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Blowflies as vectors of *Bacillus anthracis* in the Kruger National Park

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Anthrax, caused by *Bacillus anthracis*, is endemic in the Kruger National Park (KNP). The epidemiology of *B. anthracis* is dependent on various factors including vectors.

The aims of this study were to examine non-biting blowflies for the presence of *B. anthracis* externally and internally after feeding on an anthrax-infected carcass and to determine the role of flies in disseminating *B. anthracis* onto the surrounding vegetation.

During an anthrax outbreak in 2014 in the endemic Pafuri region, blowflies associated with two 2–3-day-old anthrax-positive carcasses (kudu and impala) as well as surrounding vegetation were collected and investigated for the presence of *B. anthracis* spores.

The non-biting blowflies (n = 57) caught included *Chrysomya albiceps*, *Ch. marginalis* and *Lucilia* spp. *Bacillus anthracis* spores were isolated from 65.5% and 25.0% of blowflies collected from the kudu and impala carcasses, respectively.

Chrysomya albiceps and *Ch. marginalis* have the potential to disseminate *B. anthracis* to vegetation from infected carcasses and may play a role in the epidemiology of anthrax in the KNP. No *B. anthracis* spores were initially isolated from leaves of the surrounding vegetation using selective media. However, 170 and 500 spores were subsequently isolated from *Abutilon angulatum* and *Acacia* sp. leaves, respectively, when using sheep blood agar.

Conservation implications: The results obtained in this study have no direct conservation implications and only assist in the understanding of the spread of the disease.

Introduction

Anthrax is a serious zoonotic disease affecting mainly herbivores. It is caused by the soil-borne, Gram-positive, spore-forming organism *Bacillus anthracis*. In unfavourable conditions, *B. anthracis* forms endospores that can remain dormant in the environment for long periods of time surviving outside the host. The spores are ingested by a susceptible host, where germination and multiplication occur *in vivo*. The host dies and the carcass is opened by scavengers or human, resulting in the vegetative cells being exposed to oxygen and sporulating; remaining in the soil until another host ingests the spores and the cycle is repeated (Dragon & Rennie 1995). Anthrax occurs endemically in the northern Kruger National Park (KNP) and the Northern Cape Province (Ghaap plateau area) in South Africa (Hugh-Jones & De Vos 2002). The disease cannot spread from one living animal to the next; therefore, the life cycle is dependent on various factors including rainfall, temperature, type of soil, animal densities and the presence of susceptible hosts (Dragon & Rennie 1995).

A number of abiotic and biotic vectors such as insects (biting flies, blowflies, mosquitoes and ticks) (Blackburn et al. 2010; Fasanella et al. 2010; Graham-Smith 1913; Turell & Knudson 1987), scavengers (Pienaar 1961, 1967), soil (Hugh-Jones & Blackburn 2009) and water (Hugh-Jones & De Vos 2002; Pienaar 1961) have also been implicated in the dissemination of anthrax. The main insect vectors implicated with anthrax in the KNP are the blowflies *Chrysomya albiceps* and *Ch. marginalis* (Braack 1985) as these species are the first (and most abundant) insects to arrive shortly after death. These two species consume infected bodily fluids and are probably the principal vectors in the dissemination of *B. anthracis* (Braack 1985; Braack & Retief 1986). *Chrysomya albiceps* and *Ch. marginalis* have been hypothesised to spread the bacteria over short distances despite the fact that blowflies have the ability to travel great distances (35 km – 65 km).

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They normally rest on nearby vegetation when engorged and deposit most of the contaminated defaecation and/or vomit droplets close to the carcass (Braack & De Vos 1990; De Vos 1990) of vegetation (reviewed by De Vos 1994), which pose a risk of infection to susceptible herbivores.

In KNP, outbreaks were usually associated with the driest periods of the year (winter and early spring) or during climatic dry cycles (Pienaar 1967). However, since the 2010 outbreak onwards, outbreaks mainly occurred in wet periods (summer months; E.H. Dekker [Skukuza State Veterinary Office] pers. comm., 2014). This is of importance as insect activity and abundance on a carcass is influenced by climate. In the rainy season, the insect abundance (especially the blowflies) will increase, which was speculated by Braack (1985) to increase *B. anthracis* dissemination by blowflies to vegetation during an outbreak. Scavengers have also been speculated to disseminate spores at drinking holes after opening and feeding on anthrax-infected carcasses (Pienaar 1961). Research conducted by Turner et al. (2014, 2016) indicated that grazing seems to play a significant role in the dissemination of *B. anthracis* in Etosha National Park. Similar investigations must be performed in KNP to determine the factors influencing the epidemiology of anthrax as well as the amount of spores found on flies and the bacterial load defaecated or regurgitated by flies on vegetation.

In this study, we investigated the possible role of blowflies in carrying and/or spreading *B. anthracis* during an anthrax outbreak in the KNP. This aim was investigated by quantifying the presence of *B. anthracis* on the exterior and interior of blowflies after feeding on an anthrax-contaminated carcass. Furthermore, the role of blowflies in the dissemination of *B. anthracis* onto the surrounding vegetation during the anthrax outbreak in the KNP was also investigated.

Materials and methods

Blowfly collection and identification

In March 2014 – April 2014, blowflies were collected from two confirmed anthrax-positive carcasses (a kudu and an impala) during an anthrax outbreak in the endemic Pafuri region of the KNP. The adult male kudu carcass was approximately 2–3 days old, lying in the open with clear indications that scavengers (vultures and/or hyenas) had started to feed on it. The adult male impala carcass infected with anthrax was about three days old, lying under a low thorn tree (*Acacia* sp.). The carcass was mostly intact, except for a large opening in the abdomen region. A net trap was used to collect the blowflies on the carcass. Trapped flies were collected and placed in liquid nitrogen for 30 seconds and transported at 4°C to the National Institute for Communicable Diseases (NICD) for further bacteriological analysis. All blowflies were kept, while other flies were discarded. Each blowfly was identified and sexed using the identification method described by Zumpt (1965). Blowflies that were not phenotypically or morphologically distinguishable were identified by sequencing of the ribosomal DNA internally transcribed spacer 2 (ITS 2) region that differentiates *Calliphoridae* as described by Koekemoer et al. (2002).

Isolation of *Bacillus anthracis* from blowflies

Each blowfly was tested for the presence of *B. anthracis* vegetative cells and spores on the interior and exterior surfaces. To detect the presence of *B. anthracis* on exterior surfaces, each blowfly was washed in 1 mL sterile saline to remove any bacteria. The presence of *B. anthracis* vegetative cells was determined by plating 10-fold dilutions (10^0–10^8) from the external wash onto polymyxin–EDTA–thallium acetate (PET; modified polymyxin-lysozyme-EDTA-thallium acetate [PLET] where lysozyme was omitted) agar plates followed by incubation at 37 °C for 48 hours. The presence of *B. anthracis* spores was determined by plating 100 μL heat-treated (62.5 °C for 15 min) undiluted external wash on PET agar, followed by incubation at 37 °C for 48 h.

After the exterior wash step, each blowfly was disinfected with 1 mL 0.1% (v/v) peracetic acid for 1 h to inactivate any residual bacteria left over on the exterior of the blowfly after the wash step. The 0.1% peracetic acid (v/v) was neutralised by 1 mL 0.8% sodium bicarbonate – sodium chloride (v/v) solution for 1 h. One-hundred microliters of the neutralised external wash was inoculated onto 5% sheep blood agar (SBA) and incubated at 37 °C for 24 h to ensure that no bacteria were present on exterior surface of the blowflies.

The presence of internal (ingested) *B. anthracis* was determined by homogenising each blowfly in 1 mL saline. The homogenised blowfly was briefly centrifuged (500×*g* for 5 s) to separate the blowfly debris from the supernatant. Tenfold serial dilutions (10^0–10^8) of the supernatant were prepared and plated on PET agar and incubated at 37 °C for 48 h. The presence of *B. anthracis* spores was determined by plating 100 μL heat-treated undiluted supernatant onto PET followed by incubation at 37 °C for 48 h.

Isolation of *Bacillus anthracis* from vegetation

Leaves from shrubs with blowfly defaecation or discard droplets around the infected carcasses were collected and transported at 4 °C to the NICD. Each defaecation or discard droplet was removed from the leaves by using a transport swab pre-wetted with saline, followed by the swabs being rinsed in 1 mL sterile water. *Bacillus anthracis* was detected by plating 100 μL undiluted wash before and after heat treatment on PET and SBA plates followed by incubation at 37 °C for 48 h. Leaves without any defaecation or discard droplets were sent to Prof. K. Eloff at the University of Pretoria for identification and to determine antibacterial activity. The minimum inhibitory concentration (MIC in mg/mL) of acetone extracts of the plant was tested against five bacterial organisms (*Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *B. anthracis* Sterne) using the protocol as previously described by Eloff (1998).

Confirmation of *Bacillus anthracis*

Bacillus anthracis isolates were identified based on the characteristic colony morphology (ground glass appearance, fairly flat, more tacky and white or grey-white), no haemolytic activity, penicillin sensitivity and gamma phage sensitivity after 24 h incubation at 37 °C on 5% SBA as described by World Health Organization (2008). Each confirmed *B. anthracis* colony of vegetative cell and spores was counted and recorded, followed by the calculation of total colony forming units per fly (CFU/fly).

Results

The two carcasses were confirmed to be anthrax-positive through the presence of bacilli and endospores in a Giemsa-stained blood smear as well as through the isolation of *B. anthracis* through culture. Twenty-nine blowflies were collected from the kudu carcass and 28 blowflies from the impala carcass (Table 1). *Chrysomya marginalis*, *Ch. albiceps* and *Lucilia* spp. females were collected from the kudu carcass (with a female:male ratio of 3:1), whereas only *Ch. marginalis* males and females were collected from the impala carcass (female:male ratio of 2:5) (Table 1).

Only *B. anthracis* spores were isolated from the blowflies. A total of 26 (46%; 26/57) blowflies were positive for the presence of *B. anthracis* spores, with 66% (19/29) of blowflies collected from the kudu carcass and 25% (7/28) of the blowflies collected from the impala carcass (Table 2). The *Ch. marginalis* blowflies collected from the kudu carcass had an average internal and external spore count of 128 CFU/fly. The *Ch. albiceps* blowfly collected from the kudu carcass was only positive on the interior with 20 CFU/fly. A low *B. anthracis* spore count of 10 CFU/fly was isolated from the exterior of the two *Lucilia* spp. (Appendix 1). The *Ch. marginalis* blowflies collected from the male impala carcass had an average internal and external spore count of 26 CFU/fly (Appendix 2).

TABLE 1: Summary of blowfly species collected from two anthrax carcasses during an anthrax outbreak in March 2014 in the Kruger National Park.

Fly species	Sex	Kudu carcass		Impala carcass	
		%	Total number positive out of total collected	%	Total number positive out of total collected
Chrysomya albiceps	Female	10.4	3/29	-	-
	Male	-	-	-	-
Chrysomya marginalis	Female	48.3	14/29	28.6	8/28
	Male	24.1	7/29	71.4	20/28
Lucilia spp.	Female	17.2	5/29	-	-
	Male	-	-	-	-

The shrub where blowflies defaecated or regurgitated after feeding on infected carcasses was identified as *Abutilon angulatum* approximately 2.5 m from the kudu carcass and leaves were collected with blowfly defaecation or regurgitation spots. An average of ten defaecation or regurgitation spots was found per *A. angulatum* leaf. No *B. anthracis* was isolated from the *A. angulatum* shrub leaves using selective medium combined with or without heat treatment, but 170 spores were isolated from one defaecation or regurgitation spot cultured on 5% SBA with no heat treatment. From a single thorny stem of an *Acacia* tree, 500 spores per droplet for three out of eight defaecation or regurgitation spots were isolated on 5% SBA without heat treatment. Because of the absence of spores on non-selective medium, the antimicrobial activity of the *A. angulatum* shrub was tested and the results indicated that the MIC of the *A. angulatum* acetone extract was most effective against *E. coli* (0.08 mg/mL) and had a moderate inhibitory effect towards *E. faecalis*, *S. aureus* and *B. anthracis* (Sterne) with 0.31 mg/mL.

Discussion

Chrysomya albiceps, *Ch. marginalis* and *Lucilia* spp. were collected from two *B. anthracis* carcasses and their abundance confirms that these carcasses were 2–3 days old as indicated by the investigation of carcass-attending insect communities in the KNP of Braack (1985). *Chrysomya marginalis* usually arrives within a couple of minutes to hours after death of animals and stays at a carcass for approximately 4 days, whereas *Ch. albiceps* arrives a couple of hours after the death and remains at a carcass for about 3 days in the KNP (Braack 1985). *Lucilia* spp. usually arrives at the same time as *Ch. marginalis*, but in far less numbers. The major blowfly species collected from anthrax-infected carcasses was *Ch. marginalis* of which 88% was *B. anthracis* culture-positive. No viable *B. anthracis* was isolated from any of the vegetation using selective media and could only be isolated using SBA. Based on the culture results using selective (PET) and enrichment (SBA) media, it seems that the selective medium has an inhibitory effect on *B. anthracis* spore germination from leaves, which was not the case with germination from the blowflies themselves. Because of initial negative results of the selective media from the *A. angulatum* leaves, we tested for antibacterial activity and found a moderate inhibitory effect on the non-virulent Sterne strain of *B. anthracis* by phytocompounds within *A. angulatum*.

TABLE 2: Blowflies positive for *Bacillus anthracis* spores collected from the confirmed anthrax kudu and impala carcasses during an anthrax outbreak in the Kruger National Park in March 2014.

Variable	Only internal positive		Only external positive		Internal and external positive		Negative	
	%	Total number positive out of total collected	%	Total number positive out of total collected	%	Total number positive out of total collected	%	Total number positive out of total collected
Kudu carcass (*n* = 29)	10.40	3/29	17.20	5/29	37.90	11/29	34.50	10/29
Impala carcass (*n* = 28)	17.90	5/28	7.10	2/28	0.00	-	75.00	21/28
Total (*n* = 57)	**14.00**	**8/57**	**12.30**	**7/57**	**19.30**	**11/57**	**54.40**	**31/57**

Only spores, and no vegetative organisms, from *B. anthracis* were isolated from the interior and exterior of blowflies, which could be attributed to various factors that could have killed the more labile vegetative organisms. These factors include the freezing of the flies in liquid nitrogen, collection time and the period between collection and processing of the flies. Graham-Smith (1913) fed *Musca domestica* and *Calliphora erythrocephala* on *B. anthracis* vegetative cells and spores and recovered only *B. anthracis* spores for up to ten days from fly legs and wings and seven days from both the gut and the crop. Vegetative cells were present on legs and wings for up to 24 h, lending further evidence to our findings. In this study, the collected flies were frozen in liquid nitrogen. Haines (1938) found that 90% *B. mesentericus* spores were viable after –70 °C freezing with varying loss of viability of the vegetative cells and Young et al. (1968) suggested that vegetative cells of spore-forming bacteria are more susceptible to damage from freeze-thawing than vegetative cells of non-spore-forming bacteria. Therefore, it is presumed that the freezing of the flies in liquid nitrogen did not influence the spore count significantly, as we only found spores. The absence of vegetative cells could be because of the use of liquid nitrogen to kill the blowflies.

Various researchers have determined that flies can potentially transmit *B. anthracis* to their surroundings and that these flies are carriers of the bacteria for numerous days (Blackburn et al. 2010, 2014; Hugh-Jones & Blackburn 2009; Von Terzi et al. 2014). According to De Vos (1990), explosions of blowfly populations preceded the anthrax outbreaks in the KNP in 1960 and 1970. Blackburn et al. (2010) speculated that blowflies and flesh flies are responsible for the spread of an anthrax outbreak in white-tailed deer in the USA, which was later confirmed by isolating *B. anthracis* DNA from leaves collected 21 days post-outbreak as well as viable *B. anthracis* from blowfly larvae and one adult that can potentially infect the surrounding vegetation (Blackburn et al. 2014). This coincides with the findings of this study where *B. anthracis* could not be isolated from leaves using selective medium. This could explain why only *B. anthracis* DNA on leaves was detected by Blackburn et al. (2014). The suppressive nature of selective media on *B. anthracis* from environmental samples needs further investigation.

Two recent studies investigated fly species as potential mechanical or biological vectors of *B. anthracis.* Fasanella et al. (2010) allowed the common housefly (*M. domestica*) to feed on *B. anthracis*–infected rabbit carcasses or *B. anthracis*–contaminated blood and indicated the presence of *B. anthracis* spores in defaecation or vomit spots as well as hypothesised that anthrax spores are able to germinate and replicate in the gut content of *M. domestica*. These authors hypothesise that the housefly has the potential to act as a biological vector for *B. anthracis*. Von Terzi et al. (2014) demonstrated that *B. anthracis* vegetative cells are unable to multiply inside the gut of *Calliphora vicina* and cannot survive for longer than a week. These authors' results show high numbers of viable *B. anthracis* indicating the role of *C. vicina* as only mechanical vectors as previously hypothesised (Blackburn et al. 2010; Hugh-Jones & Blackburn 2009; Hugh-Jones & De Vos 2002).

The *Ch. albiceps* had less spores per fly (20 CFU/fly) compared to *Ch. marginalis* (128 CFU/fly) collected at the kudu carcass. Only *Ch. marginalis* was caught at the impala carcass with 25 CFU/fly. The reason for the larger spore count present on flies at the kudu carcass could be attributed to the bacterial load of the host, or more sporulation as the kudu carcass was opened by scavengers compared to the partially opened impala carcass that might have been protected from scavengers by the Acacia shrub. The total CFUs isolated per fly reflect the potential infectious load of a fly with an average of 87 *B. anthracis* CFU/fly depending on the blowfly species. It is not known how many spores were defaecated onto the surrounding vegetation by flies, but 170 spores were isolated from a defaecated spot on the *A. angulatum* leave. Our results show that *B. anthracis* is present in and on blowflies that actively fed on an anthrax carcass and that the faecal or discard droplets left on leaves are infective. This can then lead to the assumption that blowflies can aid in the dissemination of *B. anthracis* to susceptible host species.

As anthrax carcass sites are the primary sources for future infections (Turner et al. 2014, 2016), it will depend if the spore doses from surrounding infected vegetation are lethal. Considering the 170–500 spores per leaf or twig (that weighed approximately 1 g – 2 g) found in this study, a lethal dose of around 10^5–10^7 for gastrointestinal anthrax (Turner et al. 2016) can easily be ingested during anthrax outbreaks as impala ingests 0.9 kg – 1.9 kg per day (Furstenburg 2002) and kudu 3.7 kg – 5.0 kg per day (http://www.wildliferanching.com/content/kudu-tragelaphus-strepsiceros). However, because of the small sample size (host species and blowflies), further research is necessary to investigate the spore load present in the different host species and CFU on and defaecated by the different blowfly species.

Despite the unique findings of this study, specifically in the KNP, it only involved two carcasses of different species with blowflies being collected at one specific time point. To obtain a clearer view of the infectious load of blowflies feeding on a carcass under different climatic conditions, a temporal study over a longer time period should be conducted. Ideally, blowflies should be collected daily from carcasses and the surrounding vegetation until the carcasses are completely decomposed giving insights into the extent of the role that blowflies may play in anthrax transmission. While most studies indicate that blowflies play a role as a vector of *B. anthracis*, the epidemiological significance of this role is difficult to determine because various factors need to be considered, including the survival of the spores on or in blowflies, the spore load in defaecation or vomit spots of blowflies, and hence, on the vegetation, the latter depending on climatic conditions and antimicrobial activity of the plant(s), and last but not least the effective dose required by susceptible host species.

Conclusion

In conclusion, the results obtained from this study indicate that blowflies feeding on an anthrax-contaminated carcass can contaminate surrounding vegetation and aid in the continuous cycle of *B. anthracis*. If there are high numbers of blowflies feeding on a carcass, it can lead to high bacillus or spore numbers being deposited on the surrounding vegetation. This can then lead to a new infection of a susceptible host through the ingestion of contaminated vegetation.

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Competing interests

The authors declare that they have no financial or personal relationships that may have inappropriately influenced them in writing this article.

Authors' contributions

L.B. did the experiments and wrote the manuscript. Knowledge transfer of techniques to L.B. used in this study was done by A.H., A.D. and A.G. A.H. did most of the vegetation experiments. A.G. was responsible for the polymerase chain reaction (PCR) identification of the blowflies. W.B., J.R. and H.V.H. participated in the design of the study and co-supervised the first author. W.B. has been the principal investigator of the DFG project BE2157/4-1. H.v.H. and W.B. were provided funding through AgriSETA and the Deutsche Forschungsgemeinschaft, respectively. L.B. received a National Research Foundation (NRF) Freestanding Innovation Scholarship. All authors participated in critical revision of the manuscript.

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Appendix 1

TABLE 1-A1: Presence and number of colony forming units of *Bacillus anthracis* on and/or in blowflies collected from the confirmed anthrax-positive kudu carcass (2–3 day old) during anthrax outbreak in Kruger National Park in 2014.

Species name	Sex	External or internal positive	Vegetative cells or spores detected	External counts (CFU/fly)	Internal counts (CFU/fly)
Ch. albiceps	Female	Internal	Spores	0	20
Ch. marginalis	Male	Internal	Spores	0	10
Ch. marginalis	Female	Both	Spores	60	90
Ch. marginalis	Female	External	Spores	10	0
Ch. marginalis	Female	Both	Spores	20	20
Ch. marginalis	Female	Both	Spores	200	10
Ch. marginalis	Female	External	Spores	90	0
Ch. marginalis	Female	Both	Spores	20	90
Ch. marginalis	Female	Both	Spores	550	280
Ch. marginalis	Male	Both	Spores	90	100
Ch. marginalis	Female	Both	Spores	40	20
Ch. marginalis	Female	Both	Spores	60	100
Ch. marginalis	Female	Both	Spores	50	40
Ch. marginalis	Female	Both	Spores	10	10
Ch. marginalis	Female	Internal	Spores	0	30
Ch. marginalis	Female	External	Spores	10	0
Ch. marginalis	Female	Both	Spores	20	10
Lucilia spp.	Female	External	Spores	10	0
Lucilia spp.	Female	External	Spores	10	0

CFU, colony forming units per fly.

Appendix 2

TABLE 1-A2: Presence and number of colony forming units of *Bacillus anthracis* on and/or in blowflies collected from the confirmed anthrax-positive impala carcass (2–3 days old) during anthrax outbreak in Kruger National Park in 2014.

Species name	Sex	External or internal positive	Vegetative cells or spores detected	External counts (CFU/fly)	Internal counts (CFU/fly)
Ch. marginalis	Female	Internal	Spores	0	30
Ch. marginalis	Male	Internal	Spores	0	10
Ch. marginalis	Female	External	Spores	30	0
Ch. marginalis	Male	Internal	Spores	0	20
Ch. marginalis	Male	External	Spores	20	0
Ch. marginalis	Male	Internal	Spores	0	50
Ch. marginalis	Male	Internal	Spores	0	20

CFU, colony forming units per fly.