Namibian farmland cheetahs (*Acinonyx jubatus*) demonstrate seronegativity for antibodies against *Bacillus anthracis*

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INTRODUCTION

The cheetah (*Acinonyx jubatus*) is a vulnerable species, with estimates of only 6700 animals left in the wild. Namibia, an anthrax-endemic country, is home to the world's largest and most viable free-ranging population (~3000 animals), which predominantly resides on unprotected private farmlands (Durant, 2015).

For over 170 years, anthrax has been reported in African wildlife species with sporadic outbreaks across Namibia (Beyer *et al*., 2012). Anthrax is regularly reported from zebra (*Equus quagga*), hartebeest (*Alcelaphus buselaphus*), springbok (*Antidorcas marsupialis*) and kudu (*Tragelaphus strepsiceros*) (Turner *et al*., 2014; Wafula, Patrick & Charles, 2007); all cheetah prey species. Anthrax epidemics occur annually in Namibia's Etosha National Park (ENP), whereas the establishment of a government mandated livestock vaccination programme in 1973 reduced the occurrence of anthrax on the surrounding farmlands (Bellan *et al*., 2012; Schneider, 1994; Turner *et al*., 2013). However, sporadic epidemics still occur on private farmlands throughout Namibia (Shaanika, 2013). Recently, changing land-use trends in these regions, where game ranching has become more popular, may be creating conditions more similar to those found in anthrax-endemic ENP. Game ranches tend to congregate unvaccinated ungulates, including species preyed upon by cheetahs. If exposed, anthrax could spread quickly through these unvaccinated game herds and extend to free-ranging wildlife.

Serological analysis has rarely been used to study anthrax in wild ungulate populations since the disease often causes sudden death in herbivores and it was presumed that ungulates would not survive long enough to mount an antibody response (Lembo *et al*., 2011). However, recent studies have shown that species vary in their susceptibility to anthrax and have identified seropositive herbivores (Cizauskas, Bellan, Turner, Vance & Getz, 2014; Lembo *et al*., 2011). Lembo *et al*. (2011) found that 49% of buffalo (*Syncerus caffer*) and 19% of wildebeest (*Connochaetes taurinus*) in the Tanzanian Serengeti were seropositive for anthrax. The same study found 0% seropositivity among zebras, which are highly susceptible to lethal anthrax infections. However, Cizauskas *et al*. (2014) demonstrated that zebras are able to survive sublethal exposure and develop short-lived antibody titres. While herbivores typically show low seroprevalence and high mortality in the face of anthrax outbreaks, most carnivores demonstrate high seroprevalence and low mortality (Lembo *et al*., 2011).

Cheetahs are unique among carnivores in that they appear to experience high and rapid mortality upon infection with *B. anthracis* (Lindeque, Nowell, Preisser, Brain & Turnbull, 1998). Jackals (*Canis mesomelas*) frequently scavenge from anthrax-infected carcasses, yet are typically asymptomatic (Bellan *et al*., 2012). In lions (*Panthera leo*), clinical signs of anthrax are rarely observed but, when seen, usually presents with cellulitis and oedema of the head (Hugh-Jones & de Vos, 2002). Although several anthrax-related deaths have been recorded in lions, jackals and wild dogs (*Lycaon pictus*), such mortalities are rarely observed in carnivores relative to herbivores in the same environment (Creel *et al*., 1995; Lembo *et al*., 2011; Tubbesing, 1997; Turnbull, Doganay, Lindeque, Aygen & McLaughlin, 1992). Cheetahs differ in that they become septic and die rapidly. Mortality is characterized by exudation of tarry blood from the body orifices, splenomegaly and gelatinous infiltration of the subcutaneous tissues (Tubbesing, 1997).

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In 1986, several captive cheetahs died in Namibia's Gobabis district (Fig. 1) after being fed meat from a baboon (*Papio hamadryas*) with cutaneous anthrax (Jager, Booker & Hubschle, 1990). In 2009, several captive cheetahs on game farms near Windhoek died after being fed anthrax-infected oryx (*Oryx gazella*) meat (Beyer *et al.*, 2012). At least 13 more captive cheetahs in Namibia's farmlands died after eating anthrax-infected meat and reportedly suffered acute septicaemia and 100% mortality within hours (Tubbesing, 1997). In ENP, extensive cheetah mortality from anthrax has also been documented (Lindeque *et al.*, 1998). In Botswana, where anthrax is endemic, three captive cheetahs died acutely after being fed infected meat during an outbreak in Jwaneng National Park (Good, Houser, Arntzen & Turnbull, 2008).

Because anthrax infections in cheetahs generally present as acute mortalities, the presence of antibodies to *B. anthracis* would suggest that some individuals are able to survive exposure (Hugh-Jones & de Vos, 2002). In ENP, two out of seven free-ranging cheetahs with low *B. anthracis* antibody titres have been detected (Lindeque *et al.*, 1998). Antibodies to *B. anthracis* are commonly found in other predators living in anthrax-endemic Etosha, including lions, hyaenas and jackals (Bellan *et al.*, 2012; Turnbull *et al.*, 1992).

The elevated risk of an anthrax outbreak in this traditionally quiescent livestock farmland area made it important to evaluate the natural level of resistance in this crucial cheetah population. Therefore, this study aimed to determine whether the Namibian farmland cheetah population possesses antibodies to *B. anthracis* in order to determine their susceptibility and predict the impact of a large-scale epizootic.

METHODS

This study utilized frozen wild cheetah serum collected between 1993 and 2005 throughout the Namibian farmlands by the Cheetah Conservation Fund (Fig. 1). Serum was kept frozen in aliquots at

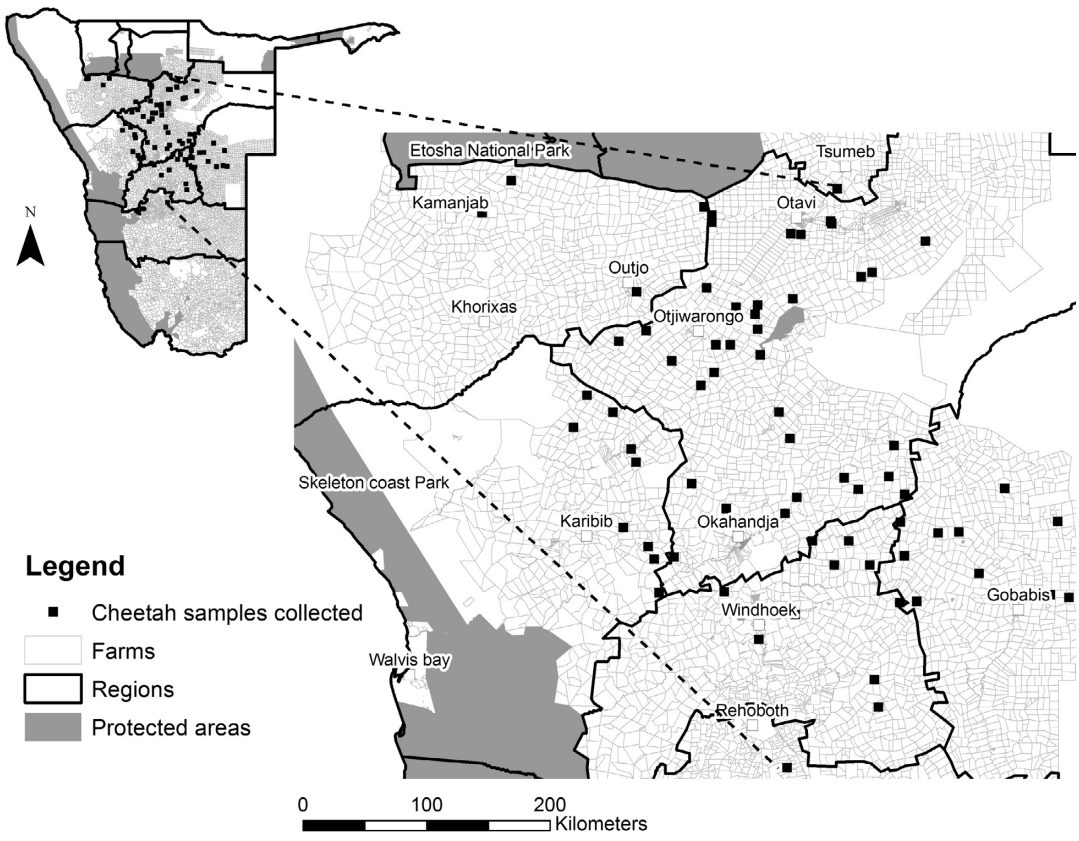

Fig. 1. Range of cheetah sample collection in relation to Etosha National Park and unprotected farmland regions.

–80°C until analysis. We chose 115 individuals that equally represented sex, age distribution and season of sampling (Table 1). Serial samples from five cheetahs were run to assess changes in titres over time.

Antibodies to the protective antigen (PA) of *B. anthracis* were detected using the QuickELISA Anthrax-PA Kit (Immunetics, Inc., Boston, MA) at the Centers for Disease Control and Prevention. Anthrax-PA Kit was designed for the qualitative detection of total anti-PA antibodies in a non-subtype, non-species specific manner, which allows for its application to a wide range of mammals since there is no species-specific conjugate (Bagamian *et al.*, 2014; Lembo *et al.*, 2011). Recent studies of wildlife, livestock and domestic canids from Tanzania (Lembo *et al.*, 2011) and wild boars (*Sus scrofa*) from Ukraine (Bagamian *et al.*, 2014) have employed this assay using the manufacturer's protocol. In Ukraine, each experiment was run with blinded positive and negative controls from vaccinated and unvaccinated bison (*Bison bison*) (Bagamian *et al.*, 2014). Using the manufacturer's protocol, Lembo *et al.* (2011) was also successful in detecting anti-PA antibodies in lions and African wild dogs. In our study, cut-off values were calculated for each run based on the mean absorbance values of controls at 450 nm, corrected by 620–650 nm background subtraction, yielding positive threshold optical density (OD) values that ranged from 0.110–0.113 net OD-450. For quality control, previously identified positive and negative sera taken from three lions and four zebras were run along with the cheetah sera.

Table 1. Summary of categorical data from cheetahs sampled.

Cheetah demographics (n = 115)	Number of samples (121 total*)	% of samples (121 total)
Sex		
Male (n = 59)	63	52.1%
Female (n = 56)	58	47.9%
Age group		
Large cubs (6 months – 1 year)	5	4.1%
Adolescents (1–1.5 years)	21	17.4%
Independents (1.5–2 years)	18	14.9%
Young adults (2–2.5 years)	30	24.8%
Prime adults (2.5–4 years)	40	33.1%
Old adults (4–8 years)	5	4.1%
Very old adults (12 years or older)	2	1.7%
Year		
1993	13	10.7%
1994	9	7.4%
1995	9	7.4%
1996	7	5.8%
1997	8	6.6%
1998	9	7.4%
1999	11	9.1%
2000	11	9.1%
2001	6	5.0%
2002	11	9.1%
2003	9	7.4%
2004	9	7.4%
2005	9	7.4%
Season		
Wet season (November–April)	58	47.9%
Dry season (March–October)	63	52.1%

*121 samples were analysed in total; six were serial samples taken from five cheetahs.

RESULTS

None of the 115 cheetahs included in the study had measurable antibodies to *B. anthracis.* The OD values of the cheetah serum samples ranged from 0.005–0.042 net OD-450, all values falling below the calculated threshold for positivity of 0.110–0.113 net OD-450. Quality control sera samples all produced the expected results: zebra sera (0.020, 0.017, 0.021 and 0.017 net OD-450) and one lion sample (0.019 net OD-450) were negative, while the two remaining lion sera were positive (2.375 net OD-450) and over the upper limit of detection of the assay (>3.5 net OD-450), respectively (Table S1 in online supplement).

DISCUSSION

Our data suggest that the farmland Namibian cheetah population lacks antibodies to *B. anthracis*. One explanation is that this cheetah population was not exposed to *B. anthracis* during or prior to the study period. Although anthrax is endemic in Namibia, incidence among livestock decreased dramatically after the government vaccination programme (Schneider, 1994); but anthrax in livestock is still reported (Beyer *et al.*, 2012). There may also be a behavioural explanation for lack of exposure; unlike other carnivores, cheetahs traditionally do not scavenge and have less contact

with anthrax-infected carcasses, and therefore may have not evolved genetic adaptations conferring resistance (Lindeque *et al*., 1998). Alternatively, cheetahs may have been exposed to *B. anthracis* through prey, become infected and suffered acute mortality before they were able to mount a measurable antibody response (Lindeque *et al*., 1998). However, it is also plausible that any immune response was too rare and/or short lived to detect with the sampling depth used in this study.

The reoccurrence of anthrax epizootics may be a limiting factor in the cheetah's population size in ENP, but Namibia's farmlands have been considered relatively anthrax-free since the livestock vaccination mandate (Lindeque *et al*., 1998). However, in a recent study, Beyer *et al.* (2012) conducted nationwide sampling for *B.anthracis*, and identified 28 cases of anthrax in livestock and wildlife in the Namibian farmlands during the 12-year period in which cheetahs were sampled for this study. Isolates were identified on 21 private ranches, 19 of which are located in the same region where the cheetahs were sampled for this study (Beyer *et al*., 2012). Efforts to conserve cheetahs are already hindered by the tenuous relationship between cheetahs and humans in the farmland regions, where livestock farmers often respond to cheetah depredation with lethal methods of predator control (Durant, 2015). Given the high mortality of cheetahs exposed to anthrax, a major epizootic in a naïve Namibian farmland population could cause yet another setback for the sustainability of this key population. Since the conclusion of this study in 2005, Beyer *et al.* (2012) has reported 19 more cases of anthrax-infected animals in the Namibian farmlands. Recent outbreaks have killed humans, livestock and cheetahs (L. Marker, pers. comm. 2015) in the agricultural regions of Omaheke and Oshikoto (Shaanika, 2013). The recent reemergence of this disease outside of ENP suggests that the Namibian farmland cheetah population may be at heighted risk of anthrax exposure.

We were not able to test the sensitivity of the QuickELISA with known positive cheetah serum, such as vaccinated animals, because the test is currently commercially unavailable. It is plausible that some cheetahs may have had titre concentrations too low to detect with this kit. To date, sensitivity and specificity for this assay have been limited to those published by the manufacturer (Bagamian *et al*., 2014). Therefore, it is possible that we underestimated sub-lethal exposure in cheetahs. However, Bagamian *et al.* (2014) confirmed blinded bison samples were successfully defined as positive or negative respectively in two separate laboratories, suggesting that the assay is sensitive enough to detect low-titre vaccine levels.

Demonstration of natural immunity among wild cheetahs is extremely uncommon. To date only two cheetahs in Namibia have been identified that demonstrated low levels of antibodies to *B. anthracis* (Lindeque *et al*., 1998). In Botswana, only one cheetah was identified with a measurable level of antibodies out of 23 wild-caught cheetahs (Good *et al*., 2008). It has been shown that cheetahs can develop protective immunity to anthrax in response to multiple vaccinations with the Sterne strain 34F2 vaccine (Turnbull *et al*., 2004). This suggests that vaccination against anthrax may confer protective immunity in cheetahs. Since the Namibian farmland cheetahs are at risk of exposure and lack detectable antibodies against anthrax, a cheetah vaccination programme may be warranted. However, due to the logistical issues of recapturing cheetahs in order to administer vaccine boosters, efforts may be better placed on encouraging the vaccination of livestock and game on private farmlands in order to reduce the overall risk of an anthrax epidemic in this region. The Namibian farmland cheetahs represent one of the last viable cheetah populations and therefore conservation of this population is crucial for the survival of this vulnerable species.

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Table S1. Optical density values for QuickELISA Anthrax-PA Kit study samples and controls

Sample type	Optical density values (net OD-450)
QuickELISA Anthrax-PA Kit controls	
Negative controls	0.010–0.018
Low positive controls	0.470–0.829
Positive controls	2.857–3.464
Calculated threshold cut-offs	0.110–0.113
Animal serum	
Zebra serum (negative control)	0.017–0.021
Lion serum (positive control)	2.375–Over
Cheetah serum	0.005–0.042