per gram of soil. The selection of carcasses for the behavioural study further depended on several factors, such as the carcass died recently (to capture both carnivores and herbivores species activities), traveling costs, safety and accessibility of the sites where video camera traps were positioned as it was planned that camera traps were inspected monthly. Off-road driving is restricted in the park unless accompanied by law enforcement officers or Ministry of Environment, Forestry and Tourism staff members, thus chosen carcasses were accessible from the road network.

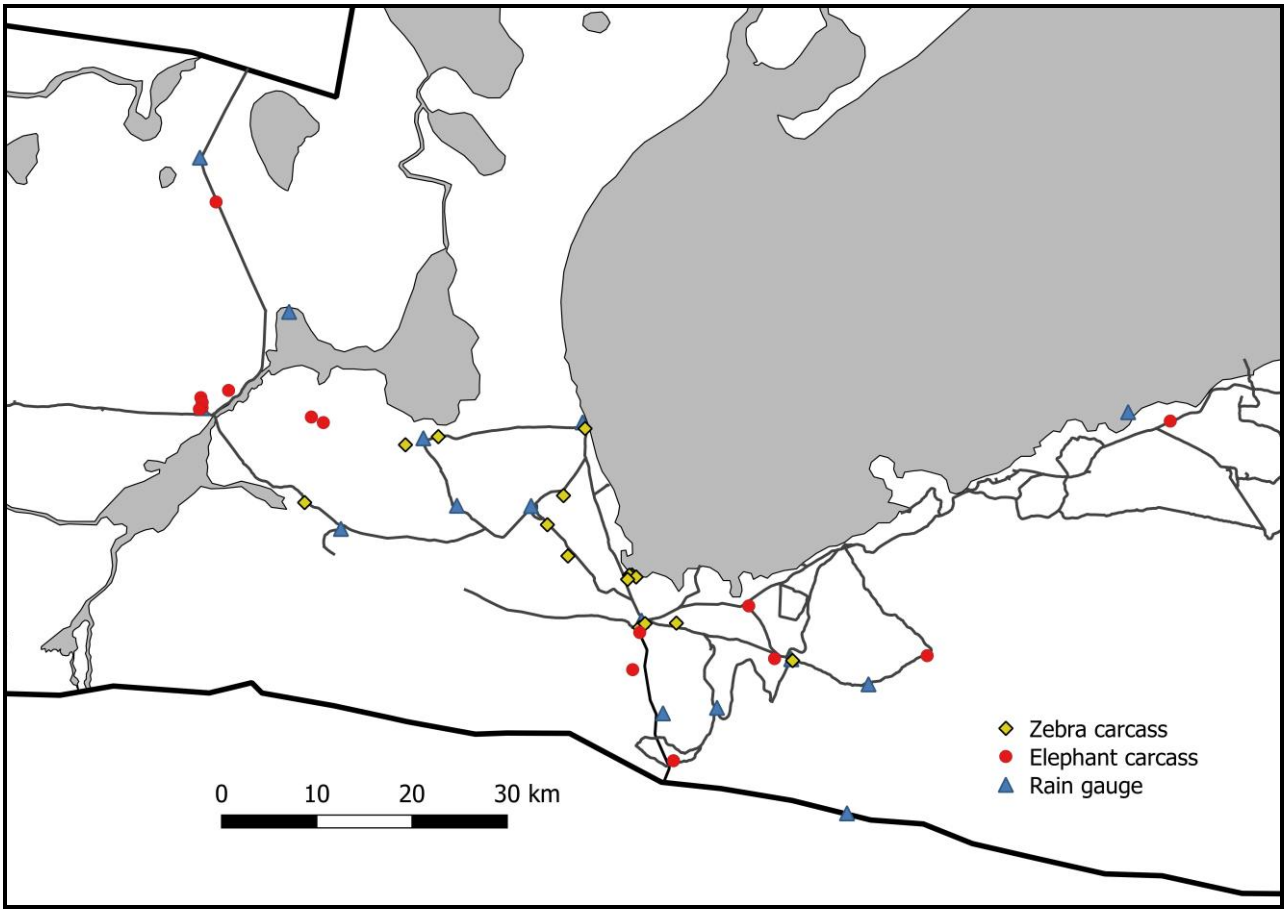


Figure 6. Map of central Etosha National Park showing nearest rain gauges (blue) to sampled carcass sites, elephant (red) and zebra (yellow) carcasses sites for disturbed soil area, vegetation cover and soil sampling for the study (Credit: Yen Hua Huang).

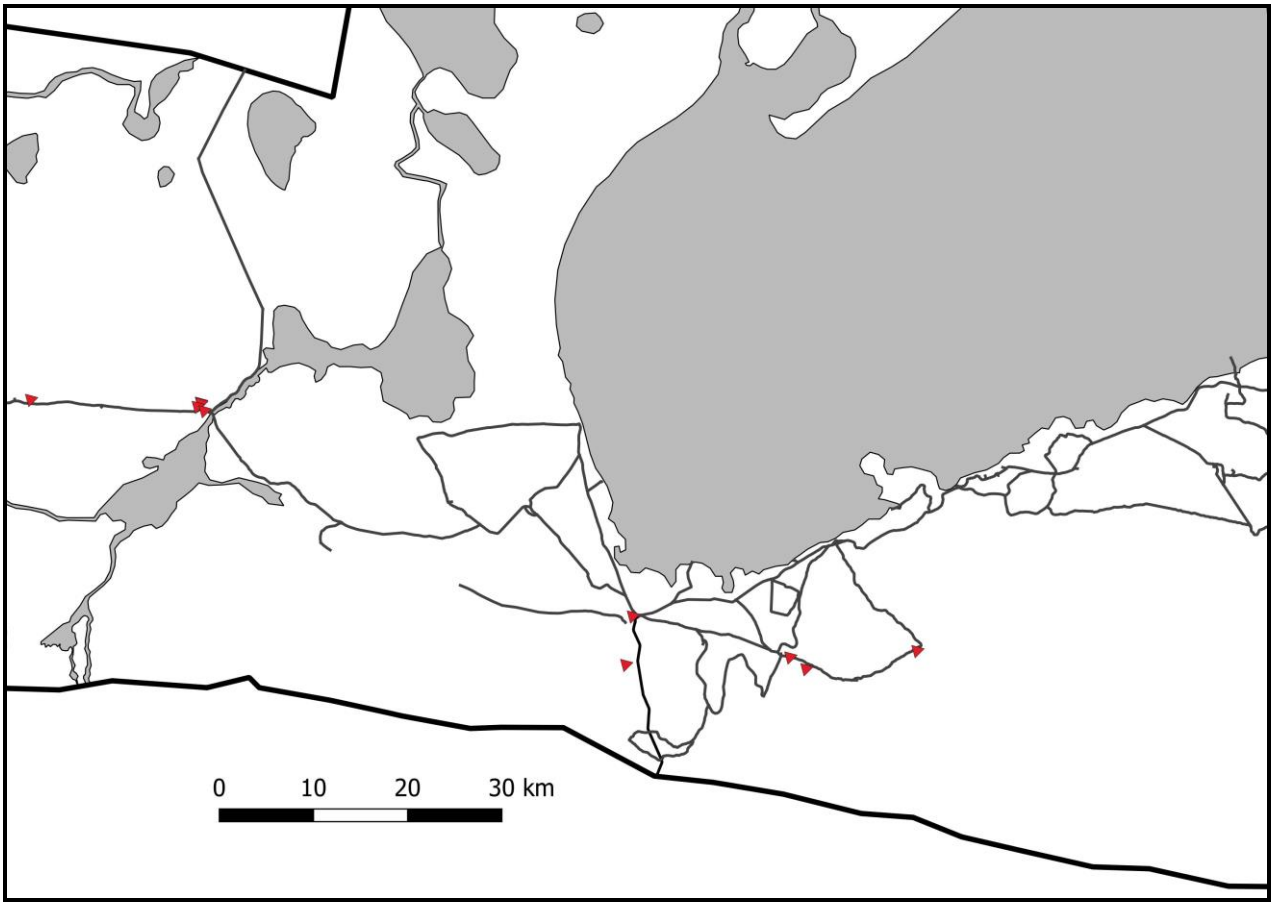


Figure 7**.** Map of central Etosha National Park showing elephant carcass sites with video camera traps for behavioural data for the study in ENP. N= 10 camera sites (Credit: Yen- Hua Huang).

3.4 Procedures

3.4.1. Scale of soil disturbance at large animal carcass sites.

Fifteen elephant carcass sites of different ages were sampled for denuded soil area in this study. Data were collected every three months (January, April and July) in 2020, from carcass sites chosen in 2019. These sampling periods correspond to early in the growing season (January), after the peak of the growing season (April) and in the dry season (July). Carcasses were classified according to carcass age, five carcass sites per carcass age (as described above). For this study, a 50m measuring tape was used to measure the longest part of the denuded area in meters (length), and the radius perpendicular to the length, to estimate the disturbed soil area around the carcass (Figure 8).

Figure 8. The area of soil disturbance at an African elephant carcass site. Image from a carcass sample video**.** Measurements were done assuming a carcass site approximated the shape of an ellipse. The arrow (blue) illustrates the length of the long axis of the ellipse, and in black is the radius of the short axis of the ellipse. This site was 1 year old when this photo was taken.

3.4.2 Vegetation cover at large animal carcass sites

Thirteen adult elephant carcass sites were sampled for vegetation cover in 2020. Data was collected every three months (January, April and July) to have samples at different points in the vegetation growing season. Carcasses were classified according to carcass age, with five carcass sites per carcass age (as described above). Starting at the centre of the carcass sites, following the four cardinal directions (north, south, west & east), four 30m transects were established at elephant carcass sites to estimate vegetation cover using a 2m by 2m quadrant alternating at a 5m interval (Figure 9). The modified Braun-Banquet method (Podani, 2006) was implemented to estimate the percentage of vegetation cover. The vegetation species for each transect were recorded but percentage cover was only estimated for total vegetation, not for each species in each quadrant. In addition, the most dominant species in each quadrant was recorded. Annual rainfall data were obtained from the permanent field rain gauges (Engert, 1997) in the area closest to the carcass site from the Etosha Ecological Institute (Figure 10). These gauges collect accumulated rainfall over the rainy season, and are only measured once per year. The annual rainfall at Okaukuejo Station in central Etosha around

was 440.5 mm for the 2020 rainfall season. The 2019 drought (83.7 mm at Okaukuejo station) is evident in the rainfall data, with rainfall around or below 100m at these seasonal rain gauges. The year in which vegetation sampling was conducted (2020) was an average to above-average rainfall year at these sites.

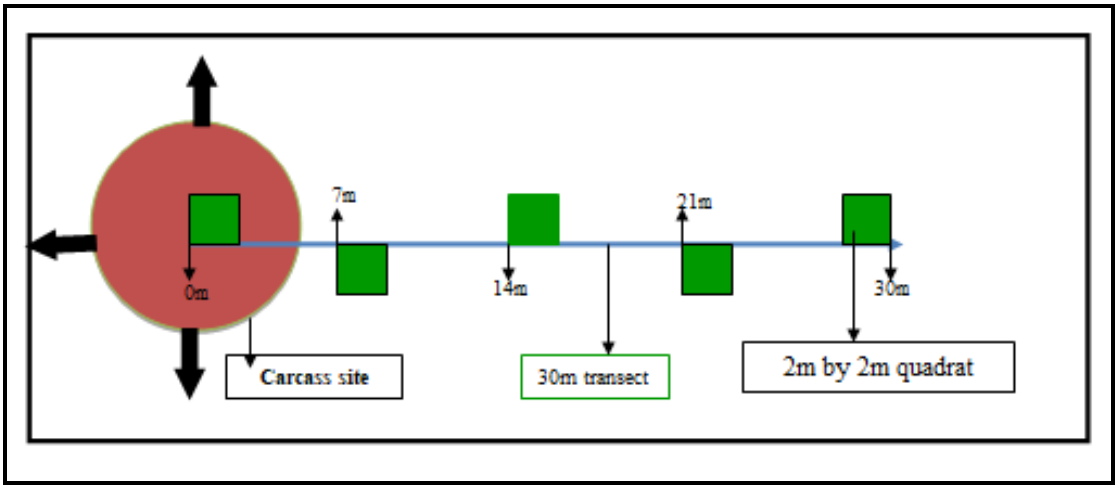


Figure 9. Illustrations of vegetation cover sampling design at elephant carcass sites. Transects were collected in four directions from the carcass site centre. The quadrats were set up at equal distances along transects and were alternating. An example of one transect is shown in grey (not drawn to scale) (Figure 9).

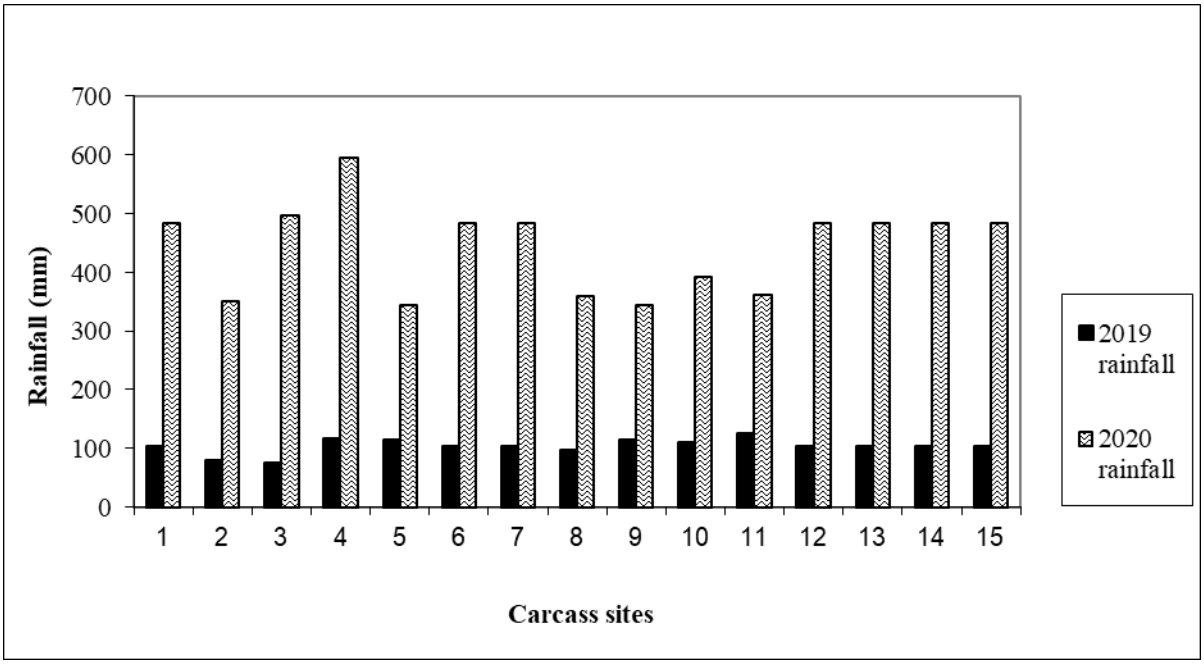


Figure 10. Rainfall recorded from field rain gauges closest to elephant carcass sites in Etosha National Park. Number 1-15 represents different elephant carcass sites sampled in this study.

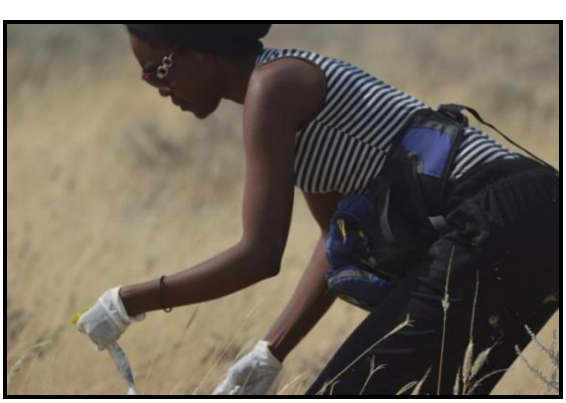
3.4.3. Isolation of *Bacillus anthracis* from the soil at carcass sites

3.4.3.1 Soil sampling from carcass sites

Twenty-eight soil samples were collected from two host species carcass sites; fifteen from medium-sized (zebra) and thirteen from large-sized (elephant) animal anthrax carcass sites. Sample collection was carried out every three months in the year 2020 (January, April and July) yielding three replicates of each site, in different seasons. Carcass age classification was according to the categories as described above. Disinfected spoons were used to dig five scoops (5g per scoop) on the soil surface of 1-2cm depth around the centre of the carcass site. One scoop in the carcass gut cement, (i.e., gut contents and blood released and compacted into the soil; (Barandongo *et al*., 2018) and four scoops around the carcass gut cement, for both species (Figure 11). Soil collected was stored in a sterile WhirlPark® bag and labelled with the date of collection and the identity of each site. These were stored at 4°C at the Etosha Ecological Institute (EEI) until cultured on growth media to quantify the

number of *B. anthracis* colonies per gram of soil to estimate spore concentration. Protocols for culturing for *B. anthracis* were after WHO (2008), with details below.

(A) (B)



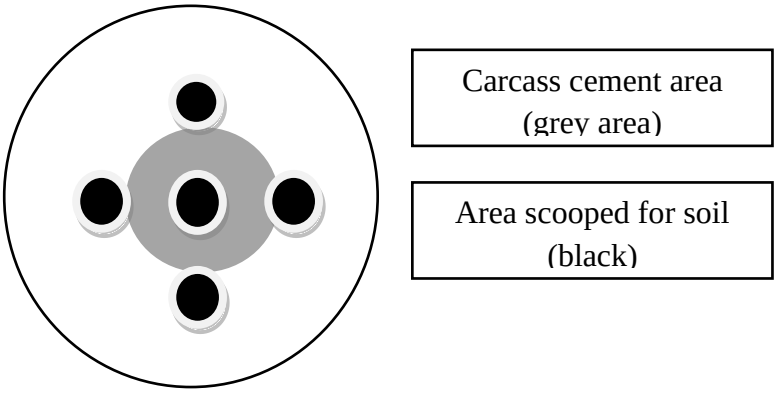


Figure 11. A) Soil sampling at carcass site; B) An illustration of soil sampling at carcass sites (not drawn to scale): (Source for (A): Yen-Hua Huang).

3.4.3.2 Preparation of media to culture *B. anthracis* from soils collected at carcass sites

The preparation of Polymixin-ethylenediaminetetraacetic acid (EDTA)-thallous acetate (PET) agar and culturing of soil samples was done in the anthrax laboratory at the EEI. According to WHO (2008), PET agar of (Knisely, 1966) is the best selective media for the isolation of *B. anthracis* from clinical materials or environmental samples heavily contaminated with other bacteria. The combination of EDTA and thallous acetate cations permits the growth of *B. anthracis* strains but generally inhibits the growth of other *Bacillus* species (Dragon *et al.,* 2001).

The working bench was disinfected using 10% F10 (Quatemary ammonium and biquanidine compounds (0.072%) and propellant) and Disinfectant Aerosol Fogger, SA. Selective (PET) culture media was prepared by adding 78 g of Brain Heart Infusion agar, 0.45 g (EDTA) and 1 ml thallous acetate into 1.5 L distilled water in a 2 L Erlenmeyer flask. The solution was heated using a hot plate stirrer (PC-351) until all particles dissolved whilst being stirred using a magnetic stirrer. The solution

was thereafter autoclaved for 45 mins at 125 °C and 15 psi and terminal cooling was done for 1 hour to ensure the solution was at 50 °C as that was the required temperature at which antibiotics could be added. The agar was removed from the autoclave and 400µl of polymyxin B sulphate solutions was added to the agar using a sterile micropipette tip for each solution, and the mixture was gently mixed by swirling. Using a sterile 25 ml pipette, 20 ml of the PET agar was transferred into 90 mm Petri plates, whilst avoiding forming bubbles. Once completed the agar plates were left on the bench to solidify and stored in a 4℃ refrigerator until ready for use.

3.4.3.3 Bacterial culture of soil samples

The protocol for culturing the soil samples was adapted from the protocol used by Turner *et al.* (2016) with a few minor changes (i.e., PET was used in this study instead of PLET). Sterile distilled water was used for the dilution series. Plates were incubated at 37 °C, and any colonies of *B. anthracis* were counted at 48 and 96 hours. The detailed protocol that was followed is outlined below.

Soil samples from each carcass and each sampling time point were analysed as individual samples. The sampled soils were homogenised by shaking and inverting the Whirlpark bags, then 5 g of soil was placed into a 50 ml falcon tube. The following health and safety procedures were observed at the time of collecting soil samples: wearing protective goggles and a 3m N95 particulate respirator mask, and use of nitrile gloves and a laboratory coat.

The soil was then suspended in 45 ml of 0.1% sodium pyrophosphate and vortexed for ten minutes to discharge and loosen spores from the soil particles (Ganz *et al*., 2014). After discharging the soil particles, the mixture was centrifuged at 0.3x 1000 rcf for 2 minutes to keep spores in suspension but allow the soil to settle. After the supernatant was transferred into a new 50ml falcon tube, it was centrifuged at 3.0 x 1000 rcf for 15 minutes to pellet any spores. The supernatant was discarded and later autoclaved, and incinerated with the trash from the laboratory after centrifuging for the second time. The pellet was re-suspended in 5 ml sterile distilled water. It was vortexed to get spores in

suspension before transferring 1.0 ml to 1.5 ml Eppendorf tubes. Four-fold dilutions of the mixture were made by transferring 100µl of the suspension to another Eppendorf tube preloaded with 900 µl of sterile distilled water using sterile micropipette tips. Dilutions of 10^{-1} to 10^{-4} were prepared. The tubes were mixed well using a vortexer before each transfer. From each dilution sample and the undiluted sample, 100µl was plated onto PET agar by spreading gently using disposable sterile spreaders for each sample. Sterile distilled water was used as a negative control, of which 100µl was plated using the same techniques as the experimental samples. A suspension of live spores of an encapsulated non-virulent strain of *B. anthracis* (Stern 34F2) was used as a positive control to assist in morphological identification of *B. anthracis* colonies, by diluting it to 10^{-5} and then plating it out. The incubation of culture plates happened at 37°C and these were evaluated for bacterial colonies read on days 2 and 4.

3.4.3.4 Identification of *B. anthracis* colonies

According to Dragon *et al.,* (2001), *B. mycoides* MU711/84, *B. thuringiensis* QC12093, *B. subtilis* 1A289 strains formed colonies on PET, so this agar is only semi-selective. However, conclusions were still focused around PET being one of the best media for the isolation of *B. anthracis. Bacillus anthracis* generally produces off-white colonies that have irregular edges and a rough "ground glass" appearance (Koehler, 2009) (Figure 12). For colonies suspected of being *B. anthracis,* these were sub-cultured and subjected to additional diagnostic tests. After 96 hours of incubation, a representative sample from the plate identified as *B. anthracis* based on morphology, and one colony from the positive control plate were picked and sub-cultured on PET by using the streaking technique. *Bacillus anthracis* is sensitive to penicillin and cherry phage, thus a penicillin G disc and 10 µl of cherry phage were added to the bacterial streak for confirmation testing. Penicillin G inhibits the growth of *B. anthracis* and forms an inhibition zone around the disc, while cherry phage lyses *B. anthracis* cells, creating a visible plaque where no bacterial growth is visible.

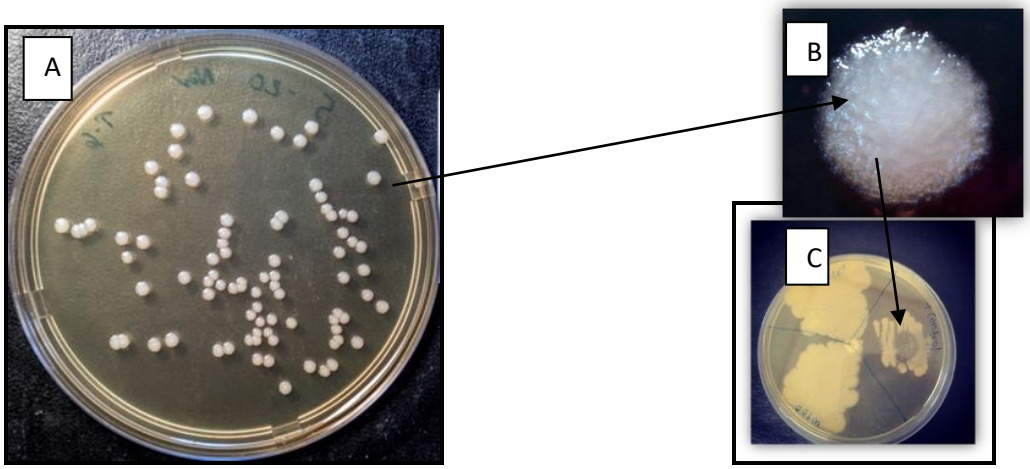


Figure 12. A) Typical *Bacillus anthracis* colonies; B) A single *B. anthracis* colony; C) Confirmation plate with Phage and Penicillin G disc**;** the left two samples are not *B. anthracis*, the right sample shows penicillin and phage sensitivity, and is considered *B. anthracis.* (Source: Dr. W.C Turner)

3.4.4. Placement of motion-sensing video camera traps at carcass sites

Camera traps were placed at or near carcass sites. These sites were identified from GPS locations of tagged vultures and carnivores that congregated around carcasses. Other carcass sites were located by driving the park around Okaukuejo, M'bari and toward the Sonderkop area as a normal procedure for park management patrol. Once a fresh elephant carcass was located that fit the selection criteria (see section 3.3 for details), a camera was placed at the site. Motion-sensing video camera traps (Browning, USA; Figure 13) were set up at 10 fresh elephant carcass sites, to record behaviours of animals that visit the carcass sites until the end of the study period. The motion-sensing video cameras were attached at least 1,2m above the ground on the pole or tree facing the carcass to monitor animal visitations to the site. The best view in the images was 1/3 of the sky and 2/3 of the ground with the cameras roughly facing south to avoid the sun affecting video quality. In addition, there must be no obstruction in the way such as branches or roads (moving cars) since they will trigger the camera. The cameras were always set or reset correctly before departing the carcass site. Each camera was set to a minimum of 20 seconds per video during the day while it was set to 20 seconds at night, with a time

lapse of 5 seconds between videos to conserve power. The videos recorded the trigger source and automatically recorded the time and date. The batteries and SD cards were replaced every month.

Figure 13. An example of a motion-sensing video camera trap box is attached to a pole. And a picture of the Browning, USA motion-sensing video camera traps used (Source: Yen Hua Huang).

The trigger sources recorded included humans, cars, wind, rain and various animal species. Videos triggered by animal species were subjected to further analysis. Each time the camera is triggered, it records a minimum of a 20-second video. If the motion-sensor detects continued activity, it will continue to record video until motion is no longer detected at the site. Thus, the length of videos recorded varied, and behavioural data were collected on a per-visitation basis. In cases where groups of animals were at or crossing a site, triggering several videos over a short period, these clusters were also treated as a single event, or a single data point for that species. However, if a single video had more than one species present, it was recorded as a separate event for each of the species present.

In this study, the activities observed in the videos were categorized into seven different behaviours. These included foraging, bone contact with the mouth, sniffing/ smelling, kicking/touching of bones, resting, walking and investigation (Table 2). Of these seven behaviours, five of them were considered potentially risky behaviours that could expose an animal to *B. anthracis* at an anthrax carcass site.

These risk behaviours included foraging, bone contact with the mouth, smelling, kicking, or touching of bones, and resting at the carcass site.

Table 2. The definitions of the 7 behavioural activities recorded at elephant carcass sites in this study.

Behaviours	**Definitions**
Foraging	When an animal bent its head below the knees for more than 3 seconds and moved its mouth on vegetation (herbivores) or meat (carnivores).
Smelling/ Sniffing bones	When an animal placed its nose along a bone at the surface of the soil at a carcass site to detect a smell or for other reasons.
Bone contact with the mouth	**Chewing bones:** When an animal lifted a bone to its mouth and chewed on it. **Licking bones**: When an animal moved its tongue over and around the surface of the bone at a carcass site without necessarily biting or chewing on it. **Picking up bones**: When an animal lifted, moved, or raised a bone upward from the soil surface at least 1cm from its original position.
Touching/ Kicking bones	When an animal struck a bone at a carcass site with its foot in a forceful way repeatedly with an attempt to move or roll it over. (In elephants: placed, moved, or rested their trunk on bone or skin repeatedly).
Resting	When an animal stood still, sat, or lay down in one position for more than 3 minutes.
Walking/ Running	When an animal walked or ran through the carcass site without stopping to engage in any other activities.
Investigating	When an animal walked around the carcass sites area with the head upright or facing down, as if in search of something.

Table 3**.** Summary details of setting up of camera traps at elephant carcass sites. Age of carcass = age of carcass from death when cameras were first placed at the carcass site. Camera ID is a unique identifier for each carcass site monitored.

Camera ID	**Age of carcass (months)**	**Start date of data collection**	**End date of data collection**	**# of Months video data collection**	**Location**	**Area**
LA01	0.0	24/07/2017	2017-11-27	4.5	S19.21043˚E016.18815˚	W-drive
LA02	12.0	04/07/2017	2020-06-30	35.3	S19.22368˚E015.91028˚	Okaukuejo
LA03	0.0	26/10/2018	2020-06-30	20. 4	S18.97299˚E015.50448˚	M'bari
LA04	0.0	24/12/2018	2020-06-30	18.7	S19.21321˚E016.04414˚	Gemsbokvlakte
LA05	0.0	8/04/2019	2020-06-30	14.2	S19.22774˚E016.08220˚	Gemsbokvlakte
LA06	0.0	27/5/2019	2020-06-30	13. 5	S18.97385˚E015.50487˚	M'bari
LA11	0.0	6/12/2019	2020-06-30	6. 3	S18.97694˚E015.50148˚	M'bari
LA12	1.0	10/1/2020	2020-06-30	5.2	S18.98105˚E015.50800˚	M'bari
LA13	0.0	6/1/2020	2020-06-30	5. 3	S18.97029˚E015.34244˚	Sonderkop
LA14	0.0	10/1/2020	2020-06-30	5.2	S19.21653˚E016.06686˚	Gemsbokvlakte

3.5 Data analysis

Statistical analyses were conducted using SPSS (version 20) (IBM Corporation, Armonk, New York, USA). The differences were considered statistically significant at $p < 0.05$. Before analysis, all data were tested to ensure that they met the assumption that they are normally distributed. Normality tests for data were done using the Shapiro-Wilk test ($p > 0.05$). If the p-value is greater than 0.05, the null hypothesis of the test indicates that the data are normally distributed. Any data with a p-value less than 0.05 was considered not normally distributed. In cases where the data were not normally distributed, appropriate non-parametric tests were conducted. If data were considered normally distributed, parametric tests were used.

Linear regressions were used to compare the change in denuded soil area around the carcass sites between carcasses of different ages over time. The dependent variable was the area of disturbed soil at each carcass site, and the independent variable was the age of the carcass sites. The area of soil disturbance was log-transformed $\log_{10}$ (area + 1). A separate linear regression was conducted for each of the sampling points (early growing season, peak growing season, late growing season). In cases where the carcass had no bare soil patches and the whole site was covered with vegetation, a zero was recorded. The following formula was used to calculate the area of soil disturbance at the elephant carcass sites, *s*, based on the area of an ellipse, where *l* is the length of the long axis of bare soil at the carcass site, and *r* is the radius of the short axis of bare soil at the carcass site (see Figure 8 for how measurements were collected).

$$s = 2\,\pi r\,(l/2)$$

To compare changes in vegetation cover at carcass sites, a multiple regression test was used. The dependent variable was the vegetation cover (m^2), and the independent variables were the age of the carcass site (in categories recent, old, and very old), the distance from the centre of the carcass site (an ordered variable from 1 to 5 for each sampling distance from the site centre), and the season of sampling (in categories early, mid and late growing season).

To estimate *B. anthracis* spore concentrations in soil, the following formula was used to calculate the number of colony-forming units (CFU) per gram of soil:

$$\mathbf{CFU} = (n \times d \times v)$$

Where n is the number of colonies counted on the culture plate with the lowest dilution factor, d, that was countable, and v is the total volume of the sample cultured. To compare the concentration of *B. anthracis* spores in soils at large (elephant) versus medium-sized (zebra) carcasses over time, a linear regression test was used. The CFU count data were log-transformed, as $\log_{10}(\text{CFU} + 1)$.

Given how little is known about animal behaviour at large animal carcass sites, basic summary data are presented from the video camera traps to demonstrate which species visit the carcass sites and the types of behaviours observed for each species. The behaviours were classified into seven different categories (Table 2), and further grouped into two groups (i.e. anthrax risk, if it increased the risk of exposure to anthrax, or non-risk behaviours). Anthrax risk behaviours included (foraging, bone contact with mouth (chewing bones, licking bones and picking up of bones), smell/sniffing, touching / kicking and resting at the carcass site. Non-risky behaviours included investigating and walking/running by carcass site. This study focused on anthrax risk behaviours. The number of individuals of each species detected at carcass sites for every trigger was recorded and totaled across all cameras. Behaviours were recorded as occurrences (present or absent) for the species recorded during a visitation. These occurrences were then totalled across all visitations and cameras, to get the frequency of occurrence for each behaviour by species. From the frequencies of occurrences of behavioural activities, the proportion of all recorded risky behaviours was calculated for each species, to compare the relative frequency of behaviours between species. This gives an indication of which routes of exposure may be most common for a particular species.

3.6 Research ethics

Ethical clearance was obtained from the University Research Ethics Committee. The research permission was obtained from the Centre of Post-Graduate Studies (CPGS) and the research permit from National Commission on Research Science and Technology (NCRST) (permit authorization 2017070704). Health and safety precautions, following WHO (2008) and UNAM Health and Safety guidelines, were adhered to during data collection and sample analysis.

CHAPTER 4: RESULTS

4.1 Scale of soil disturbance at large animal carcass sites

The denuded soil area declined significantly with site age for all sampling seasons at elephant carcass sites (linear regression; early season: R^2 = 0.312, F (1,13) = 5.902, t = -2.429, p = 0.030; mid-season: R^2 = 0.339, F (1,13) = 6.663, t = -2.581, p = 0.023; late season: R^2 = 0.340, F (1,13) = 6.506, t = -3.551, p = 0.024; Figure 14).

The study reveals that carcass sites of animals that died recently have a significantly larger area of disturbed soil (mean of 31.55 m^2), as compared to old (1.25 m^2) and very old carcass sites (0.22 m^2) (Figure 14). The area of denuded vegetation was largest in the first year after death, and then decreased significantly once another growing season occurred. This declining trend across years was congruent across the three sampling seasons, although higher denuded areas were recorded in the dry season than the wet season samples.

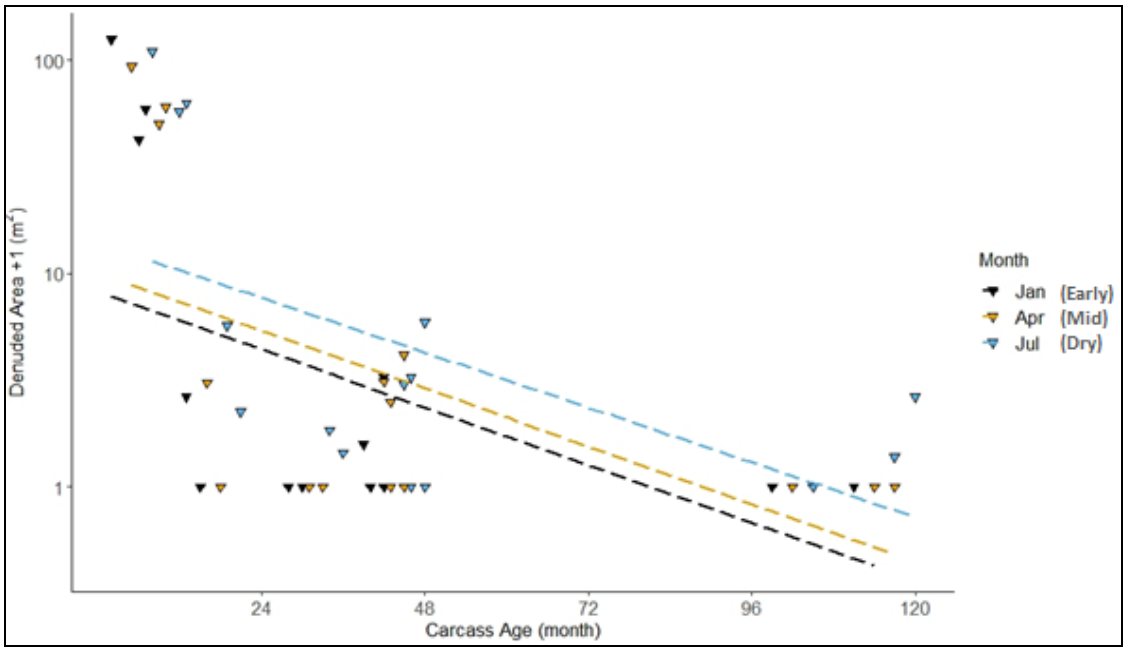


Figure 14. The relationship between denuded soil area and age of carcass sites at African elephant carcass sites in Etosha National Park: Black= early growing season, yellow= mid, and blue = dry season. (Sample size, N= 15 carcass sites).

4.2 Vegetation cover at large animal carcass sites

Twenty-one plant species were recorded at the 13 elephant carcass sites in Etosha National Park (Table 4). Vegetation species identification followed Le Roux *et al.* (1988). Vegetation was divided into the groups: herbs, grasses, shrubs and trees. The most common grass species observed at carcass sites were *Enneapagon desvauxii, Tragus racemosus, Urochloa brachyura* and *Enneapogon cenchroides,* which may attract herbivores for grazing. The most common shrubs species observed were *Leucosphaera bainesii, Catophractes alexandri, Monechma genistifolium* and *Petalidium englerianum*. Furthermore, four tree species were recorded at these sites, and never all 4 at a single carcass site (Table 4). Opportunistic herbs such as *Tribulus terrestris, Zypophyllum fabago* and *Cleome gynandra* were also observed at the carcass sites (Table 4). In addition, there were four unidentified flowering herbs observed around the gut cement at many carcass sites. After sampling three times at these sites across seasons, the study revealed that on the second or third visit to the sites, some grass species and one of the shrubs (*Leucosphaera bainesii*) were eaten by herbivores to the ground, while herbs were mostly left untouched. In this study, two carcasses were far from water sources, limiting their access by herbivores, and this change in vegetation cover was less evident at these sites.

Table 4**.** Plant species observed at the 13 elephant carcass sites in ENP from January 2020 to July 2020. The percentage is the number of times each species was seen in total out of all the carcasses sampled.

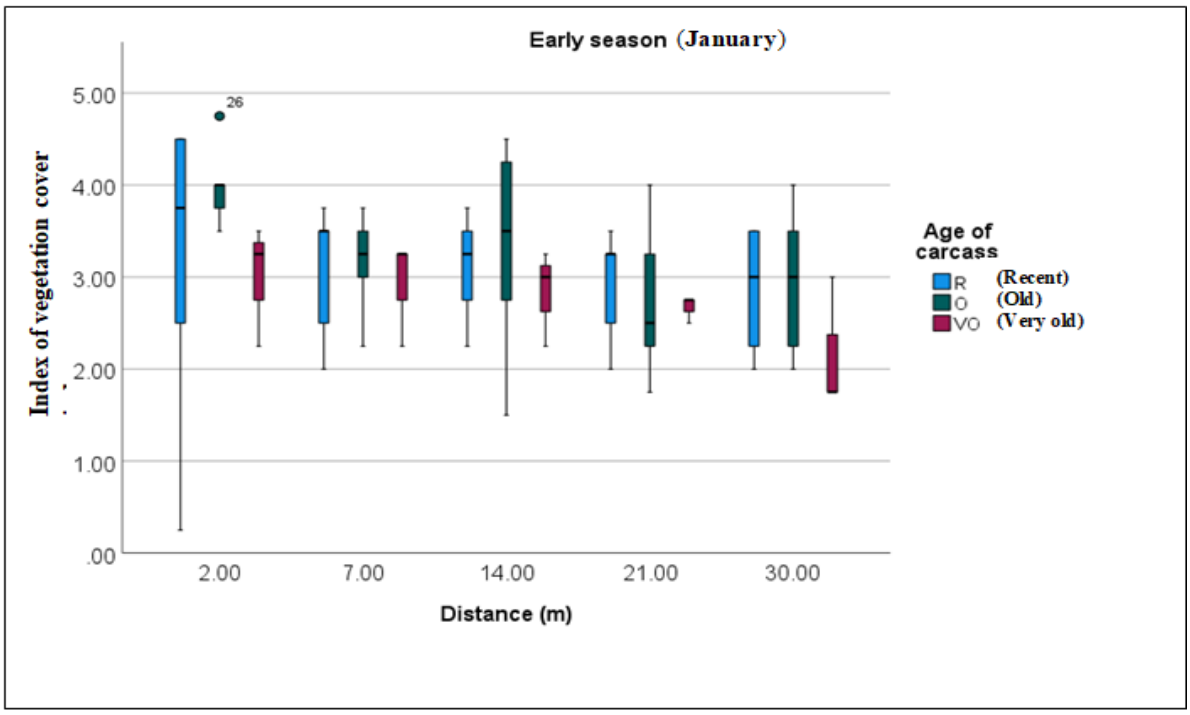
HERBS	**%**	**GRASSES**	**%**	**SHRUBS**	**%**	**TREES**	**%**
Cleome gynandra	*7.7*	*Enneapagon desvauxii*	*7.7*	*Petalidium englerianum*	*50*	*Vachellia tortilis*	*7.7*
Sesbania pachycorpa	*7.7*	*Aristida adscensionis*	*30.8*	*Catophractes alexandri*	*30*	*Vachellia nebrownii*	*38.5*
Acrotom inflate	*7.7*	*Enneapogon cenchroides*	*61.5*	*Dichrostachys cinerea*	*20*	*Colophospermum Mopane*	*46.2*
Tribulus terrestris	*46.2*	*Chloris virgata*	*38.5*	*Leucosphaera bainesii*	*80*	*Ziziphus mucronata*	*7.7*
Amarathus thunbergii	*23.1*	*Cenchrus ciliaris*	*23.07*	*Monechma genistifolium*	*70*		
Zypophyllum fabago	*46.2*	*Eragrostis nindensis*	*38.5*	*Mundulea sericea*	*30*		
Momordica humilis	*7.7*	*Eragrostis porosa*	*15.4*	*Pechuel- Loeschea leubnitziae*	*20*		
Psedogaltonia clavata	*23.1*	*Setaria verticillata*	*38.5*	*Brachiaria deflexa*	*20*		
Citrullus lanatus	*23.1*	*Stipagrostis uniplunis*	*30.8*	*Cythula lanceolate*	*20*		
		Tragus racemosus	*84.6*	*Salsola etoshensis*	*15*		
		Urochloa brachyura,	*38.5*				
		Sporobolus spicatus	*23.1*				

4.2.1 Vegetation cover at elephant carcass sites

Vegetation cover declined significantly with distance from the centre of the carcass site (multiple regression, distance from carcass centre: t = -2.033, p = 0.0434; N = 13; Figure 15). The age of carcass sites affected vegetation cover such that, cover was significantly higher for old carcasses than recent carcasses (t = 3.430, p = 0.0007; Figure 15). However, there was no significant difference in vegetation cover between recent and very old carcass sites (t = -1.284, p = 0.2009). Vegetation cover

was also significantly higher (out of a scale of 5) during the mid - growing season (April) (3.9 m^2) compared to the early growing season (3.0 m^2) and the dry season (3.1 m^2; mid versus early: $t = 5.486$, $p < 0.0001$; dry versus early: $t = 0.655$, $p = 0.5113$; Figure 15). During the dry season, signs of foraging on vegetation around the carcass sites were observed. However, vegetation cover at two carcasses that were only 700m apart did not change much throughout the three seasons and these two sites were far from water than other sampled sites.

A)



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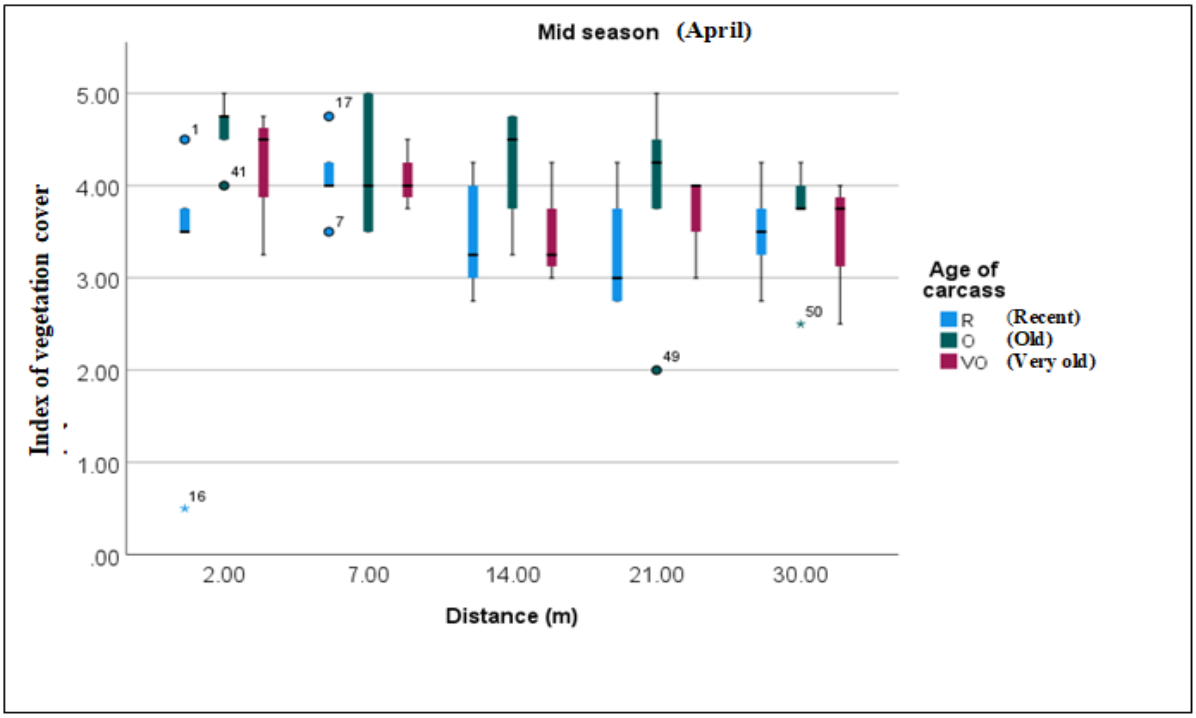

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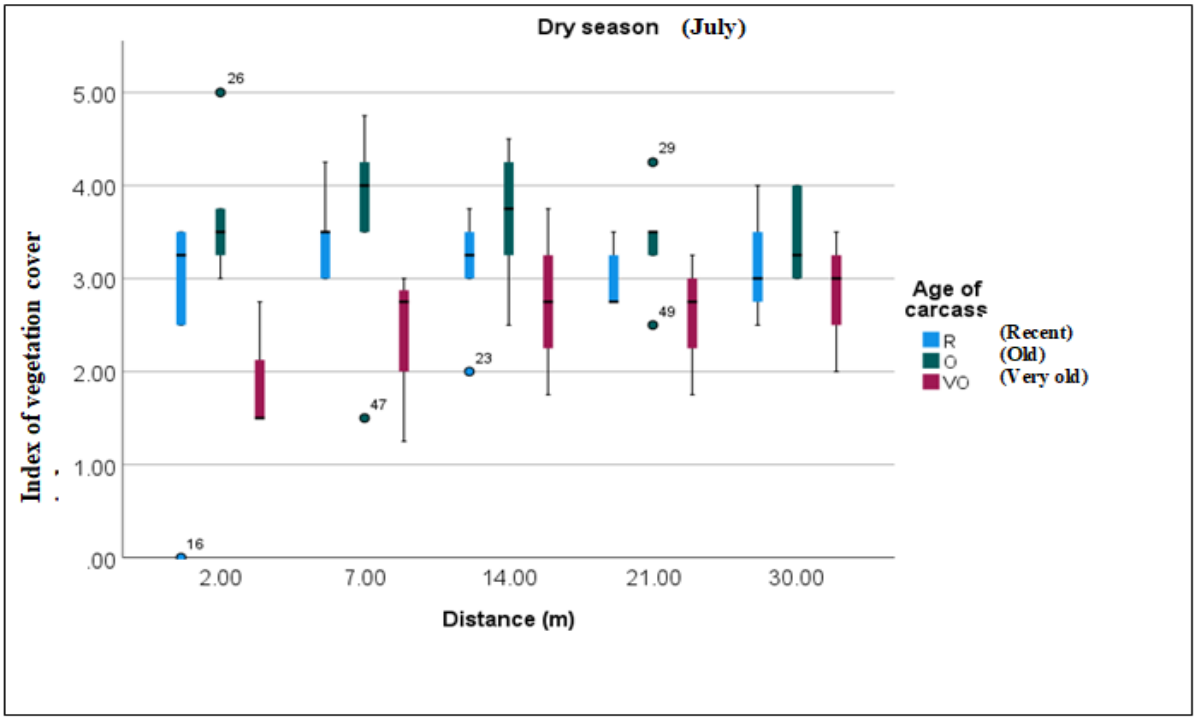


Figure 15. Vegetation cover (m^2) at elephant carcass site by sites age and distance from the carcass centre for the three sampling seasons (early, mid and dry season) in ENP: (Sample size, *N*= 13 carcass sites).

4.3 Concentration of *B. anthracis* spores present in the soil at carcass sites

Although *Bacillus anthracis* spore concentrations, measured as colony forming units per gram (CFU/g), showed a decreasing trend with site age, these trends were not statistically significant for either zebra carcass sites (linear regression: R^2 = 0.018, *F (1, 13)* = 0.239, *t* = -0.488, *p* = 0.633; *N*=28; Figure 16), or elephant carcass sites (linear regression: R^2 = 0.019, *F (1, 11)* = 0.211, *t* = -0.459, *p* = 0.655; Figure 16).

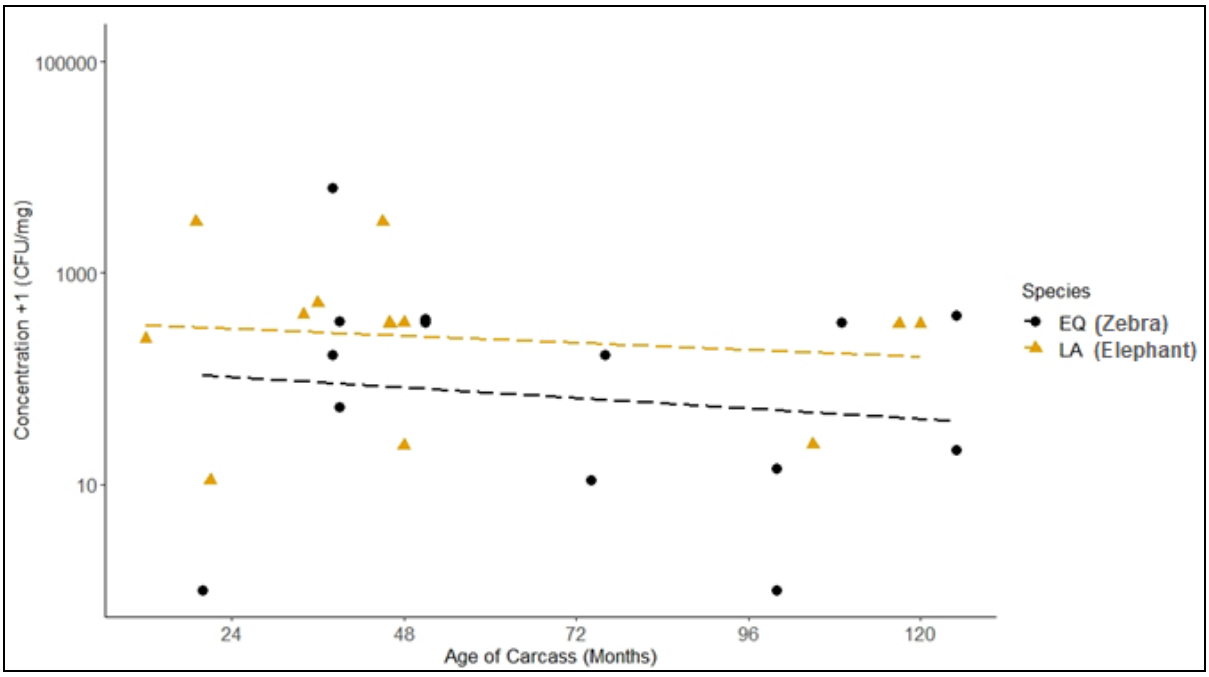


Figure 16. The relationship between the concentrations of *Bacillus anthracis* spores (measured as colony forming units per gram (CFU/g) in the soil at zebra and elephant carcass sites and the age of carcass sites in ENP. (Sample size, *N* = 28 carcass sites).

4.4. Behavioural activities displayed by species at elephant carcass sites

Behavioural observations using video cameras at adult elephant carcass sites covered a total of nearly 7 years of observations, with individual sites monitored for an average of 13.8 months (range 4.5-35 months; Table 3).

The cameras recorded 31,068 videos during this study. Only 12,728 videos showed animals at the carcass sites (41%). The rest of the 18,340 videos were triggered either by wind, rain and small birds flying around (59%). In total, animals had 1,984 visitations recorded at the 10 carcass sites displaying different behavioural activities (Table 5). The study focused only on large mammals (and not on smaller mammals). Twenty-six animal species visited or passed by the 10 monitored elephant carcass sites (Table 5). Of these, 15 were herbivores, 8 carnivores, and 3 bird species. Carnivores such as hyena and jackal visited carcass sites when soft tissues were still present on the carcasses. In addition, herbivores started to visit the carcass sites after all flesh had been removed from the bones of the carcass.

Table 5. The species that visited elephant carcass sites and their frequency of occurrence.

Frequency of occurrence represents the total number of times a species visited the carcass site displaying behaviour at the 10 carcass sites.

Species that visit carcass sites					
Herbivores	**Frequency of occurrence**	**Carnivores**	**Frequency of occurrence**	**Birds**	**Frequency of occurrence**
Elephant *(Loxodonta africana)*	153	Jackal *(Canis mesomelas)*	419	Vulture (*Gyps africanus/ Torgos tracheliotos)*	29
Springbok *(Antidorcas marsupialis)*	215	Lion (*Panthera leo)*	31	South African ostrich (*Struthio camelus australis)*	28
Gemsbok (*Oryx gazella)*	205	Brown hyena *(Parahyaena brunnea)*	96	Secretary bird *(Sagittarius serpentarius)*	2
Blue wildebeest *(Connochaetes taurinus)*	6	Spotted hyena *(Crocuta crocuta)*	352		
Plains zebra *(Equus quagga burchellii)*	43	Leopard *(Panthera pardus)*	4		
Black rhino *(Diceros bicornis)*	103	Honey badger (*Mellivora capensis)*	1		
Red hartebeest *(Alcelaphus buselaphus caama)*	1	African wildcat *(Felis silvestris lybica)*	3		
Giraffe *(Giraffa Camelopardalis)*	39	Small spotted genet *(Genetta genetta)*	1		
Black-faced impala *(Aepyceros melampus petersi)*	61				
Kudu (*Tragelaphus strepsiceros)*	156				
Eland (*Taurotragus oryx)*	3				
Warthog *(Phacochoerus africanus)*	1				
Scrub hare *(Lepus saxatilis)*	19				
Steenbok *(Raphicerus campestris)*	8				
Ground squirrel *(Xerus inauris)*	1				

Animal species that visited the carcass sites displayed a range of different activities. In this study behaviours at carcass sites were recorded in seven different categories (Figure 17). The behaviours recorded most often at elephant carcass sites included investigating and passing by the site (more than 600 sightings), both of which were considered low risk for anthrax exposure. Of the behaviours classified as risky for anthrax exposure, the most commonly recorded ones were foraging, sniffing/ smelling and bone contact with mouth (more than 200 sightings). Kicking/touching bones and resting at carcass sites were recorded relatively rarely (100 sightings or fewer).

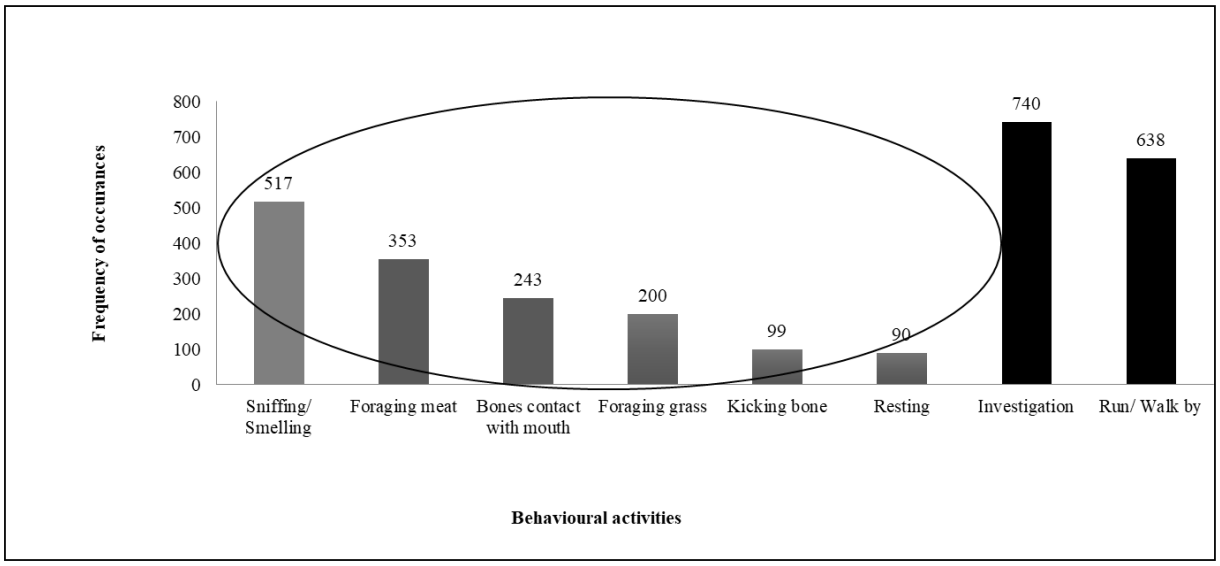
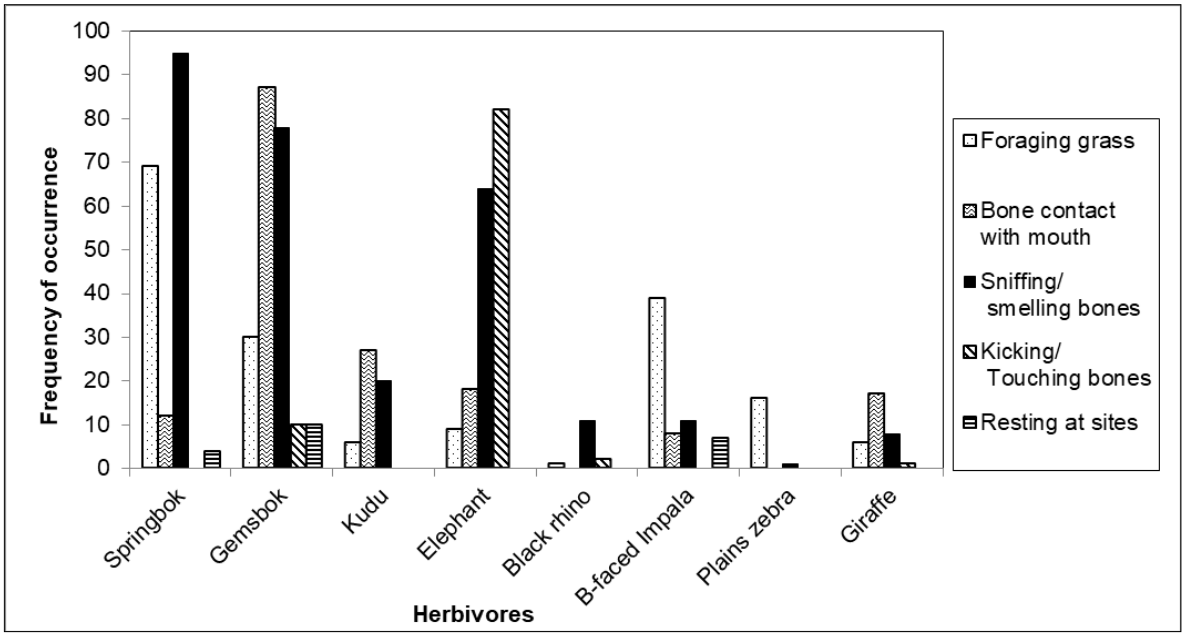


Figure 17. The frequency of occurrences of all behavioural activities displayed by all species combined at elephant carcass sites during their visit summed over all visits for the 10 carcass sites. Behaviours are presented in descending order of frequency within anthrax high/low risk categories.

Species varied in the most common behaviours observed at elephant carcass sites. Behaviours such as running/walking by and investigating were displayed by all species and with higher frequency of occurrences than others behaviours**.** Foraging of grass behaviour was recorded in zebra, gemsbok, springbok and foraging of meat was observed in jackal, hyena and vulture, while some of the behaviours were specific to only certain animal species. For instance, chewing of bones (osteophagia) behaviour (in the bone contact with mouth category) was recorded only for giraffe, gemsbok and kudu. In addition, risky behaviours were recorded in springbok, elephant, zebra and black rhino and these are some of the known anthrax host species in ENP. (Figure 18, Appendix 3, Table 6).

A) Herbivore species



B) Carnivore and scavenger species

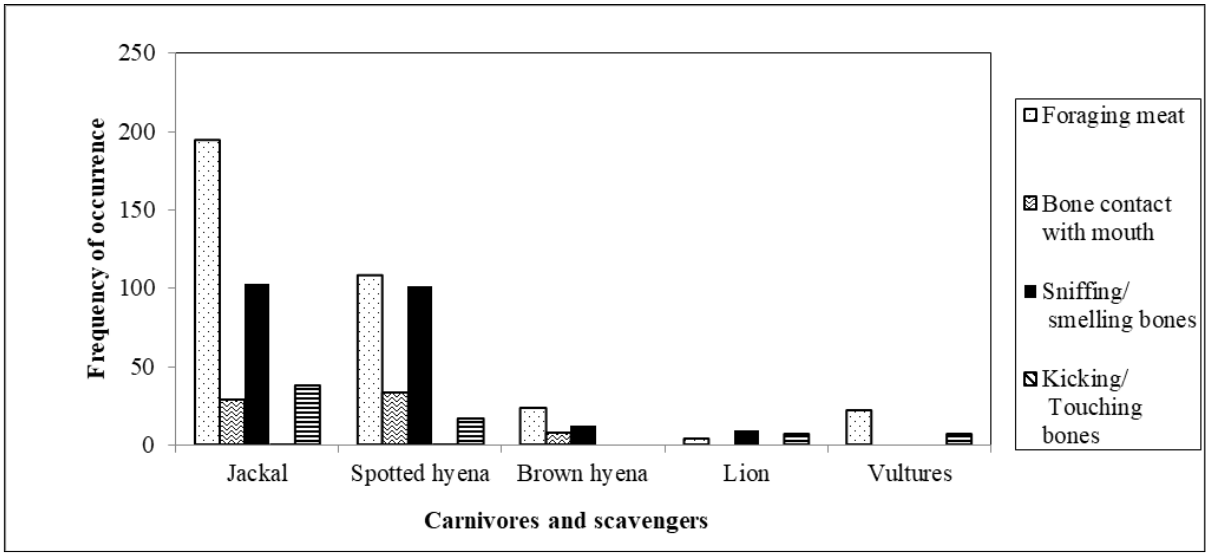


Figure 18. The frequency of occurrences of behavioural activities, considered risky for anthrax exposure, displayed by animal species at elephant carcass sites. A) Frequency of occurrences of behaviours displayed by herbivores and B) frequency of occurrences of behaviour displayed by carnivore and scavenger species.

In a natural environment, herbivores visiting anthrax carcass sites display different behavioural activities, some of which may expose them to the pathogen and others not, and these behaviours vary among species. A behaviour that involved contact with the carcass remains (fresh or bones), or the potential for inhaling of spores were considered high risk. In the present study, behaviours which were classified as high risk for anthrax transmission, either through ingestion or inhalation, included the following: foraging, bone contact with mouth, sniffing and smelling, kicking and touching and resting. While, running/walking by and investigation of carcass sites were classified as low risk behaviours. Results of the present study (Figure 19, Appendix 4, and Table 7) reveal that, among the herbivores, black-faced impala, zebra, and springbok had a high risk of potential exposure through foraging. In addition, gemsbok, kudu, giraffe had a higher risk of exposure through bone contact with mouth than other species. Elephant had the highest risk of exposure through kicking and touching of bones. Gemsbok and black-faced impala were also observed with a risk of exposure through resting at carcass site even though the risk for resting is expected to be lower than for the other risky behaviours (Figure 19, Appendix 4, and Table 7). Furthermore, almost all herbivores species face a risk of anthrax exposure through smelling of bones (Figure 18, Appendix 4, and Table 7).

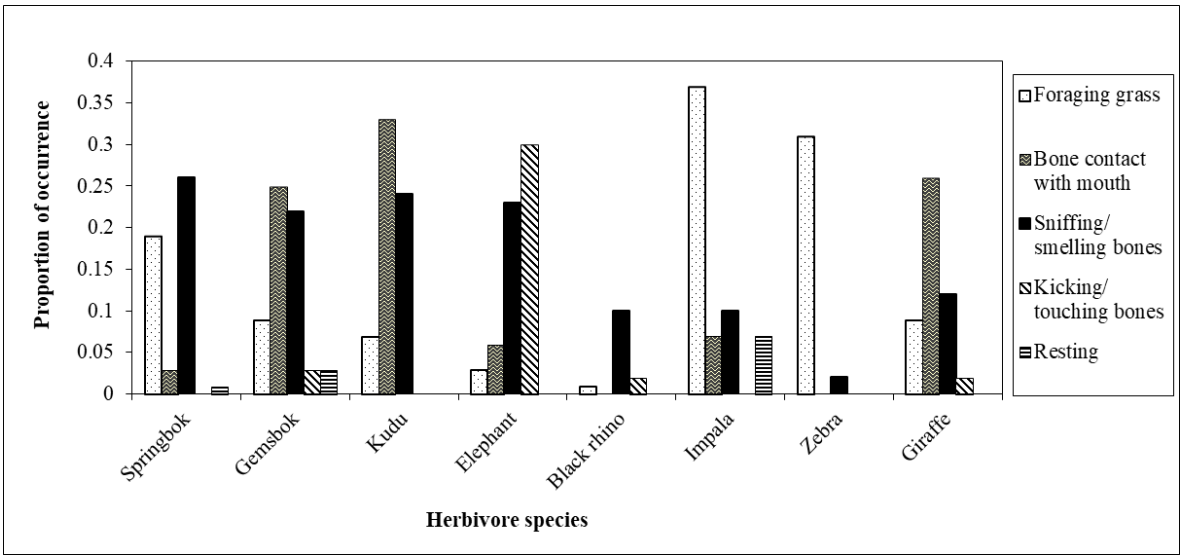


Figure 19. Proportion of occurrences of behaviours (considered risky for anthrax transmission) displayed by herbivores at elephant carcass sites (From Appendix 4, Table 7).

In this study springbok, elephant, gemsbok, kudu, impala, jackals, hyena and vulture visited carcass sites with a large number of individuals detected (Figure 20). Wildebeest and leopard were the least commonly detected at elephant carcass sites, with the fewest number of individuals.

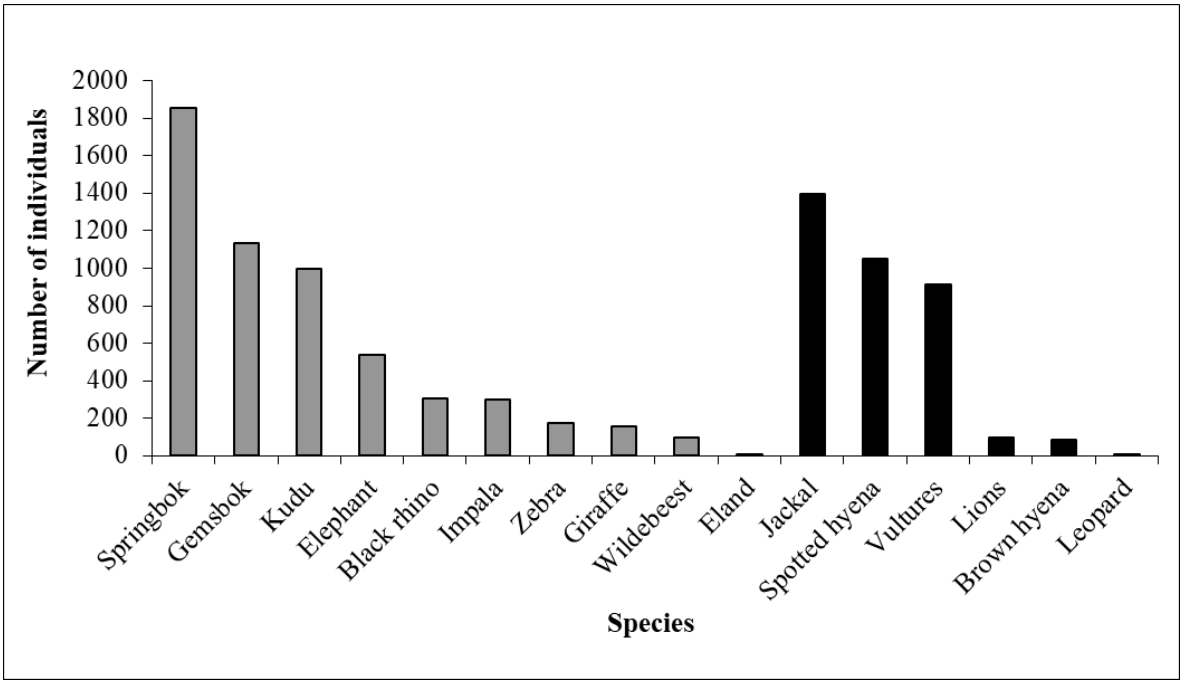


Figure 20. Total number of individuals of all species that visited the African elephant carcass sites in Etosha National Park: (Sample size, *N*= 9,118). Species are grouped by herbivore or carnivore, and presented in descending order of frequency at elephant carcass sites.

CHAPTER 5. DISCUSSION

5.1 Scale of soil disturbance at elephant carcass sites

The present study revealed that the area of disturbed soil around an elephant carcass site decreased significantly with the age of carcasses. The oldest carcass at the beginning of sampling was 114 months old and the most recent was 1 month old. The area of bare soil was largest within the first year of death, and decreased considerably in subsequent years (Figure 14). After the first year, the size of the denuded areas decreased to an average of only 1.25m^2 for the older carcass age category, and then to less than 1m^2 for the very old carcass sites. Thus, the age of elephant (large-sized animal) carcass sites can be a significant predictor affecting the area of disturbed soil around the carcass site. In a previous study at medium-sized carcass sites (plains zebra) the average area of soil disturbance shortly after death was 32.56 m^2 (W. C. Turner unpublished data, 2014). This seems similar to results of this study for recent elephant carcass sites at which an area of the soil of about 31.55 m^2 was disturbed around the sites. Nevertheless, this may be due to the fact of a smaller sampling size of elephant carcass (13) sites compared to the 54 sites for zebra carcass sites.

The significant decline in the area of disturbed soil at the carcass site with age can be due to different contributing factors. When an animal dies and the carcass is scavenged, the gut contents and blood are released into the soil and churned up by the activity of scavenging animals (Turner *et al.*, 2014). As a result, the vegetation around the carcass is trampled and the soil at a carcass site can become hardened and resist even plant growth (Barandongo *et al.,* 2018*).* At medium-sized animal carcass sites (zebra) gut cement was observed at all of the sites, a pattern that persisted for at least four years after the death of the animal (Barandongo *et al.,* 2018). In this study, and for large-sized carcass sites, the “gut cement” was observed both at recent and some of the old carcass sites and not observed at very old carcass sites (5-10 years old). Hence this pattern of gut cement at carcass sites can persist at least for 4 years after death. One may hypothesize that rainfall is likely to significantly decrease the size of the

bare soil patch at carcass sites, allowing grasses and herbs to colonize the open space. During every growing season, there is both vegetation regrowth at carcass sites and consequent utilisation by herbivore species (Turner *et al*., 2014). These disturbed patches at carcass sites are not completely recolonised, however, perhaps due to hardened soil resisting colonisation or continued disturbance by animals visiting these sites. Animals that live and travel in groups (Wittemyer *et al*., 2007), when visiting these carcass sites contribute to the further disturbance of the soil more so than lone visiting individuals. In this study springboks, impalas, elephants, vultures, zebras, kudus, jackals and spotted hyenas were observed to visit carcass sites with a large number of individuals detected, either passing by the carcass or foraging on vegetation/carcass remains or performing other activities when visiting the carcass site (Figure 20). During their visits, these animals were also observed to be in groups of different sizes and sometimes alone. Elephants were also observed to repeatedly visit the carcass sites in groups. This, however, does not apply to bulls, which tended to travel by themselves.

5.2 Vegetation covers at elephant carcass sites of different age class

The results showed that vegetation cover was higher at old (2-5 years old) carcass sites than recent or very old carcass sites (Figure 15). The increase in vegetation cover from recent to old sites is likely due to recent sites having larger areas of bare soil due to the trampling effect of scavengers and herbivores, and the older sites showing a fertilizing effect of carcass nutrients on vegetation (Turner *et al*., 2014). A possible fertilizing effect of the carcass site is evident in the decrease in vegetation cover with increasing distance from the carcass site centre (Figure 15). In this study it was observed that at a distance of 30m from the carcass centre, vegetation cover was lower at the further distances than the close ones (near the carcass remains) and 30m is likely beyond the area affected by the carcass nutrients. Thus, the age of carcass sites and distance from carcass centre may be amongst significant predictors to affect vegetation cover at elephant carcass sites. Interestingly, this peak in vegetation cover, at 3-5 year old sites, was delayed in comparison to the peak in biomass recorded at zebra

carcass sites. At zebra carcass sites the highest biomass was recorded one year after death (Turner *et al*., 2014).

Such observed changes in vegetation cover can be due to different contributing factors. Firstly, the type of soil at the carcass site and whether it can support vegetation growth. A study carried out by Afzal *et al.*(2011) showed a reduction in seed germination and biomass in sandy soil as compared to loamy soil. Loamy soil provides better habitat for plant growth than sandy soil (Afzal *et al*., 2011). This can be due to differences in organic carbon content and high cation exchange capacity, indicating high nutrient levels that potentially boost plant growth (Weissenhorn, 1996; Easo *et al.,* 1999; Carrenho *et al.*, 2007). In contrast, sandy soils are usually more porous, warmer, drier and less fertile, thus limiting plant growth (Carrenho *et al*., 2007). Although this study did not assess soil type at elephant carcass sites, it is clear that different soil type have different impact on vegetation growth.

Secondary, changes in vegetation cover may be influenced by the type of vegetation colonizing the carcass sites, and whether it is palatable enough to attract herbivores for grazing. Herbivores utilize a wide variety of plant species, but only a few species are most important in terms of biomass consumed. Herbivores selectively forage on vegetation species throughout the year but increase their consumption of certain species over others during the wet season when they are more readily a with seasonal changes in preferred forage (Havarua *et al.,* 2014). In the present study, *Enneapagon desvauxii, Leucosphaera bainesii, Catophractes alexandri, Tragus racemosus, Eragrostis nindensis, Urochloa brachyura, and Enneapogon cenchroides* (Table 4) were considered dominant vegetation at these sites all combined. These species were observed at most of the sampling sites in comparison to other species. A previous study indicated that *Enneapagon desvauxii* and *Eragrostis nindensis* were some of the grass species preferred by zebra (Havarua, 2011), the main anthrax host species in ENP. The main component of zebra diet will vary across ecosystems based on the species composition of grasses and their palatability. A previous study in Kruger National Park by Grobler (1983) and Nowak (1999) also report that diet of zebra consisted of these major grass species *(Enneapagon*

desvauxii and *Eragrostis nindensis*). In contrast, a study done in Kiboko Range Research Station, Kenya found out that *Digitari milanjiana* was the most preferable grass species for zebra (Ngethe, 1976). This was out of all the four other grass species studied (*Eragrostis caespitosa*, *Themeda triandra*, *Cynodon dactylon* and *Cymbopogan pospischilii*) (Ngethe, 1976). While in another study in Kenya by Casebeer and Koss, (1970) found *Themeda triandra* to be the most abundant diet component.

Furthermore, carcass patches create "micro disturbances" in the ecosystem providing new plant growth opportunities (Bump *et al*., 2009). New plant growth includes exotic and weedy plant species promoted through seed dispersals, or enrichment of naturally infertile soils (Barton *et al*., 2016). The presence of these weedy species might preclude opportunities for competitively suppressed native species to establish on carcass sites (Barton *et al*., 2016). For example, a study done in North America reported that vegetation at bison and cattle carcass sites in tall-grass prairies remained different from the surrounding grassland after five years due to settlement by annuals and a reduction in perennial grasses (Towne, 2000). These studies indicated the potential of carcass sites to introduce longer-term heterogeneity into ecosystems beyond the initial decomposition phase (Barton *et al*., 2016).

During the sampling year, ENP received good rainfall and vegetation growth was plentiful which led to new growth and regrowth of many plant species. In the present study, herbs and annual grasses were recorded around the carcass gut cement, while perennial grasses, trees and shrubs were recorded away outward from the carcass site. It was noted that exotic annuals (for example *Zypophyllum fabago, Amarathus thunbergii* and two flowering herbs that were not identified) were most prevalent close to the carcass site while perennial grasses (such as *Enneapagon desvauxii and Enneapogon cenchroides*), shrubs (*Catophractes alexandri* and *Leucosphaera bainesii*) and trees (*Vachellia nebrownii* and *Colophospermum mopane*) where more prevalent further away from the centre of the carcass. Such colonization pattern by annuals at the carcass sites introduced heterogeneity at these sites.

The distance of the carcass sites to the nearest water source may be another important factor affecting vegetation cover. When water is a limiting resource, animals are less likely to utilize areas far from a water source despite an abundance of vegetation in the area. In this study, two carcass sites (located west of the Adamax area) had little change in vegetation cover, by season or distance from the carcass site. These two sites were the farthest from water sources, about 13km, and showed little evidence of foraging when visited for sampling. In a study by Turner *et al.* (2014), the frequency of visitations and grazing events at zebra carcass sites were related to seasonality and distance to a water source but no predictive models of visitation rates could be built using distance to water as a meaningful predictor. This suggests that more visitation and grazing events are likely to occur at zebra carcass sites closer to water sources, and possibly during the growing season than other seasons. Further research is needed to investigate the relationship between carcass sites, distance to water, and selection by herbivores.

Since animals forage more intensively at nutrient hot spots which can include carcass sites (Ben-Shahar and Coe, 1992), higher vegetation consumption would be likely to occur at carcass sites than other vegetation patches. For instance, ENP herbivores forage at contaminated carcass sites and may contract anthrax in the process (Turner *et al.*, 2014). If zebra were to forage so intensively at a carcass site, they are likely to have a higher exposure to *B. anthracis* than if they foraged less intensively (Havarua *et al.*, 2014). Foraging studies of zebras indicate they are likely to have the highest exposure to *B. anthracis* during wet seasons, which matches the infection patterns observed (Havarua *et al.*, 2014). This may then apply to other plain ungulates that come to forage at other contaminated sites, such as elephant carcass sites, and may further explain the seasonality of anthrax outbreaks in Etosha. Although this study did not assess the selection of grasses at elephant carcass sites versus other grassland sites, foraging was observed by many herbivore species at the carcass sites.

5.3 Concentration of *B. anthracis* spores at carcass sites

Results of the present study showed that the concentration of *B. anthracis* spores in carcass site soils for both elephant and zebra carcass sites did not change significantly over time. There was little to no change in the concentration of *B. anthracis* spores at carcass sites of both species. Thus, the size and age of a carcass site alone were not significant predictors of the concentration of *B. anthracis* spores. These results are contrary to what has been reported in other similar studies in Etosha National Park.

Cloete (2013) revealed that all soil types in ENP could support the persistence of *B. anthracis* in the environment, of which soils of central ENP had the highest *B. anthracis* spore counts and an indication that soil was not a limiting factor for the concentration of *B. anthracis* in this study. In addition, Cloete (2013) stated that soil collected from fresh elephant carcass sites was reported to test positive for *B. anthracis* more often than soil from old elephant carcass sites. A study carried out by Turner, *et al.* (2014) for zebra carcasses, stated that the concentration of *B. anthracis* on the aboveground component of grasses decreased significantly from recent to older carcass sites, which dropped to near zero on 3-year-old sites. Furthermore, Lindeque (1991), Coker (2002), Turner *et al.* (2016) found out that the numbers of *B. anthracis* spores declined overtime at carcass sites (more at fresh and less at old carcass sites). This indicated that anthrax spores are found more at fresh carcass sites than older carcass sites and it is likely that this can also apply to the carcasses of all other host species. Therefore, a decline in the concentration of spores along the age spectrum at elephant and zebra carcass sites was expected in the present study. However, the result of the present study showed no statistically significant decline in concentrations of anthrax spores for both species.

Various factors can affect the fate of *B. anthracis* in soil over time. *Bacillus anthracis* spores are formed upon the release of vegetative cells into the environment through terminal haemorrhaging (Lindeque, 1991). Thus, the presence of *B. anthracis* can be detected at the site where the terminal bleeding occurred (Lindeque, 1991). Spores can be moved around by the dispersal of spores at the original site due to wind and water (Lindeque *et al*., 1998). Even though the *B. anthracis* spores are rarely found in soils outside of anthrax carcass sites (Lindeque and Turnbull, 1994), there is still a

possibility to be found at an uncontaminated area as the *B. anthracis* could be moved away from carcass sites to other locations where animals could be exposed through grazing, browsing or drinking. This can be by local movements such as wind (Turnbull *et al.,* 1989) water runoff or faecal deposition, or movement by spore adherence to animal fur or feathers on scavengers (Dragon *et al.,* 2005). These factors would further reduce the concentration at the original sites of contamination over time.

The lack of a significant negative trend in spore concentrations at these carcass sites can be attributed to many factors. Firstly, relatively few young sites were sampled, and these would be expected to have the highest concentrations. Thus this sample may have under-represented higher-concentration young sites. Secondly, the sample size was relatively small across the age spectrum of sites. Hence a larger sample size, across the age spectrum and including younger carcass sites, would help clarify the decline over time and if differences exist between the two species. Resampling these sites over time would also help clarify patterns with site age, since individual variability in spore concentrations among sites can be large (Turner *et al.,* 2016). In this study, the sampled sites were not previously marked, thus the choice of sample sites was guided by features such as the presence of elephant bones or gut cement. However, the gut cement was not found intact in very old and some old carcasses even if the exact location where the animal died was provided, which may introduce more variability.

5.4 Behavioural activities displayed by species at elephant carcass sites

Carcass sites serve as potential locations where *B. anthracis* can be transmitted to new hosts. Hence the distribution of spores in the environment and the interaction between host behaviour and carcass sites becomes the crucial link in transmission (Turner *et al.,* 2016). Animal species perform different behavioural activities at carcass sites, some of which pose a risk of anthrax transmission, especially at an infectious site. It has been reported that many herbivores in ENP for example, plains zebra, blue wildebeest, and springbok are likely to be exposed to anthrax through ingestion of *B. anthracis* spores at zebra anthrax carcass sites (Turner *et al.,* 2014). However, African elephants were rarely detected at these zebra anthrax carcass sites (Turner *et al*., 2014). These findings lead to many questions one can

ask. For example, how does the different size of carcasses from different host species contribute to anthrax transmission? How do animals interact with carcass sites of their own, versus other species? These questions suggest that species differ in the way in which they acquire anthrax and that the specific behaviours of host species may contribute to anthrax transmission in different ways.

Carcass sites are likely to serve as a primary source of infection, hence the probability of anthrax exposure at contaminated carcass sites depends on how often potential hosts visit infectious carcass sites, and how, or if, they interact with potentially infectious material at these sites. When herbivores forage at carcass sites, the chance of infection depends upon their foraging intensity at that site (Havarua *et al.,* 2014). Hence, an animal foraging intensively at a site is more likely to contact *B. anthracis* at a carcass site than one with low foraging intensity. Linking foraging behaviours to carcass sites assumes that herbivores are not deterred from foraging at or visiting these sites despite the visual cues from the carcass. Besides foraging, there are other behaviours that animals may engage in at carcass sites that could also expose them to pathogens. These however have received less research attention. These behaviours include but are not limited to chewing bones, smelling, touching of bones or the carcass, and resting at carcass sites, which host species may also display at contaminated carcass sites.

There are species-specific differences in exposure risk, and exposure pathway, at different carcass sites, and these differ based on the species of carcass. In the present study, springbok, impala and zebra displayed a higher proportion of foraging behaviours at carcass sites and a lower proportion of other risky behaviours (Figure 19). These 3 species are known as anthrax host species (WHO, 2008), although few impala cases are recorded in ENP. According to Turner *et al.* (2014), herbivore species such as zebra, springbok and wildebeest commonly graze at zebra anthrax carcass sites up to 1.5 years after the death of a host, providing evidence that these species are most likely to contact the pathogen by foraging at anthrax carcass sites. Wildebeests regularly visit zebra carcass sites, and forage (Turner *et al.,* 2014), but were rarely detected at elephant carcass sites. Zebra and springbok are potentially exposed at carcasses of both species, but wildebeest only at zebra carcass sites. However, zebra

visitations to elephant carcass sites were relatively infrequent—these sites were visited more often by black rhinos than zebras. Gemsbok and elephant have little risk of exposure at zebra carcass sites (Turner *et al.,* 2014) but performed risky behaviours at elephant carcass sites such as osteophagia (gemsbok) or contacting bones (elephant; Figure 19).

Havarua (2011) stated that high numbers of anthrax spores are only likely to be encountered at sites of anthrax mortalities. This may put grazers at higher risk when they graze at sites where previous anthrax-positive victims died. Many anthrax cases have been reported in zebra (grazers) and springbok (mixed feeders) in ENP (Berry, 1993), and these species foraged at both carcass types. Hence, their foraging behaviour at these sites may put them at more risk of exposure than browsers, or other grazers who avoid foraging at some carcass sites. According to Havarua (2011) and Clegg *et al.* (2007), the chance of contact with the pathogen in the soil is likely to be higher when host species graze close to the ground on short grasses. Even though black-faced impala displayed a high proportion of foraging behaviour at elephant carcass sites, few cases of anthrax-infected have been reported in ENP. There is not much historical information about their exposure to anthrax because their population size has been small. Black-faced impala current population in ENP grew from an initial population of 180 animals captured in the Kunene region and were released in western Etosha between 1968 and 1971 (Green and Rothstein, 1998). In addition, the black-faced impala population is a fraction of springbok in ENP, approximately 1,500 are found in Etosha National Park (Green and Rothstein, 1998), versus 15,000-30,000 springbok, depending on estimates. Low reported anthrax cases of black-faced impala may also be due to difficulty in detecting carcasses from a small-bodied animal that prefers denser woodland habitats (Matson et al., 2005). Furthermore, they do not often use the open areas that zebra prefer, so they are unlikely to encounter many zebra carcass sites. Therefore, habitat preferences of different potential host species will affect which species' carcass sites they encounter and their level risk based on how they use carcass sites within that habitat. Open grasslands in ENP have higher anthrax risk than more closed habitats, and grazing herbivores generally have higher anthrax risk than browsing species in ENP (Huang *et al.,* 2021). Although this disease primarily

affects grazing hosts, elephants are suspected of transporting the disease to different habitats across the park(Lindeque, 1991). Elephants may thus serve as a bridge species Caron *et al.* (2015), moving the disease into different habitats, and then exposing a wider range of host species to this disease.

Most animals, in comparison to human, appear to show less interest in the remains of dead conspecifics (McComb, Baker and Moss, 2005) although animals like dolphins (Dudzinski *et al*., 2003) and elephants (Douglas-Hamilton *et al*., 2006) were described as concerned with dead members of their species. In this study, elephant, springbok, gemsbok, kudu, black rhino, impala and giraffe were commonly observed investigating and sniffing the carcass remains. Elephants recorded a high proportion of occurrences of the behaviour smelling and touching of the carcass remains of their species throughout the decomposition process. There could be many contributing factors to such behaviours. Firstly, Douglas-Hamilton *et al.* (2006) suggest that this could be due to evolutionary thinking, providing a selective advantage, especially if it increases the fitness of the surviving kin. Secondly, this may be viewed as an elephant, perhaps displaying "mourning behaviour" suggested by Douglas-Hamilton *et al.* (2006). Elephants spend significantly more time exploring the remains of dead elephants than other large herbivore species (Payne, 2003; Douglas-Hamilton *et al.*, 2006). Douglas-Hamilton *et al.*, (2006) have observed the death of a matriarch and the responses by her own family and other four unrelated families to the carcass at Samburu National Reserve, in Kenya. In addition, the elephant in Addo Elephant National Park, South Africa also expressed interest in elephant bones, by turning and touching them (Merte, Gough and Schulte, 2009). This may be attributable to the fact that, just like humans, elephants tend to have close relationships with their relatives, and hence are interested in the sick, dying, or dead individuals irrespective of genetic relationship (Douglas-Hamilton *et al*., 2006), although helping of unrelated families amongst elephant has also been reported (Douglas-Hamilton, 1972). Similar guarding of unrelated individuals has also been observed in Samburu during radio-collaring operations (Douglas-Hamilton *et al*., 2006). These observations suggest that elephants have a generalized response to the suffering and death of conspecifics and that this is not restricted to kin.

This study also revealed that some behaviours displayed at carcass sites were only specific to certain species. For example, the behaviour of bone contact with the mouth involved chewing and licking of bones. Gemsbok, kudu, giraffe and elephant proportionally displayed more bone contact than foraging behaviour at elephant carcass sites (Figure 19). Hence, they spent a larger proportion of encounters on this behaviour than other species, suggesting that if these species were to acquire anthrax at carcass sites it could be through this behaviour instead of foraging. Some studies have reported ungulate species chewing bones, antlers, and horns (generally termed osteophagia). Examples of such species include the eastern Sahara camel (*Camelus dromedarius*: (Johnson and Haynes, 1985), and several African herbivores, most notably giraffe (*Giraffa camelopardalis*: (Kok and Opperman, 1980; Langman, 1978), gemsbok (*Oryx gazella*) and kudu (*Tragelaphus strepsiceros*) (Sutcliffe, 1973). It has been suggested that the consumption of bones, antlers, and horns by these non-carnivorous mammals is likely motivated by the need to obtain essential minerals absent from a largely vegetarian diet (Bowyer, 198; Wika, 1982). A second possible factor is the maintenance of proper phosphorus/calcium ratios in the body (Barrette, 1985) largely for antler growth and maintenance of velvet (Barrette, 1985). Thirdly, the consumption of bone may reduce the incidence of osteoporosis (Wika, 1982; Barrette, 1985). This however is not typically done unless necessary, as bone chewing may injure teeth and the mouth or cause botulism, hence is hazardous to ungulate health (Wika, 1982; Barrette, 1985).

Animals such as impala, springbok and gemsbok, displayed resting behaviour at elephant carcass sites (Figure 19). Resting with the head near the soil surface at a carcass site could pose a possible risk of inhaling anthrax spores, leading to inhalational anthrax infection. The anthrax spores may be moved by wind and possibly, on occasion, carried very long distances before deposition (Turnbull *et al*., 1998). However, the concentrations of spores moved in this way are likely to be very low, and are unlikely to cause a lethal dose as there is a clear pattern of declining numbers of *B. anthracis* spores with distance from the centres of the sites (Turnbull *et al.*, 1998). Hence, exposure through resting does not apply to animals resting some distance from the carcass site.

Scavenging carnivores forage on dead animals, including those that die of anthrax. Thus, during an anthrax outbreak, lions, vultures, jackals, and spotted hyena scavenge on anthrax-infected carcasses. Moreover, these carnivores have acquired an immunity to anthrax (Lindeque, 1991; Bellan *et al*., 2012), facilitating their survival and increase in numbers. The timescale over which animals develop and lose immunity to *B. anthracis* remains unknown. Lindeque and Turnbull, (1994) reported that anthrax spores were found in jackal (72 %), hyena (60 %), and in vulture (50 %) faeces collected around anthrax carcass sites. In the present study, carnivores such as lions, jackals, hyenas, and vulture were observed at the elephant carcass sites displaying behaviours such as eating, sniffing, and investigating a carcass site. Among carnivores, jackals recorded the highest number of visitations to the carcasses, with the highest frequency of occurrence in foraging (N = 194). This is supported by a previous study in which jackals were more frequently observed at anthrax carcass sites than other mammalian scavengers (Bellan *et al*., 2012).

CHAPTER 6: CONCLUSION AND RECOMMENDATION

6.1 Conclusion

This study had 4 objectives namely: to determine the scale of soil disturbance caused by large-sized animal carcasses over time, to determine the effect of large-sized animal carcasses on vegetation cover around the carcass site over time, to determine and compare the concentrations of *Bacillus anthracis* spores present in the soil at large-sized and medium-sized animal anthrax carcass sites over time and to describe behaviours displayed by animals at large-sized animal carcass sites and relate these to potential pathogen exposure.

The results of the present study showed that recent elephant carcasses may exert a considerable impact on the environment, leading to a significantly higher disturbance of the soil around the carcass site which declined with the age of carcass sites. This is likely due to the trampling of vegetation around the carcass and the soil at a carcass site becomes hardened and resists even plant growth. Regarding the effect of large-sized animal carcasses on vegetation cover around the carcass site over time, the results showed a significant decline in vegetation cover with distance from the centre of the carcass site across site ages. Such is attributed to recent sites having larger areas of bare soil (trampling of vegetation and soil) than older sides.

Older elephant carcass sites (3-5 years old) showed higher vegetation cover than recent or very old sites. This implies that these sites may have a delay in attractiveness to herbivores as compared to zebra carcass sites, but from this study we do not yet have data with which to test this. The concentrations of *Bacillus anthracis* spores in the soil at large-sized and medium-sized animal anthrax carcass sites did not differ significantly over time suggesting that these sites can remain infectious for many years. There was no strong support that the size of the carcass affected the potential infectivity of the site. Large-bodied carcass sites (of elephants) had a somewhat higher concentration of *B. anthracis* spores than medium-sized animal's carcass sites, but this was not a significant pattern and more data are necessary before making conclusions about species differences in site infectivity. Based

on this study, elephant carcass sites of all ages and sizes may have the potential to become anthrax host spots for herbivores in Etosha National Park, and these sites may have a delayed attraction to herbivores, as compared to zebra carcass sites.

In this study, animals displayed different behaviours at carcass sites, ranging from merely investigating the site, walking by the carcass site, sniffing or smelling the bones, licking or contacting the bones with their mouth and resting by the carcass site. Some of the behaviours displayed by both herbivores and carnivores that visited the carcass sites may expose the animals to the risk of contracting anthrax. For example, many anthrax host herbivore species foraged around elephant carcass sites; a behaviour that would pose a risk of exposure to anthrax. Some of the risk behaviours were unique to specific herbivores species (chewing bones for example in kudus, gemsbok and giraffe or resting for example in springbok, gemsbok and impala) while sniffing and smelling of the carcass remains was displayed by all species that visited carcass sites except for vultures. Therefore, this suggests that there is a possibility that different species differ in the way they acquire anthrax in the environment, and not just through foraging.

All in all, the results of the present study provide important insights into variation in transmission risk for this multi-host pathogen that can inform epidemic control efforts in managed livestock (by farmers) and wildlife populations beyond ENP. In addition, the results also provide park management with insights into which areas and host species to give more focus on for disease control. The study lends support that anthrax outbreaks in herbivores are a result of complex interactions among host physiological status (that influenced their feeding behaviour), age of carcass sites, host behaviour at contaminated sites, level of spore concentration at contaminated sites, and environmental conditions. Thus, anthrax transmission in nature is challenging to predict as it is not caused by one factor but a contribution of many inter-related factors. The present study together with past research conducted in ENP provides information on possible ways in which anthrax susceptible host species may or may not contract the pathogen, and through which routes of infection. Hence, in future, these could add more information to the formulation of guidelines in surveillance and control of this enzootic disease.

6.2 Recommendation

The following recommendations are suggested based on the findings of the present study:

(a) A study on how vegetation changes with distance from the centre of the carcass site across elephant carcass sites (e.g., annual to perennial, or exotics/natives vegetation species), should be carried out. *Bacillus anthracis* spores are known to be highly concentrated at carcass site centres. Knowledge of the types of species colonizing the disturbed area at carcass sites will enable us to understand interactions between vegetation responses, the palatability of colonizing species, and how these are selected, or avoided, by potential host species encountering these sites.

(b) In addition, in the search to understand the transmission of anthrax to animals through oestephagia behaviour, it will be useful to conduct a new study to evaluate the risk associated with bone chewing, by testing the concentration of *B. anthracis* spores present on bones, and compare how these change with bones age.

(c) The mortality surveillance conducted at ENP has been enormously useful in understanding the ecology and epidemiology of this disease. This surveillance effort should continue, to support management and research efforts of this and other mortality sources, and how they change over time and space. Initiating or supporting these efforts elsewhere can help with disease management and species conservation.

(d) A study on a variety of species' carcasses and how they affect the environment, *B. anthracis* spore concentrations, and animal interactions with these sites would be valuable for further understanding this multi-host disease.

(e) A long term study on how vegetation changes with distance from the centre of the carcass site across elephant carcass sites (e.g., annual to perennial), should be carried out. This will enable us to understand interactions between vegetation responses, vegetation palatability and the preferences by potential host species visiting these sites.

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LIST OF APPENDIX

Appendix 1. Scale of soil disturbance at large animal carcass sites

1. Disturbed soil area for the early season in January (Linear regression)

Model Summaryb					
Model	R	R Square	Adjusted R	Std. Error	Durbin-Wa
1	.559a	0.312	0.259	0.63917	2.078
a Predictors: (Constant), Age of carcass					
b Dependent Variable: area of disturbed soil					

ANOVAa						
Model		Sum of Sq	df	Mean Squa	F	Sig.
1	Regressior	2.411	1	2.411	5.902	.030b
	Residual	5.311	13	0.409		
	Total	7.722	14			
a Dependent Variable: area of disturbed soil						
b Predictors: (Constant), Age of carcass						

Coefficientsa								
Model		Unstandardized Coefficients		Standardized Coefficients	T	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	0,908	0,253		3,585	0,003	0,361	1,456
	Age of carcass	-0,011	0,005	-0,559	-2,429	0,03	-0,021	-0,001
a Dependent Variable: area of disturbed soil								

2. Disturbed soil area for the Mid-season in April (Linear regression)

Model Summaryb					
Model	R	R Square	Adjusted R	Std. Error	Durbin-Wa
1	.582a	0.339	0.288	0.6079	2.288
a Predictors: (Constant), Age of carcass					
b Dependent Variable: area of disturbed soil					

ANOVAa						
Model		Sum of Sq	df	Mean Squa	F	Sig.
1	Regressior	2.462	1	2.462	6.663	.023b
	Residual	4.804	13	0.37		
	Total	7.266	14			
a Dependent Variable: area of disturbed soil						

Coefficientsa								
Model		Unstandardized Coefficients		Standardized Coefficients	T	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	1,003	0,251		3,996	0,002	0,461	1,545
	Age of carcass	-0,011	0,004	-0,582	-2,581	0,023	-0,021	-0,002
a Dependent Variable: area of disturbed soil								

3. Disturbed soil area for the Dry season in July (Linear regression)

Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Durbin-Watson
1	.578[a]	0.334	0.282	0.58217	2.136

a. Predictors: (Constant), Age of carcass

b. Dependent Variable: area of disturbed soil

ANOVAa						
Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	2,205	1	2,205	6,506	.024b
	Residual	4,406	13	0,339		
	Total	6,611	14			
a Dependent Variable: area of disturbed soil						
b Predictors: (Constant), Age of carcass						

Coefficientsa								
Model		Unstandardized Coefficients		Standardized Coefficients	T	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	1,141	0,25		4,559	0,001	0,6	1,682
	Age of carcass	-0,011	0,004	-0,578	-2,551	0,024	-0,02	-0,002
a Dependent Variable: area of disturbed soil								

Appendix 2. Concentration of *B. anthracis* spores present in the soil at carcass sites

1. Concentration of *B. anthracis* spores at zebra carcass sites by age (Linear regression)

Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the	Durbin-Watson
1	.134[a]	0.018	-0.058	1.082	2.021

a. Predictors: (Constant), Age of carcass site (months)
b. Dependent Variable: concentration (CFU/mg)

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	0.279	1	0.279	0.239	.633[b]
	Residual	15.223	13	1.171		
	Total	15.503	14			

a. Dependent Variable: concentration (CFU/mg)
b. Predictors: (Constant), Age of carcass site (months)

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	2.110	0.655		3.221	0.007	0.695	3.525
	Age of carcass site (months)	-0.004	0.008	-0.134	-0.488	0.633	-0.022	0.014

a. Dependent Variable: concentration (CFU/mg)

2. Concentration of *B. anthracis* spores at elephant carcass sites by age (Linear regression)

Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the	Durbin-Watson
1	.137[a]	0.019	-0.070	0.763	1.832

a. Predictors: (Constant), Age of carcass site (months)
b. Dependent Variable: concentration (CFU/mg)

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	0.123	1	0.123	0.211	.655[b]
	Residual	6.398	11	0.582		
	Total	6.521	12			

a. Dependent Variable: concentration (CFU/mg)
b. Predictors: (Constant), Age of carcass site (months)

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	2.537	0.386		6.568	0.000	1.687	3.387
	Age of carcass site (months)	-0.003	0.006	-0.137	-0.459	0.655	-0.016	0.011

a. Dependent Variable: concentration (CFU/mg)

Appendix 3. Frequency of occurrences of behavioural activities

Table 6**.** The frequency of occurrences of behavioural activities displayed by different species at elephant carcass sites**.** Each number recorded represents the frequency of occurrence by species exhibiting a particular behaviour within a single visitation, summed over all visitations. Forging/eating for herbivores was eating vegetation at the carcass site, while for carnivores was consuming the carcass. "Other species" are the birds (crows and koribustard) and small mammals that were rarely detected (i.e. fewer than 20 detections in videos), such as hare, spotted genet, warthog and steenbok.

Species	Foraging / Eating	Bone contact with mouth	Sniffing/ smelling bones	Kicking/ Touching bones	Run/ Walking by sites	Resting at sites	Investigating the site	**Total frequency of occurrence**
Ungulate herbivores								
Springbok	69	12	95	0	128	4	59	367
Gemsbok	30	87	78	10	70	10	63	348
Kudu	6	27	20	0	8	0	22	83
Elephant	9	18	64	82	45	0	63	281
Black rhino	1	0	11	2	61	0	35	110
Impala	39	8	11	0	19	7	22	106
Zebra	16	0	1	0	32	0	3	52
Giraffe	6	17	8	1	18	0	16	66
Carnivores								
Jackal	194	29	103	1	64	38	259	688
Spotted hyena	108	34	101	2	115	17	139	516
Brown hyena	24	8	12	0	22	0	13	79
Lions	4	0	9	0	8	7	13	41
Vultures	22	0	0	0	0	7	17	46
Other species								
Others	25	3	4	1	48	0	16	97

Appendix 4: Proportion (%) of risky behaviours displayed by host species at elephant carcass sites

Table 7. Proportion (%) of visitations to elephant carcass sites by a species in which certain potentially risky behaviours were recorded. Proportion was calculated out of every total frequency of occurrence per species (from the last column of Table 5).

Species	Foraging/ eating	Bones contact with mouth	Sniffing/ smelling bones	Kicking/ touching bones	Run/ walking	Resting	Investigating	Total Proportion
Ungulate herbivores								
Springbok	0.19	0.03	0.26	0	0.35	0.01	0.16	1
Gemsbok	0.09	0.25	0.22	0.03	0.2	0.03	0.18	1
Kudu	0.07	0.33	0.24	0	0.09	0	0.27	1
Elephant	0.03	0.06	0.23	0.3	0.16	0	0.22	1
Black rhino	0.01	0	0.1	0.02	0.55	0	0.32	1
Impala	0.37	0.07	0.1	0	0.18	0.07	0.21	1
Zebra	0.31	0	0.02	0	0.61	0	0.06	1
Giraffe	0.09	0.26	0.12	0.02	0.27	0	0.24	1
Carnivores								
Jackal	0.28	0.04	0.15	0	0.09	0.06	0.38	1
Spotted hyena	0.21	0.07	0.2	0	0.22	0.03	0.27	1
Brown hyena	0.3	0.1	0.15	0	0.28	0	0.17	1
Lions	0.1	0	0.21	0	0.2	0.17	0.32	1
Vultures	0.48	0	0	0	0	0.15	0.37	1
Rare sighting								
Others	0.26	0.03	0.04	0.01	0.5	0	0.16	1